Mononuclear phagocytes in head and neck carcinoma patients

In vitro studies focusing on the clinical relevance of monocyte and macrophage responses in tumour cell interaction

Kenneth Wilfried Kross

Dissertation for the degree of philosophiae doctor (PhD)
University of Bergen, Norway
2008

UNIVERSITETET I BERGEN

Mononuclear phagocytes in head and neck carcinoma patients

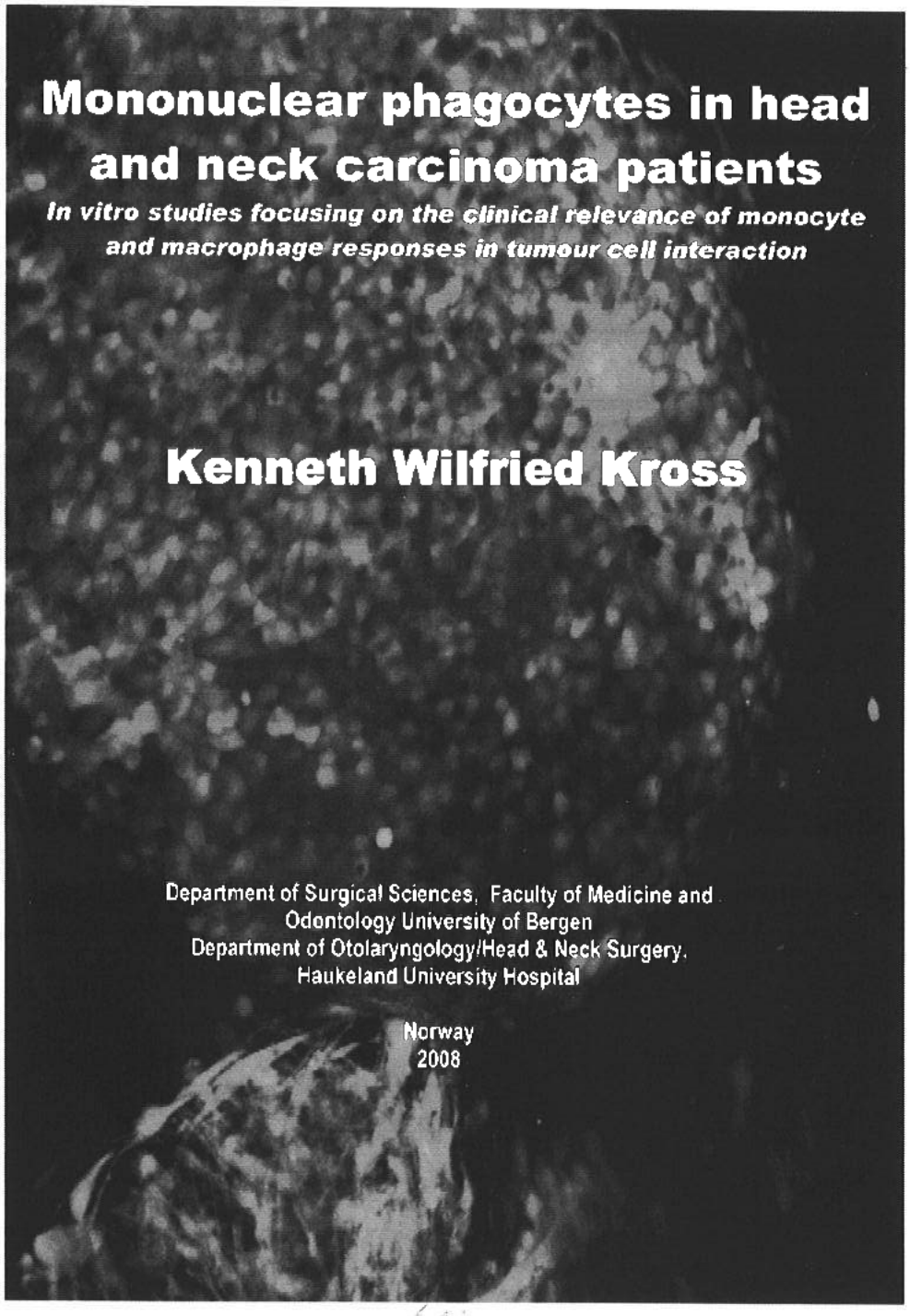
In vitro studies focusing on the clinical relevance of monocyte and macrophage responses in tumour cell interaction

Kenneth Wilfried Kross

Dissertation for the degree Philosophiae doctor (PhD) at the University of Bergen, Norway

Mononuclear phagocytes in head and neck carcinoma patients

In vitro studies focusing on the clinical relevance of monocyte and macrophage responses in tumour cell interaction



Kenneth Wilfried Kross

Department of Surgical Sciences, Faculty of Medicine and Odontology University of Bergen
Department of Otolaryngology/Head & Neck Surgery, Haukeland University Hospital

Norway
2008

CONTENTS

1.0 LIST OF ORIGINAL PUBLICATIONS

1. Kross KW, Heimdal JH, Olsnes C, Olofsson J and Aarstad HJ. Head and Neck Squamous Cell Carcinoma spheroid- and monocyte spheroid-stimulated IL-6 and monocyte chemotactic protein-1 secretion are related to TNM stage, inflammatory state and tumour macrophage density. Acta Otolaryngol 2005 Oct;125(10):1097-104.

2. Kross KW, Heimdal JH, Olsnes C, Olofsson J and Aarstad HJ. Tumor associated macrophages secrete IL-6 and MCP-1 within Head and Neck Squamous Cell Carcinoma (HNSCC) tissue. Acta Otolaryngol. 2007 May;127(5):532-9.

3. Heimdal JH, Kross KW, Klementsen B, Olofsson J, and Aarstad HJ. Stimulated monocyte IL-6 secretion predicts survival of patients with head and neck squamous cell carcinoma. BMC Cancer. 2008 Jan 30;8(1):34.

4. Kross KW, Heimdal JH, Olsnes C, Olofsson J and Aarstad HJ. Co-culture of head and neck squamous cell carcinoma spheroids with autologous monocytes predicts prognosis. Scand J Immunol. 2008 Apr;67(4):392-9.

2. ACKNOWLEDGEMENTS

Writing acknowledgments to thank fellow workers and authors might be one of the most difficult parts of writing this thesis. I would like to try anyway.

Clinical director & professor, dr. med. Jan Olofsson: without your trust in my capabilities and me I would not have been where I am now. Prof. dr. med. Hans-Jørgen Aarstad: thank you for your optimism and hard work with the statistics and for our discussions on immunology. John-Helge Heimdal: I would like to thank for you for your constructive criticism and you always had time for me no matter how busy you were. Thank you Carla Olsnes for your quality work, computer skills and proofreading. Helen Stavang for your excellent laboratory analysis. I am very grateful to Dagny Ann Sandness for teaching me, with a lot of patience, basic laboratory skills. Thank you also for your excellent lab analysis. Thanks to Broegelmann Laboratory, under the supervision of Prof. Dr. R. Jonsson, for giving us a place to perform our ELISA analyses.

Special thanks go to dr. Robert Maat, with whom I shared the office and had many hours of discussions on the nice things in life. You are a real friend.

Thanks to my friends in the L.H.D. Cobra for all the good times we had so far.

Most important I want to thank my family for always being there for me. Thank you Joke, Rob, Chindy, Ruud, Martijn, Laura, Thom and Silke.

Last, but certainly not least, Håvard Valvatne, my greatest gift. Without your continuous support I would have given up a long time ago.

3. ABBREVIATIONS

APC(s)	Antigen presenting cell(s)
B	Benign
BRM	Biological Response Modifiers
CD	Cluster of Differentiation
CRP	C-reactive protein
CTL(s)	Cytotoxic T lymphocyte(s)
EGFR	Epithelial growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ECM	Extracellular matrix
ESR	Erythrocyte sedimentation rate
F-	Fragment
HNC	Head and Neck Cancer
HNSCC	Head and Neck Squamous Cell Carcinoma
IFN-	Interferon-
IL-6	Interleukin-6
LLME	L-Leucine Methylester
LPS	Lipopolysaccharide
Mφ(s)	Macrophage(s)
M	Malignant
MIP-1β	Macrophage inflammatory protein-1β
MHC	Major histocompatibility complex
MO(s)	Monocyte(s)
MCP-1	Monocyte chemoattractant protein-1

MNP(s)	Mononuclear phagocyte(s)
NCR	Natural cytotoxicity receptors
NF-κB	Nuclear Factor-κB
NK	Natural Killer Cell
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
STAT-3	Signal transducer and activator of transcription 3
TAA	Tumour-associated antigen
TAM	Tumour-associated macrophage
TIL	Tumor-infiltrating lymphocytes
TCR	T-cell receptor
TNF-α	Tumor necrosis factor-α

4. GENERAL SUMMARY

This study was performed in order to explore and study the functions of mononuclear phagocytes in head and neck squamous cell carcinoma. Unravelling the interactions between mononuclear phagocytes and head and neck squamous cell carcinoma might form a basis for an adjuvant therapy in the treatment of this type of cancer, which still has a 5-year survival rate of about 50%. Mononuclear phagocytes can secrete many different substances, such as interleukin-6 and monocyte chemoattractant protein-1. These substances have been shown to play a role in the development and metastasis of cancer. Interleukin-6 co-regulates cell differentiation, survival and apoptosis. Monocyte chemoattractant protein -1 is a chemokine responsible for recruiting mononuclear phagocytes to tumours. Mononuclear phagocytes have the ability to secrete these substances in substantial amounts.

We have used an organ culture model whereby fragment spheroids were established from head and neck squamous cell carcinoma tissue and autologous benign squamous tissue. These fragment spheroids served as vectors for tumour cells in co-cultures with autologous monocytes. Fragment spheroids developed within 14 days of *in vitro* culture whenever adequate tissue was available. We studied the composition of fragment spheroids and densities of tumour-associated macrophages. We found that fragment spheroids do not secrete tumour necrosis factor-α and that interleukin-6 and monocyte chemoattractant protein -1 production by monocytes, in co-culture with spheroids, correlated with density of tumour-associated macrophages both within the spheroid and correlated to each other. Furthermore, monocyte

function was suppressed upon the presence of enhanced "inflammation" in the body, as expressed by the erythrocyte sedimentation rate.

The study was taken further where a method was developed for diminishing tumour-associated macrophages in spheroids. Tumour tissue was incubated with L-leucine methylester before allowing it to further develop into fragment spheroids. With this method, we succeeded in partial removal of mononuclear phagocytes from the spheroid, without significantly intoxicating the tissue and maintaining viability and integrity of the spheroid. We found that tumour-associated macrophages are the main producers of interleukin-6 and monocyte chemoattractant protein -1 in head and neck squamous cell carcinoma tissue.

As level of tumour-associated macrophage infiltration has been associated with prognosis, we wanted to study the presence of an association between monocyte function and survival in head and neck cancer patients. Monocytes were isolated from patients with head and neck squamous cell carcinoma and a control group of patients operated for benign conditions. We found that monocyte responsiveness was increased in cancer patients compared to controls and that patients, whose monocytes had high interleukin-6 production after stimulation with lipopolysaccharide, had a reduced disease-specific survival compared to patients with low monocyte interleukin-6 production, when adjusted for age, gender and TNM stage. This indicates that activated mononuclear phagocytes may, in fact, increase rather than reduce tumour cell aggressiveness in head and neck cancer.

Co-culture-derived interleukin-6 and monocyte chemoattractant protein -1 production was correlated to survival and recurrence rate in a cohort of

patients operated for head and neck squamous cell carcinoma. In comparison to patients without recurrence, patients with recurrence had higher interleukin-6 production when monocytes were co-cultured with benign and malignant fragment spheroids. Furthermore, a high value of benign fragment spheroid-derived interleukin-6 secretion predicted survival while interleukin-6 production from monocytes in co-culture with malignant spheroids correlated to recurrence.

This thesis has focused on the interactions between monocytes and head and neck squamous cell carcinoma tissue and related mononuclear phagocyte function, as expressed by cytokine secretion in an organ culture model, to clinical parameters such as survival, recurrence and serum factors. Tumour-associated macrophages produce interleukin-6 in head and neck squamous cell carcinoma, thus forming consequences in prognosis. Future therapy strategies might be directed towards mononuclear phagocytes and their cytokine production.

5. INTRODUCTION

5.1 Head and Neck cancer

Head and Neck Cancer (HNC) is a relatively rare cancer disease in the Western world. In the United States, where the population is approximately 300 million, an estimated 40 500 new cases of HNC arose in 2006, with an estimated mortality of 11.170 persons[1]. Worldwide, 644 000 new cases of HNCs are estimated to be diagnosed each year, with almost two-thirds occurring in developing countries[2]. In some of these countries, HNC can be one of the most common malignancies. This is mostly due to factors such as poor oral hygiene, chewing betel nuts, smoking and drinking alcohol[3] but other factors such as genetic susceptibility and virus infections may play a role in the carcinogenesis of HNC[4]. Over 90% of malignancies in the upper aero-digestive tract are squamous cell carcinomas (HNSCC)[5],which will be discussed further in this thesis.

5.2 Treatment of HNSCC

HNSCC is usually treated with surgery and radiotherapy either combined or one modality alone. In the last years, advances in surgical treatment have been mainly made on the field of organ preservation and more effective means of reconstruction[6]. Advances in radiotherapy have been seen in recent years to include altered fractionation radiotherapy and radiotherapy combined with chemotherapy in patients with locally advanced tumours [7, 8]. However, this combined treatment is associated with severe side effects[9]. Despite advances in diagnosis and treatment, the survival rate has not significantly changed over the last 20 years and remains rather low.[10] The

highest 5-year survival is for lip cancer, at 91% but just 31% for hypopharyngeal cancer[5]. There is, therefore, an urgent demand for new therapies to improve the survival of HNSCC patients.

5.3 Challenges in treatment of HNSCC

Many advances have been made in understanding the carcinogenesis and biology of cancer and metastasis. Several new areas of research have emerged involving biologic compounds targeting specific regions in the cancer cell. One area of special investigation is the Epithelial Growth Factor Receptor (EGFR) which is expressed in more then 90% of HNSCC tumours and is associated with a poor prognosis[11]. EGFR inhibitors hold promise in two important ways: (1) further improve efficacy for patients at risk for recurrence and (2) decrease treatment-related toxicities by replacing toxic cytotoxic drugs without jeopardizing survival[12]. Several clinical studies have been performed where monoclonal antibodies, directed against the extracellular domain of EGFR, are administered, or several small molecule tyrosine kinase inhibitors are given[13] These substances are administered combined with radiotherapy or chemotherapy[14]. Results are promising despite severe side effects but a new standard of treatment has yet to be found in the clinic[14].

The innate immune system may interact with cancer cells, both as an inhibitor[15] and facilitator[16] of tumour growth. This interaction allows for the use of immunomodulating therapies of cancer by more or less generalized stimulation of mononuclear phagocytes (MNP). Killed bacterial toxins[17], along with bacillus Calmette-Guerin (BCG)[18], β-glucan[19], interferons[20] and monoclonal antibodies[21] are examples of applied biological response

modifiers (BRMs), used as immune stimulators in tumour treatment.

In Japan, it has been a long-standing tradition to use penicillin-killed lyophilized *Streptococcus pyogenes*, denominated OK-432 or picibanil, as a BRM in cancer treatment[22]. Sakamoto *et al.*[22] published in 2001, a meta-analysis where the benefits of immunochemotherapy with OK-432 were assessed in patients with resected non-small-cell lung cancer. The meta-analysis was based on data from 1,520 patients enrolled in 11 randomized clinical phase III trials. The 5-year survival rate for all eligible patients in the 11 trials was better with OK-432 treatment, i.e. 51.2% in the immunochemotherapy group versus 43.7% in the chemotherapy group. Furthermore, Oba et al[23]have shown that patients with resectable gastric carcinoma treated with adjuvant immunochemotherapy, with OK-432 as one component, may improve their survival (performed was a meta-analysis on 8009 patients from 8 randomized clinical phase III trials). There are also reports suggesting that patients with other cancers, such as HNSCC[24], may benefit from OK-432 treatment. OK-432 may also be used as a maturation factor for dendritic cells (DCs) as part of vaccination therapy of cancer patients[25]. Despite these convincing results for OK-432 as cancer treatment, OK-432 treatment has not gained any important role as a cancer drug in Europe or USA.

Other new therapies might be directed towards other target receptors on the cancer cell such as the Fas receptor[26]. This, as well as other new (gene) therapies, has not been properly developed nor have there been, to our knowledge, performed any clinical studies.

Therapy may be further improved but the potential effect of traditional therapeutic strategies seems to have been almost reached with respect to HNSCC patients. This forms a rationale for searching for other therapeutic strategies, and immunotherapy is one interesting option. Several strategies might be feasible as to immunotherapy, e.g, cancer vaccine-based immunotherapy should enhance immunosurveillance and prevent and protect against growing tumours. Several trials have been done with tumour-associated antigens (TAA) and peptides though objective clinical responses are still rare[27] . Other strategies involve passive transfer of immunological active cells, generated *ex vivo*, into patients in order to mediate tumour regression either directly or indirectly. Transfer of gene modified antigen presenting cells (APCs) might reverse tolerance to tumour antigens, e.g., by using autologous APCs with tumour tissue-derived DNA[28]. Cytokines might also be used as therapy modus by direct intratumoral or locoregional delivery or by using autologous cells to secrete cytokines, thereby inducing, e.g., T-cell activation.[28].

5.4 THE IMMUNE SYSTEM

The immune system may be divided into two major compartments: innate immunity that is antigen non-specific and adaptive immunity that involves antigen specific humoral and cellular immunity. Cells of the two compartments interact in joint regulations of immune responses.

5.4.1 Innate immunity

Innate immunity constitutes antigen-independent immune mechanisms generally involving: surface barriers, MNPs, polymorphonuclear phagocytes (neutrophils, basophils and eosinophil granulocytes), natural killer (NK) cells, DCs and complement activation. MNPs, NK cells as well as DCs, have been the focus of studies in tumour immunology/immunotherapy.

Cells of the MNP develop from myeloid progenitors in the bone marrow. DCs can also develop from the lymphoid progenitor[29] and are described separately later in this chapter. Cells of the MNP system are important for innate immunity but also play a central role in specific adapted immunity. In addition, MNPs participate in several biological processes, e.g., disposal of dying cells, ingestion of aged erythrocytes, regulation of inflammatory processes, e.g., tissue repair, initiating coagulation and stimulation of chemotaxis[30-32].

After maturation and subsequent activation MNPs achieve varied morphological forms. Monocytes (MOs) may, after 2-3 days in circulation at baseline conditions, move into different tissues and further differentiate into macrophages (Mφs). Mφs associated with tumours are called tumour-associated macrophages (TAMs). MNPs, in general, may act as defence

cells and phagocytes even in the absence of activation. MNPs are effective as APCs and are involved in the regulation of immune responses via interaction with lymphocytes and as effector cells in adaptive immune response[33]. The functional capacity of MNPs depends on the activation level of the cells. The original proposed term of Mφ activation was defined as resistance of Mφs against *Listeria monocytogenes* infection[34]. This activation was dependent on the presence of a T lymphocyte product that was later shown to be interferon (INF-)-γ[35]. Another example of a cytokine that regulates the response of MNPs to activating substances is the macrophage colony-stimulating factor (M-CSF)[36]. Today, a more broad definition of activation is used, where any amplification of MO/ Mφ function is designated as activation[32]. Furthermore, in extension of this broadened definition of activation, MNP priming may be used as a term for describing a state of MNP functional regulation that perpetuates a secondary, enhanced MNP response upon subsequent stimulation[37]. Several substances are known to activate MNPs and these cells may respond differently to given stimuli depending on the status of MO-Mφ differentiation[38]. A frequently used stimulus for MNPs in *in vitro* studies is lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell wall. LPS is a very potent MNP *in vivo* stimulator, acting as a central component in the pathogenesis of sepsis[39].

NK cells develop in the bone marrow from common lymphoid progenitor cells and circulate in peripheral blood but can also be found in lymph nodes and the spleen. They represent a unique subset of lymphocytes, distinct from T and B cells and contribute to host antimicrobial and antitumour defence reactions[40]. NK cells are able to kill cells without being activated by APCs

and do not need to recognize major histocompatibility complex (MHC) class I molecules. Cytotoxic granules are released at the target cell. These proteins penetrate the cell membrane thereby causing lysis. NK cells used to be considered a homologous group of cells though intensity of CD56 expression (dim or bright) as well as presence or absence of CD16 epitopes, suggesting functional differences and further subdivision[41]. $CD56^{bright}$ NK cells have predominantly immunoregulatory properties and possess a strong cytokine producing capability whereas $CD56^{dim}$ NK cells have a marked cytotoxic function[40]. Furthermore, NK cells may become activated by various stimuli such as contact with MOs and DCs, MHC class I-negative cells, binding of antibody complexes, direct binding to receptors on the cell surface by tumour-associated molecules or pathogen-derived products. The activity of these cells then increases by several fold[29]. NK cells may also be activated upon stimulation with cytokines such as interleukin (IL)-1, IL-2, IL-12, IL-15, IL-18, IL-21 and IFN-α and IFN-β[41]. NK cytotoxic receptors are collectively named natural cytotoxicity receptors (NCR)[42]. Many ligands necessary for activation of NCRs are still unknown. Only two groups of ligands have been identified so far and it appears that these ligands either lack in normal cells, are expressed at particular cell sites unaccessible to NK cells or are expressed at too low surface density to allow for NK cell triggering[42].

DCs are "professional" APCs playing an important role in both innate and adaptive immune responses. Immature DCs in the peripheral blood cannot up-regulate MHC class II molecules and other co-stimulatory molecules, thus leading to inefficient signaling. After DCs have captured foreign material, they

mature and transport the modified material to lymphoid follicles delivering it to B lymphocytes or presenting it as antigens to T lymphocytes.

5.4.2 Adaptive immunity

The adaptive immune system constitutes of B and T lymphocytes. B cells are involved in humoral immune responses whereas T cells are involved in cell-mediated cytotoxicity. A specific immune response is normally triggered when an individual is exposed to a foreign antigen. The features of a specific immune response are; specificity, diversity, self-regulation, tolerance to self, and memory[43]. The specificity of the immune response is attributed to lymphocytes capable of recognizing and distinguishing different antigen determinants. Induction of efficient primary adaptive immune responses requires two different signals: one being the direct interaction of lymphocytes with mature antigens (presented by APCs) and the other being provided by "helper cells" or "accessory cells" in a pro-inflammatory environment. The extreme diversity of antigenic determinants is mirrored by the variability in the structures of antigen binding sites. Self-regulation is ensured by brief presentation of antigens and feedback regulation of the immune response. Discrimination of self from non-self is ensured through a selection of lymphocytes during the maturation process followed by a secondary rapid immune response, due to the presence of "memory" cells that have previously responded to that specific antigen.

B cells may, upon direct contact with antigen via their surface receptor, differentiate into plasma cells to produce antibodies against antigens. B cells produce cytokines and chemokines which can activate and maintain a chronic

inflammatory reaction and produce antibodies which might mediate recruitment of innate immune cells via activation of complement cascades or engagement of FcR expressed on resident immune cells[15]. In this manner, the adaptive immune system may play a role in the regulation of inflammation-associated cancer development via B-cell activation and production of soluble mediators.

Receptors on T lymphocytes (T-cell receptor(TCR)) recognize antigens presented by MHC on cell surfaces. Class II molecules (MHC-II) present peptides derived from proteolysis of extracellular antigens on, e.g., APCs, whereas class I molecules (MHCI) present peptides primarily originating from intracellular degradation, e.g., target cells. TCRs are complexly related to membrane (accessory) proteins. The interaction between these proteins is necessary for the antigen-MHC molecule recognition, and further regulates the activation as well as the effector phase of the T lymphocyte response[29]. Two subgroups of T cells can be distinguished depending on rearrangement of the T cell receptor in the thymus. During maturation and selection T lymphocytes are distinguished according to different surface glucoproteins expressed as accessory molecules, termed "cluster of differentiation" (CD), into $CD4^+$ T helper (T_H) cells and $CD8^+$ Cytotoxic T-(T_C) cells. T_H cells further form 2 subpopulations. The Th1 subset, secretes IL-2, IFN-γ and TNF-α upon activation and aids in the activation of cytotoxic T cell, and the Th2 subset, which secretes IL-4, IL-5, IL-6 and IL-10 upon activation and has a acts as a helper cell in the activation of B cells. There is a complex interplay between cells in an immune response through which secretion of cytokines from one

subset can regulate activation and secretion of cytokines from the other subset.

Cytotoxic T cells (CD8+) (CTLs) recognize antigen presented by MHC class I and can act cytotoxic upon activation by cytokines such as IL-2 and IFN-γ. They also exert their effects by releasing IFN-γ, TNF-α and TNF-β. IFN-γ can activate Mφs and induce an increased expression of MHC class I in infected cells. Furthermore, IFN- γ, together with TNF-α and TNF-β, act in a synergistic manner in Mφ activation[44]. Regulatory T-cells (T_{reg}) play a role in the regulation and suppression of self-reactive lymphocytes[29].

5.5.1 Cancer and the human Immune System

The degree of interaction between tumours and the immune system has been discussed for more than a century. Theories have been proposed with respect to general tumour immunology, as well as strategies for immunotherapy in the treatment of malignant diseases. Ehrlich observed in animal experiments that recipient mice could acquire immunity to tumour growth. He suggested that the immune system could detect and respond to cancer cells because the cancer cell was considered a foreign element by the organism (Ehrlich, 1909). Later, this view was further substantiated and systematized to become the theory we now know as the "immune surveillance" theory[45]. This theory hypothesizes that malignant cells are exposed to tumour-associated antigen determinants on their cell surface. These antigens then induce a T lymphocyte-dependent immune response that eventually eliminates tumour cells[45]. According to the theory of immune surveillance, a malignant tumour may only arise when malignant cells escape an immune response[46]. There is

experimental evidence that malignant tumours can elicit a specific T cell-mediated immune response and suppression of the immune system increases the incidence of certain types of cancers in humans[44]. Scepticism has been stated towards the general relevance of immune surveillance in humans[47] and any firm role of immune surveillance in HNSCC patients has yet to be established[44]. Still, observations from several studies indicate that immune system-related mechanisms may act either as stimulatory or inhibitory regulators on the development and spread of malignant diseases in humans[48].

Active immune mechanisms protecting the organism against distant spread of cancer are demonstrated in animal models[49, 50] and may argue that immune system related mechanisms act against tumour cell spread, also in humans. These immune mechanisms apparently fail to eliminate tumour cells in progressive cancer disease. This incapability of the immune system may be attributed to three principally distinct reasons[51]:

A. Escape by loss of recognition (loss or alteration of molecules which are important for the recognition by and activation of the immune system)
B. Escape by loss of susceptibility (escape from effector mechanisms of cytotoxic lymphocytes)
C. Induction of immune cell dysfunction

When developing effective strategies for the treatment of HNSCC, a thorough understanding of tumour cell escape mechanisms becomes fundamental. Cells of the immune system may not only fail to act against tumour growth but several reports have revealed that cells of the innate immune system may act in supporting tumour cell growth. This is, e.g., the case for MNP cells that

secret growth factors and factors contributing to neo-angiogenesis by stimulating proliferation and migration of endothelial cells and local degradation of the extracellular matrix (ECM)[52]. Immune cells should therefore be regarded as possible "friends or foes" when one discusses interactions between tumours and the immune system.

5.5.2 Inflammation, cancer and the human immune system

A relationship between cancer and inflammation has since long been discussed and epidemiological and experimental evidence points to a connection between long-term inflammation and development of dysplasia followed by development into cancer[53]. Inflammation is a very complex process where many effector cells and mediators are involved potentially facilitating tumour growth through multiple mechanisms[54]. Solid cancers generally show signs of inflammation with influx of many types of leukocyte populations. In a deficient immunosurveillance situation, a switch may occur where cells of the MNP (mainly TAMs) change their phenotype from pro-inflammatory to pro-angiogenic, contributing to tumour progression via secretion of proteases, cytokines and angiogenic factors[52]. Chronic inflammation can be caused by viruses and contribute to cellular transformation[55] while bacterial infection may play a role when an organisms produces free radicals, such as reactive oxygen and/or nitrogen species, causing DNA damage and mutations leading to tumour development. Chronic inflammation, furthermore, elevates a population of myeloid-derived immunosuppressive cells that inhibit anti-tumour immunity[56]. IL-1β is a cytokine that plays a role in this process. In a recent study, mice deficient for

IL-1 receptor(IL-1R) were inoculated with mammary cancer cells and compared to non-deficient mice. Tumour-associated inflammation as well as tumour progression was delayed in IL-1R-deficient mice as well as number of suppressive myeloid cells. Tumour progression as well as progression and number of suppressive cells could be restored by administering IL-6, a pleiotropic cytokine known as a mediator of IL-1β, and from that perspective, a contributing factor in tumourigenesis[54]. For colitis-associated cancer, an animal model has been developed that reproducibly leads to colonic neoplasia within the setting of colitis. In this model, a carcinogen is administered followed by 3 rounds of chemical colitis induced by a chemo-irritant in drinking water. After 1-2 months of treatment, nearly 100% of mice developed neoplasms in the colon. This model has shown that mucosal-derived Nuclear Factor-(NF-)κB factor is activated. NF-κB is a pleiotropic transcription factor which plays a role in both innate and adaptive immunity, and is required for the expression of several pro-inflammatory factors[57, 58]. From this perspective, (chronic) inflammation might be a key factor in cancer development. Since the head and neck area is prone to exposure to factors causing irritation of the squamous epithelium, it might be plausible that chronic inflammation is also a major cause for the development of HNSCC.

5.5.3 HNSCC and the Immune System

The HNSCC microenvironments also comprise immune cells and their secretory products. The presence of immune cells, mainly T lymphocytes and DCs but also of B cells, plasma cells, NK cells, Mφs and eosinophils, as well

as proximity to cervical lymph nodes, is likely to have an effect on the initiation, promotion and progression of HNSCC [59].

5.5.3.1 MNPs in HNSCC

Phenotypic and functional changes have been described in MOs from patients with different types of cancer, upon comparison to controls. In prostate cancer patients, peripheral blood has high levels of phenotypically immature MOs compared to healthy individuals [60]. Recent data have demonstrated that myeloid cells accumulating in cancer patients play an important role in tumour non-responsiveness via suppression of antigen-specific T cell responses[61]. On the other hand though, it has been shown that MOs from cancer patients are activated compared to healthy controls[62, 63] but contact with tumour cells can decrease function of MOs in cancer[64]. Although some of the results are conflicting, there is evidence suggesting that functional activities, as expressed by, e.g., cytokine secretion of MOs in cancer patients, are changed.

5.5.3.1 Tumor-Associated Macrophages in HNSCC

TAMs may play a dual role in the interaction with tumour cells[65]. In some situations, a symbiotic relationship may exist between TAMs and tumour cells, thereby providing support to the tumour in terms of growth and metastasising capacity. It has, e.g., been shown that a high amount of TAMs in tumours can be associated with increased neo-angiogenesis and a worsened survival rate[66]. TAMs also have the potential for acting cytotoxic towards tumour cells and some reports state an improvement in prognosis associated with high

numbers of TAMs in tumours[67]. Correlation analyses between number of TAMs and prognosis in HNSCC have, however, shown that high levels of TAMs in HNSCC may be related to worse long- term prognosis[68, 69]. This might be different in other cancer diseases[48, 66, 70-73]. In recent years, evidence has been provided on Mφs being induced by cytokines to polarise into two distinct groups. These 2 groups differ in receptor expression, cytokine and chemokine production as well as effector function[65]. Type I Mφs are effector cells which kill micro-organisms and tumour cells and produce anti-inflammatory cytokines while Type 2 Mφs regulate inflammatory responses and adaptive Th immunity, act as scavenger cells, promote angiogenesis, tissue modelling and repair. This definition, however, does not take into account that Mφs are not static and that functional and phenotypic changes occur during inflammatory responses, thereby, inducing Mφs into an evolving shift in functional activities and into an unstable, undefined and non-functional phenotype[74]. Watkins et al were, e.g., able to demonstrate this in a mouse model where functionally polarized TAMs could be converted, from a tumour-supportive and immunosuppressive phenotype to an inflammatory functional phenotype, by IL-12 treatment *in vivo* as well as *in vitro*[75].

5.5.3.3 Dendritic cells in HNSCC

DCs are able to induce an anti-tumour immune response by generating tumour-specific cytotoxic T cells. In addition to functioning as APCs, DCs have been shown to regulate the innate immune response by activating NK cells, which show cytotoxic effects toward tumour cells without MHC-class I restriction[29]. The presence of DCs, Mφs and lymphocytes in solid tumours is

regulated by local production of chemokines and inflammatory factors by tumour cells, stroma and immune cells.

Patients with HNSCC show a reduced number of mature DCs in peripheral blood with a correlation to extension and duration of disease, and an accumulation of immature DCs[76]. Low infiltration of DCs in HNSCC might be caused by loss of expression of the chemokine ligand CXCL14, which attracts immature DCs into tumours[77].

5.5.3.4 Natural Killer Cells in HNSCC

Several receptors on the NK cell are involved in the process of cytotoxicity and lysis of tumour cells. Many studies are performed in order to obtain better insight in the function of NK cells in cancer, and in finding ways of using its direct cytotoxicity in the treatment of cancer in humans. A recent study showed that low levels of circulating, invariant, NK cells predicts poor clinical outcome in patients with HNSCC[78] and several strategies have been developed for the use of NK cells against a variety of malignancies, such as administration of cytokines, e.g., IL-2, for enhancing endogenous NK-cell responses to tumours. There are also ongoing experiments employing adoptive transfer and even allogeneic transfer of NK cells[79]. Results are promising and in the future, NK cells can be used as an adjuvant modality in the treatment of cancer[40].

5.5.3.5 Lymphocytes and HNSCC

In HNSCC, stroma is infiltrated with tumour-infiltrating lymphocytes (TIL).

T-lymphocyte-stimulated proliferation decreases with increasing tumour burden in HNSCC patients[80]. T-lymphocyte function, subpopulations in peripheral blood as well as growth factor responses, may be correlated to prognosis in HNSCC[81]. A recent publication showed that total percentage of CD4+ T-cells was significantly decreased in patients versus controls and that proportion of T_{reg} cells was significantly elevated in patients relative to healthy donors. These cells seemed to downregulate subsets of CD8+ cells, which may play a role in anti-tumour responses[82]. In a recent study, Aarstad showed that that a high level of peripheral blood (PB)-derived T-lymphocyte activation *in vivo* can predict impaired prognosis with and without adjustment for TNM stage in HNSCC[83]. The amount of peripheral blood lymphocytes might be correlated to tumour load as expressed by node status.

5.6 MNPs and cytokine production

Cells of the MNP system secrete many different mediators of the immune response, such as cytokines and chemokines. When MNPs are activated, genes expressing various inflammatory mediators are induced, among these being cytokine genes. The genes encoding cytokines IL-1β, IL-6, and TNF-α are closely regulated and classified as early or immediate genes. These cytokines are denominated pro-inflammatory[84]. MNPs also secrete peptides, such as monocyte chemoattractant protein-1 (MCP-1), that have chemoattractant effects. In this study, we primarily focused on IL-6, MCP-1 and to a lesser degree, TNF-α.

5.6.1 Interleukin-6 (IL-6)

IL-6 is a 26-kDa protein that is a potent, pleiotropic, inflammatory cytokine mediating many physiological functions, including developmental differentiation of lymphocytes, cell proliferation and cell survival, as well as apoptosis. Furthermore, IL-6 has an effect on bone formation, general system metabolism, endocrine functions and can affect many cells in other tissues and organ systems[85, 86].

The IL-6 receptor (IL-6R) is composed of a ligand-binding α subunit and a signal-transducing component, designated gp130. A high production of IL-6, by MOs in cancer patients with recurrence and/or death from disease, might be linked to an IL-6/gp130 autocrine/paracrine mechanism activating Signal Transducer and Activator of Transcription-(STAT-)3 in an EGFR-independent matter[87]. STAT3 activation is associated with cell proliferation and prevention of apoptosis, thereby participating in oncogenesis[88] ,and has been shown to be up-regulated in HNSCC tissue and in the normal mucosa of the same patient[89]. Recently, IL-6 was shown to have an augmenting influence on the invasion potential of HNSCC cell lines though having an inhibiting effect on proliferation rate[90, 91].

5.6.2 Monocyte chemoattractant protein-1 (MCP-1)

Chemokines, a family of low-molecular-weight cytokines that play key roles in immune response and in development of several cell types, are classified mainly into CC and CXC subfamilies according to location of the first two cysteine residues. MCP-1 (or CCL2) was originally thought to be a selective chemokine for MOs, but later been shown to also have effects on T

lymphocytes[92], NK cells[93] and basophilic granulocytes[94, 95]. MCP-1 is structurally and genetically related to other chemokines (MCP-1, -2, -3, and -4 in humans, MCP-1, -2, -3, and -5 in the mouse) with similar properties. They all activate the same receptor, CCR2, with similar potencies[96]. Many studies have been performed relating synthesis of MCP-1 and influx of TAMs in tumour tissue and relating this influx to factors, such as angiogenesis, invasion and prognosis[68, 70, 97]. Lately, evidence has been provided on MCP-1 possibly being responsible for recruitment of myeloid suppressor cells into tumour and such, having a pro-tumour effect in itself[98]. Various types of cancer cells, including ovarian, breast and colorectal cancer cells, synthesize and express chemokine receptors[99].

5.6.3 Tumour Necrosis Factor-α

Tumour Necrosis Factor-(TNF-)α is a polypeptide of 157 amino acids and in humans, it is expressed on the cell membrane of a majority of cells, with the exception of erythrocytes and resting T lymphocytes[100]. TNF-α is a pleiotropic cytokine and plays an important role in the pathophysiology of different diseases, such as sepsis and rheumatoid arthritis. Blocking TNF-α production is very effective in reducing local and systemic inflammation in patients with rheumatoid arthritis and psoriasis[101]. TNF-α promotes killing of tumour cells through apoptosis via binding with death-domains in tumour cells, stimulating NK cells and activating CD8+ cells[102].

TNF-α molecules are membrane bound or circulate in two forms, either as biological active molecules or as immunologically detectable but inactive

peptides. There are inhibitory mechanisms that protect cells from TNF-α activity, such as circulating TNF receptors and membrane-bound "decoy" receptors[102].

Transcripts of protooncogenes (c-jun and c-fos), transcription proteins, such as the immediate-early growth-response transcription factor Egr-1 as well as subunits of NF-κB family p50 and p65, are involved in induction of TNF-α gene transcription by binding to specific sites on the TNF-α promoter region[103].

5.6.4 HNSCC-derived factors affecting the immune system

Changes observed in systemic responses such as immune responses, acute phase inflammatory protein responses and decreases in delayed-type hypersensitivity in HNSCC patients are not well understood. Still, the local and systemic nature of these responses suggests that cytokines with pro-inflammatory, pro-angiogenic and immunoregulatory activity produced by carcinomas could contribute to the pathogenesis of HNSCC disease.

Cytokines that regulate pro-inflammatory and pro-angiogenic responses are detected in tumour environment as well as systemically in HNSCC patients[104]. It might not be just tumour-derived factors which contribute to growth and metastasis of the tumour itself. Observations from several studies have linked certain subsets of the innate immune system to factors such as clinical prognosis, tumour angiogenesis and invasion[68, 75, 97, 105] and it is now clear that certain subsets of chronically activated innate immune cells promote growth and/or facilitate survival of neoplastic cells[106]. In the tumour microenvironment, as well as in circulation, there are different immune cells

that can produce cytokines having these effects. Among the cells with most active cytokine secretion are the MNPs.

6. AIMS OF THE THESIS

The general aim of the present work was to study the interactions between MNPs and HNSCC tissue by stimulating MOs with LPS and F-spheroids. We measured production of TNF-α, IL-6 and MCP-1 since these substances are secreted by MNP and have been shown to play a role in the development of cancer.

SPECIFIC AIMS

1. To study histological composition of F-spheroids and compare it to the original HNSCC tumour tissue by measuring TAM density and relating it to clinical parameters (paper 1 and 4).
2. To study to what extent TAMs are responsible for IL-6 production in HNSCC tumour tissue by using LLME, a substance that selectively can deprive tissue of MNPs (paper 2 and 4).
3. To study a correlation between secretion of IL-6 by MOs *in vitro* and prognostic factors (paper 3).
4. To correlate *in vitro* co-culture responses in HNSCC patients to recurrence and survival (paper 4).

7. METHODS AND MATERIALS

Patients

Patients that were admitted for surgery for squamous cell carcinomas of the oral cavity, pharynx and larynx were included in the study. Patients treated for autoimmune diseases or previously treated with loco-regional radiotherapy were excluded from the study. The Regional Committee for Medical Ethics at the Haukeland University Hospital approved the studies. Each patient gave written consent before participating in the study.

Clinical Parameters

Blood levels of haemoglobin, C-reactive protein (CRP), erythrocyte sedimentation level (ESR) and albumin were determined one day pre-operative and on the day of co-culture, i.e. day 14 post-operative. Levels were determined according to standard procedures and performed, as part of the general blood-sampling tests, by the Clinical Lab facility at Haukeland University Hospital.

Monocyte isolation

A method was adopted by which PBMCs were isolated by gradient centrifugation, and MOs were separated from PBMCs by *in vitro* adherence to plastic wells[107]. This method provides an easy way of isolating a substantial number of MOs in a standardized manner.

Selection of culture media

We have used autologous serum as serum source in culture media rather than allogenic serum from the blood bank or bovine serum, in order to better mimic the ***in vivo*** situation. Ideally, an ***in vitro*** culture should encompass all the natural surrounding elements that are necessary to cultured cells and serum is usually supplied to media in order to achieve an optimal growth of the cultured cells. Arguments for using autologous serum are that serum components, i.e. soluble immune complexes[108], may change depending on disease in HNSCC patients, possibly affecting the cytokine response of MOs[109]. We have also used serum-free medium as one medium condition to study cytokine production, thus examining possible serum-caused effects.

Selection of method for tumour cell culture

We have used a method based on tissue culture techniques described for maintaining airway mucosa[110] and HNSCC[111]. This method provides a way for small fragments obtained from vital tumour tissue to be cultured ***in vitro***. These tumour fragments develop, within a span of two weeks, on an agar-coated culture well to become fragment (F-) spheroids. Benign tissue from the same area can be used to develop benign F-spheroids, thereby providing an autologous control. Use of this organ culture model might provide a tool for closely simulating the ***in vivo*** situation, as tumour and benign fragments together with (tumour) stroma, are preserved and can be used for investigating interactions with MOs or other types of cells. Single-cell cultures from HNSCC are difficult to obtain[112] and lose in the process the properties they had in establishing the heterogeneity of the primary tumour. More

importantly only cells, which were able to survive and adapt to the used culture medium, continue to grow and proliferate. Incubation of (tumour-) tissue fragments with LLME, directly after cutting (tumour-) tissue, was used to diminish the amount of TAMs in fragments. LLME was originally used to deplete MNPs from bone marrow suspensions[113].

Immunohistochemistry of tumour tissue and F-spheroids

The CD68 antigen is a specific marker for MOs and Mφs. Antibodies against this marker can be used to label Mφs and other cells of the MNP system in tissue sections[114]. F-spheroids and their original (tumour) tissue were stained with anti-CD68 after which TAMs and Mφs were manually counted using a microscope.

8. SUMMARIES

Paper 1

F-spheroids and biopsies from donor tumour tissue and benign mucosa, from 14 HNSCC patients, were paraffin embedded and cut into 4.0 mm-sections. Densities of epithelial cells, fibroblasts, Mφs and TAMs were determined by microscopy. In the second part of this study, 17 HNSCC patients were included and secretion of MCP-1, TNF-α and IL-6, from MOs in co-culture with F-spheroids, were correlated with clinical parameters such as TNM stage, donor inflammatory state and F-spheroid macrophage density. Epithelial cells were shown to be partly replaced by interstitial tissue during spheroid formation and malignant (M) F-spheroids had a higher proportion of epithelial tissue compared to benign (B) F-spheroids. Percentage of fibroblast tissue and density of Mφs and TAMs was not significantly reduced during F-spheroid formation, neither from benign mucosa nor tumour tissue. MF-spheroids were shown to secrete more MCP-1 than BF-spheroids but no F-spheroid secreted measurable amounts of TNF-α MOs secreted more IL-6 when co-cultured with MF- compared to BF-spheroids. MO-MF-spheroid production of IL –6 was correlated to TAM density. MO-MF- and MO-BF-spheroid production of MCP-1 correlated with TAM density. In addition, there was an association between MF- and BF-spheroid-stimulated MO IL-6 secretion, as well as between BF- and MF-spheroid-stimulated MCP-1 secretion. An inverse relation was noted between the erythrocyte sedimentation rate (ESR) upon MO isolation and MCP-1 production from MOs in co-culture with F-spheroids.

Paper 2

In this study, function of TAMs in HNSCC tissue was examined. In the previous study, we showed a correlation between the amount of TAMs in F-spheroids and IL-6 and MCP-1 secretion from MOs in co-culture with F-spheroids. These cytokines influence tumour cell growth and Mφ influx in tumours, respectively. Furthermore, Mφs secrete copious amounts of IL-6. We wanted to study the function of TAMs in HNSCC tissue, as expressed by cytokine secretion. LLME eradicated MOs from ***in vitro*** cultures and reduced IL-6 and MCP-1 secretion from MO-derived macrophages ***in vitro*** but did not affect viability of F-spheroids. F-spheroids from LLME-treated tissue fragments, both from HNSCC and benign mucosa, secreted less IL-6 and MCP-1 secretion as compared to untreated F-spheroids. Production of IL-6 and MCP-1 from F-spheroids can be down-regulated by using LLME, thus, suggesting that TAMs are the main producers of IL-6 and MCP-1 in HNSCC tissue. We did not find a qualitative difference between function of TAMs and tissue Mφs in mucosa as measured by IL-6 and MCP-1 secretion.

Paper 3

We studied whether MO function in HNSCC patients, as measured by LPS-stimulated MO-derived IL-6 and MCP-1 secretion, correlated to prognosis and stage of HNSCC disease. MO function, as expressed by MCP-1 and IL-6 secretion, was then compared to a control group of healthy patients.

A prospective study was performed evaluating at least a five-year follow-up of patients treated for HNSCC disease in relation to their MO function. Sixty-five newly diagnosed HNSCC patients and eighteen controls were studied. Production of IL-6 and MCP-1 was measured. Survival, and cause of death was determined.

A significantly changed LPS-induced inhibition of MCP-1 secretion from MOs in cancer patients compared to controls was observed when cultures were supplied with serum-free medium, but not when cultures were supplied with autologous serum. Production of MCP-1 by MOs stimulated with LPS was reduced in patients with HNSCC. No difference was observed when MO function was compared in patients with limited versus extended HNSCC disease. Increased MO responsiveness was associated with decreased survival rate both in general and disease specific. The predictive value of MO responsiveness, as measured by IL-6, was also retained when adjusting for age, gender and disease stage, and to some extent ESR and the albumin level of patients, when measured by multivariate Cox regression survival analysis.

We have shown that MO function, as measured by LPS-induced MO secretion of IL-6 and MCP-1, was changed in HNSCC patients compared to controls and MO function can predict outcome in HNSCC patients. A high

LPS-induced MO IL-6 secretion correlates to worsened prognosis independent of staging. Thus, MO function is directly associated with HNSCC biology.

Paper 4

We studied a possible correlation between production of IL-6 and MCP-1 by MOs in co-culture with F-spheroids and prognosis of disease. In the previous study, we showed that IL-6 production in LPS-stimulated MOs indeed was associated with a worsened prognosis. This study did take into account the influence of the tumour stroma, in addition to MOs, thereby, mimicking a more accurate ***in vivo*** situation.

Analysis of the IL-6 and MCP-1 content in supernatants from MOs co-cultured with autologous MF- or BF-spheroids, in a cohort of HNSCC patients, was performed. Recurrence, survival and cause(s) of death were then established following the second part of 2005. MCP-1 levels did not predict prognosis. IL-6 secreted upon ***in vitro*** co-culture with MOs and BF-spheroids predicted recurrence and prognosis, whereas co-culture with MOs and MF-spheroids predicted only recurrence.

9. GENERAL DISCUSSION

Our goal in this thesis was to study MNP function in HNSCC. One of the main challenges in cancer research is interpreting laboratory study results and applying them to the clinic. An important goal is therefore to establish an experimental setting most closely mimicking the ***in vivo*** situation. This discussion comprises of MO function in PB and an organ culture model, as well as correlations to clinical parameters, as found in the studied HNSCC patients.

9.1 Methodological aspects

9.1.1 Use of the organ culture model

HNSCC tumours develop as a network with tumour stroma, neo-angiogenesis, fibroblasts and an influx of immune cells, mainly lymphocytes and TAMs.

An ideal study model of MNP-tumour cell interaction would be to study ***in vitro*** direct contact between individual tumour cells, and circulating- and tumour-infiltrating autologous MNPs. Co-culture of F-spheroids with MNPs might represent a model of the ***in vivo*** situation as a closing-up step. The difference between the ***in vivo*** and ***in vitro*** conditions must, however, be recognized when interpreting results of the co-culture studies. F-spheroids were established from HNSCC patients and not every attempt to generate F-spheroids was successful, causing exclusion of the patient(s) from the study. Results from spheroids in co-culture with autologous MOs and spheroid autocrine production could provide a more reliable basis for conclusions compared to cell lines, which can be established from few HNSCC patients or

from a single, surviving tumour cell. A complicating factor, in interpreting results derived from spheroids, is that a spheroid is a heterogeneous cluster of cells with inter- and intra-patient variations. We tried to eliminate some of these variations by clustering 4 to 6 spheroids per well in parallel. Furthermore, IL-6 results were always compared, after performing a standardization analysis, to background IL-6 production. In this manner, IL-6 results could be compared between patients. Such a standardization analysis was, though, not found feasible for MCP-1 production in co-culture. MCP-1 is consecutively produced by MOs when cultured *in vitro* with serum present and stimulation with LPS caused a down-regulation in its production. A standardization analysis, such as the one used for IL-6, would yield a negative MCP-1 production. For this reasons we used the absolute production of MCP-1 to study co-culture results. We could not detect a significant TNF-α production from F-spheroids, making standardization analysis obsolete.

9.1.2 Serum and Monocytes

Autologous serum was used for the co-culture studies. There might be a general factor that could account for the difference(s) in the functional properties of MOs seen between cancer and control patients. In previous studies from our group though, we showed that MO-F-spheroid co-culture response is also present under serum-free conditions, interpreting that the basic interactions between MOs and F-spheroids are serum-independent. What kind of serum factor(s) accounts for adjuvant activation of MOs in co-culture with F-spheroids should be a target for further research. MOs isolated by adherence to plastic, as studied in this thesis, are presumably primed

compared to the ***in vivo*** condition[115]. In our studies, we used the same MO isolation method for both groups of spheroids as well as in our case-control study (paper IV). The relative differences should be minimal in this way. Interpretations though, should be made with these reservations in mind. We have not excluded that the observed co-culture-induced MO responses are dependent on adherence-induced priming. Additional research is necessary to clarify this question. To investigate this further one might apply low-adherence wells. In co-culture studies MOs were isolated 2-3 weeks following surgery. At this time most patients had recovered from surgery, but still needed hospitalization. Thus, MOs may have functional changes due to performed surgery. Surgical stress, however, is likely to affect MO-derived cytokine secretion, only in the first few days after surgery[116]. Since many patients were still unfit to leave the hospital, it might implicate that MOs from these patients are still changed compared to MOs from patients at baseline conditions. This observation should be taken in account when interpreting co-culture responses.

9.1.3 Cytokines

Discrepancies have been documented in the absolute levels of cytokines estimated from samples of biological fluids analyzed by commercially available enzyme-linked immunosorbent assay (ELISA) kits[117]. In order to account for this variability in cytokine analyses, assays were performed on the largest possible series, e.g., all samples from one experiment were analyzed simultaneously. In addition, comparisons were not made between cytokine levels analyzed by different kits, unless acceptable control conditions were

established. The MO stimulatory capacity varied considerably between spheroids. Therefore, several F-spheroids were used in each co-culture well in order to minimize this heterogeneity. With this in mind, conclusions were not based on small quantitative differences as to MO-co-culture stimulated activation unless proper control conditions were established, e.g., MO co-culture responses were compared in cultures comprising the same F-spheroids at different experimental conditions.

9.2 Composition of F-SPHEROIDS

F-spheroids mainly harbour three cell types: Mφs, fibroblasts and epithelial (tumour) cells. In study I, we studied the amount of TAMs in F-spheroids and the original tumour tissue. We showed that epithelial cells are replaced with interstitial tissue during F-spheroid formation and that a higher percentage of epithelial cells are present in Malignant (M)F-spheroids compared to Benign (B)F-spheroids. Epithelial coverage in BF-spheroids was more extensive than in MF-spheroids.

Mφ density was not significantly different between MF-spheroids and the original tumour tissue and we did not find a significant difference between MF- and BF-spheroids. Therefore, in the second part of the study we studied densities of TAM in MF-spheroids. This was done by counting CD68-positive cells in slices of MF-spheroids, from different patients, used after co-culture with MOs. This TAM density was correlated to MCP-1 and IL-6 production of MOs in co-culture with F-spheroids and significant correlations were found: TAM density in MF-spheroids correlated to MO-BF-spheroid MCP-1 production and MO-MF-spheroid IL-6 production.

TAM density in MF- and BF-spheroids is not significantly different, i.e. a higher TAM content provided for a higher production of MCP-1 (BF-spheroids) and IL-6 (MF-spheroids) from MOs in co-culture with spheroids. This suggests that TAMs are in equilibrium with other MNP compartments in HNSCC patients. In other words, enhanced cytokine and chemokine production by MOs reflects the presence of more TAMs in spheroids. Indeed, one cannot conclude that TAMs and Mφs are phenotypically identical since the microenvironments they subsequently differentiated from are very different, but MCP-1 and IL-6 production from MOs isolated from PB is linked to TAM density in MF-spheroids, making it apparent that TAM infiltration is correlated with, at least, the circulating compartment of MNP. Thus, HNSCC seems to induce changes within patients beyond the actual tumour site.

Fibroblasts from benign tissue, originating from HNSCC patients, may be the sole stimulators for the production of IL-6 in co-culture, but we have previously ruled this out[111]. It has, however, been suggested that tumour-associated fibroblasts, but not un-associated fibroblasts, may stimulate MOs to aggregate in breast cancer tumours[118]. We performed co-cultures with MF-spheroids parallel to BF-spheroids and found that IL-6 and MCP-1 production was correlated, i.e. a higher production of IL-6 and MCP-1 from MOs in co-culture with BF-spheroids also showed a higher production of MCP-1 and IL-6 from MOs in MF-spheroids and vice versa. This gives arguments against tumour-associated fibroblasts playing a significant role in HNSCC.

9.3 HNSCC-derived factors affect MNPs

MCP-1 production by MOs in co-culture with F-spheroids, as well as TAM density, was inversely correlated with ESR level both at diagnosis and co-culture. In other words, presence of "general" inflammation suppresses MNP functions, as expressed by MCP-1 production. Thus, MNP function in HNSCC patients seems to be more of a functional entity related to the inflammatory state of the organism than previously recognized. Studies on the molecular counterparts of the shown interactions, such as density of lectins/lectin receptors[119, 120], density of integrins/integrin receptors[119, 120] and additional receptors, could shed further light on the MNP function in HNSCC patients. We could not define a statistical significant correlation between TNM stage and TAM density. There was a trend showing a greater density of Mφs in MF-spheroids from more advanced TNM staged tumours. Though not statistically significant, these results correspond to other reports correlating TAM density and function to prognosis in cancer patients[121] as well as a strong association between Mφ content, N stage and lymph node metastasis in oral SCC tumours[69]. F-spheroids do not secrete detectable amounts of TNF-α which strengthens the view that TAMs have a tumour supportive function in an established tumour[48]. From our results though, it is questionable to reach such a conclusion as we did not study secretion of several other potential cytotoxic cytokines, e.g., TNF-related apoptosis-inducing ligand (TRAIL) and TNF-like weak inducer of apoptosis (TWEAK)[122, 123]. These aspects should be examined in future studies. A thorough understanding of these mechanisms lends promise to future cancer therapy based on modulating interactions between tumour cells and the MNP system.

We could not determine a simple linear relationship between TAM and co-culture secretion of MCP-1/IL-6. MO-MF-spheroids IL-6 production was significantly higher than MO-BF-spheroid IL-6 production. This was not the case for the MO-derived MCP-1 production. So, MO-co-culture IL-6 response might not reflect the percentage of epithelial cells on the surface, but more likely total epithelial content of the F-spheroids. This might provide an explanation for the significantly higher production of MCP-1 by MF-spheroids compared to BF-spheroids. Production of MCP-1 by MF-spheroids was correlated to MO-MF-spheroids IL-6 production. Furthermore, IL-6 production by BF-spheroids correlated with MCP-1 production by MOs in co-culture with MF-spheroids. Thus, spheroid derived MCP-1 and IL-6 have an autocrine effect also in co-culture suggesting that HNSCC tissue activates MOs isolated from PB, separate from a general co-culture "activation".

9.4 Effect of L-Leucine-Methylester on TAMs

LLME is a substance that selectively inhibits MNP function through formation of free leucine, which causes lysis of intracellular lysosomes. It has been shown that LLME eliminate MOs and NK cells[124, 125]. Our main goal in using LLME was to determine the main source for IL-6 and MCP-1 secretion by F-spheroids. Incubation with LLME did not cause significant epithelial cell death in F-spheroids, as determined by a LIVE/DEAD kit, and did not eradicate cell proliferation, as shown by BrDU staining. On the other hand, both MF- and BF-spheroids from HNSCC patients treated with LLME showed reduced secretion levels of IL-6 and MCP-1. These results suggest that LLME can reduce tissue Mφs and TAM function as measured by IL-6 and MCP-1

secretion while surface cells on F-spheroids remain vital. The co-culture response did not change following LLME treatment of F-spheroids, which further supports that F-spheroids are viable following LLME incubation.

The main disadvantage in combining an organ-culture model with LLME lies in the fact that the spheroid is a heterogeneous structure composed of Mφs, fibroblasts, epithelial cells and interstitial tissue. Incubation of spheroids with LLME did show an impaired IL-6 and MCP-1 secretion but we did not succeed at stopping it altogether. One reason might be that LLME cannot be transported throughout the whole spheroid, thereby unaffecting TAMs and Mφs at the centre of the spheroid. Furthermore, it might be that LLME needs to be used at a higher concentration. In our experiments though, we found a conflict with the osmolality of the LLME solutions used for incubation as being too high when using concentrations higher than 30 mM.

Malignant cells as well as other cells, e.g. TIL, are also able to produce IL-6 and MCP-1 and may be responsible for production of IL-6 and MCP-1 in F-spheroids after treatment with LLME.

9.4.1 Use of LLME in studies

For HNSCC as for most other studies concerning TAM accumulation in malignant tumours, expression and secretion of cytokines in tumour tissue has been determined by the use of immunohistochemical methods and/or cell lines. Incubation of tumour tissue with LLME, before formation of F-spheroids, might be an important TAM research tool as to investigating dynamics of ***in vitro*** cytokine and chemokine secretion by TAMs.

In the last years, much focus has been drawn to studying tumour biology through microarray technology[126]. Such studies, however, mostly use whole tumour tissue. Thus, no conclusions can be reached as to whether the shown differences reflect any changes in tumour cells or various support cells, of which TAMs might be an example. Incubating tissue with LLME before processing could exclude a complicating factor and add to identifying which of the changes in mRNA construct levels actually reflect tumour cell changes.

9.5 Monocyte IL-6 production as a prognostic factor

In study III, we found that IL-6 secretion by LPS-stimulated MOs from HNSCC patients predicted prognosis. In study IV, correlation was found between amount of IL-6 produced in co-culture of MOs with F-spheroids and recurrence of disease, as well as prognosis of patients. For MCP-1 though, we could not find any major correlation as to recurrence and prognosis. This general, predictive, value of IL-6 secretion from MOs stimulated with different stimuli, such as LPS and co-culture, is interesting as well as intriguing. In LPS-stimulated MO cultures the main source of IL-6 is the MO as low levels of IL-6 in serum minimally contribute to the total amount. Still, changes in cytokine levels in serum may have input on the regulatory effects of MO when stimulated under ***in vitro*** and autologous conditions. Still, we observed no difference between autologous and serum-free culture conditions in terms of the predictive role of MO-derived IL-6 secretion.

There are parallel observations as to MO function independent of whether MOs were stimulated before any treatment (paper III) or when patients were recovering from surgical treatment (paper IV). It seems that increased MO

responsiveness, as measured by IL-6 secretion, is a negative prognostic factor both with LPS and co-culture stimulation.

MOs in HNSCC patients might be functionally changed due to several factors related to presence of malignant disease. Cytokines and growth factors, produced in or around the vicinity of the tumour, could not only affect the immune system locally but also generally, i.e. by affecting monoblast development in the bone marrow. Such an MO priming effect seems to be more qualitative than quantitative, i.e. non- linear to the tumour burden. We actually found that the MO-derived IL-6 predictive value was still valid after adjusting for gender, age, TNM stage, albumin and ESR levels. These findings strongly indicate that MOs in HNSCC patients are functionally changed as a consequence of disease and the influence of other factors, such as level of tumour burden and inflammation, are secondary to this effect. We, therefore, concluded paper III with the assumption that MO functional change is connected to HNSCC biology.

It could be suggested that functional changes observed in MOs reflect a resistance to an inflammatory response due to surgical treatment, which might explain the observed correlation between increased MO responsiveness in BF co-culture and impaired prognosis. Still, although an increased MO responsiveness could possibly also result in an increased co-culture response, the observed MO-BF-spheroid IL-6 production cannot simply be explained by such an effect, as co-culture responses were corrected by the functional maximal LPS response obtained through the RR equation. Furthermore, the co-culture experimental situation is more complex in comparison to ordinary MO cultures, due to the presence of several other

candidate cells, e.g., fibroblasts, tumour cells, TAMs in MF-spheroids, benign mucosal cells, Mφs in BF-spheroids or factors, e.g., autologous serum, being responsible for cytokine production. We have previously shown that the MO activation in co-culture experiments is dependent on both direct contact and on products derived from the spheroids[111]. In paper I, we found that MCP-1 levels were higher in MF compared to BF cultures. It is, therefore, reasonable to propose that MOs are activated or regulated differently in MF and BF co-cultures due to different levels of other stimulatory/regulatory agents. In co-cultures, MOs have contact with epithelial elements on spheroid surfaces. As shown in paper I, there are differences also in the amount of epithelial elements between BF and MF. These differences are clearly shown to have clinical relevance. When correlating co-culture responses with survival, MO-BF-spheroid IL-6, but not MO-MF-spheroid IL-6 production, predicted patient survival. A factor analysis supported the observation that the MO-MF-spheroid IL-6 response was associated with recurrence while a MO-BF-spheroid IL-6 response associated with cancer-related survival. Further studies need to be performed in order to reveal which factor(s) present in BF-spheroids and MF-spheroids are responsible for this correlation in survival and recurrence. We did not find a significant correlation between IL-6 secretion and tumour (T) or node (N) stage in this study. This might indicate that MNP function is changed at an early stadium of HNSCC disease, though this should be interpreted with limitations since all included patients were treated with surgery and most of them had additionally at least a T2 stage disease.

Benign as well as malignant co-culture responses were correlated with local-regional recurrence. Particularly, production of IL-6 from BF-spheroids is

intriguing since these should not comprise of tumour cells. Our study shows that benign squamous tissue from HNSCC patients may, in fact, give an indication as to prognosis, making it a valuable tool in follow-up studies. Our research up to this point cannot provide answers as to when this increase in IL-6 secretion occurs. To study this, a case/control prospective study should be performed. Our hypothesis is that this might be a display of field cancerization whereby changed dysplastic epithelia is found throughout mucosa of both the upper and lower respiratory tracts, as well as to some extent in the oesophagus, which in turn may give rise to cancer at multiple sites [127]. An elevated production of IL-6 from BF-spheroids could be linked with dysplastic-transformed epithelia, which stimulates MOs, as shown by an elevated BF-spheroid co-culture response in patients who perished (from cancer disease) during follow-up. Alternatively, it may a characteristic of MOs that is associated with prognosis under BF-co-culture condition. Such an explanation is in line with published studies both as to serum values of pro-inflammatory cytokines[126] and secretion of pro-inflammatory cytokines by immune cells[129].

9.6 Inflammation and cytokine production

The present thesis presents IL-6 as a negative prognostic factor. It cannot be determined whether this observation is related to the effect of IL-6 *per se* or the increased IL-6 MO responsiveness, as a result of increased inflammatory response in diseased patients. A changed inflammatory state may be present in HNSCC patients, as shown by, e.g., increased ESR and lowered albumin values in serum[130]. Still, a pro-inflammatory cytokine, such as IL-6, could

possibly enhance the inflammatory response towards the tumour in a way that could enhance tumour aggressiveness. IL-6 may activate NF-kB, a nuclear regulator in the expression of multiple genes in cells, thereby, making a possible link between infection, inflammation and carcinogens to cancer development [58]. The observed significantly higher production of IL-6 by MOs in patients with recurrence and/or death from disease might also be linked to an IL-6/glycoprotein130 stimulation, which activates STAT3 in an EGFR-independent manner[87]. STAT3 activation is associated with cell proliferation and prevention of apoptosis, thereby participating in oncogenesis [88] and has been shown to be up-regulated in HNSCC tissue and in the benign mucosa of the same patient[89]. It appears that there is a threshold level in the benign co-culture level of IL-6 secretion that is closely associated with recurrence and survival. Above this threshold, 6 out of 8 patients had recurrence and subsequently died. No surviving patient had a MO-BF IL-6 production above this threshold. Thus, the results of this response may provide us with the ability of sorting out patients posing a higher high risk of dying from disease at a later point in time. These patients could provide targets for a very cost-efficient follow-up.

10. FUTURE PERSPECTIVES

10.1 Perspectives on clinical use of results

Several studies have been performed where MNPs have been used as an adjuvant tool in treatment of HNSCC[131, 132]. Many of these studies are Phase I and II studies particularly using DCs as an adjuvant to surgery, radiotherapy and chemotherapy[132]. Several clinical studies have also focused on the use of NK cells as a form of immunotherapy where endogenous NK cells are activated by infusions of cytokines or allogeneic cells are adoptively transferred to a donor with cancer[40]. Unfortunately though, no reports have yet been published validating the use of this kind of immunotherapies as adjuvant therapy.

TAM in tumours might promote a type II stage induced in part by tumour hypoxia. In this way, TAM promotes tumour growth by inducing neo-angiogenesis. In this view, strategies could be developed to prevent TAMs from differentiating into a type 2 phenotype, thereby, inhibiting neo-angiogenesis. On the other hand, TAMs might be used as a vehicle for delivering anti-tumour "substances" to specific areas prone to hypoxia where agents, such as chemotherapeutical drugs, are less potent due to lack of blood vessels. One might also inhibit the function of TAMs themselves, e.g., cytokine secretion. IL-6 might be one of the cytokines that might be targeted in the treatment of cancer in general. Monoclonal recombinant antibodies against circulating IL-6 and the IL-6 receptor (IL-6R) have been successfully applied in the treatment of juvenile rheumatoid arthritis and Castleman disease[133]. Other strategies might be directed towards the IL-6/gp130 autocrine/paracrine mechanism that activates STAT3. Several substances are

know to inhibit IL-6-induced activation of STAT3[133], most notably steroids which are potent inhibitors of IL-6 production. LLME is a substance that reduces TAMs in F-spheroids. As a high number of TAMs is associated with poor prognosis[68, 69], use of LLME as a TAM reducer *in vivo* is, at this stage, not possible due to the lack of its use in animal-model experiments.

10.2 Research possibilities

Many interesting studies can be performed in order to clarify the interaction between MOs and HNSCC tissue. A thorough evaluation of chemokines and cytokines from supernatants of BF- and MF-spheroids might be a goal for further studies. Three CC chemokines are mainly involved in the recruitment of mononuclear cells: MCP-1, macrophage inflammatory protein-1α (MIP-1α), and macrophage inflammatory protein-1β (MIP-1β). These chemokines induce influx of neutrophils, Mφs, NK cells, and T cells in tumors. MIP-1α has been implicated in anti-tumor activity in several mouse models involving solid tumors[134, 135]. Several studies agree on TAMs having a tumour supporting role in some tumours[65]. Still a regulation mechanism for cytokine production from TAMs in HNSCC tissue is not clarified.

F-spheroids have been derived from HNSCC tissue but also from human respiratory mucosa[110]. We also performed experiments using F-spheroids developed from other tumours (results not shown) making this organ-culture model feasible also for other tumours besides HNSCC. It might be very interesting to determine whether the found mechanisms in this study can be translated to other carcinomas, such as rectal or cervix carcinomas. In particular, we could not find a significant correlation in the MO-MF IL-6

production and tumour staging. As interactions between MNPs and HNSCC are not completely disclosed, one might ponder on how the enhanced IL-6 production of MOs in HNSCC patients can be used in a clinical setting in addition to being a follow-up tool, as mentioned above. Many cells produce IL-6 and as such it is important to determine the nature of the main stimulus responsible for this raised IL-6 production by MNPs. This is an interesting and important line of exploration in future studies.

11. CONCLUSIONS

- IL-6 production by MOs isolated from PB and stimulated with LPS is a prognostic factor for survival.

- IL-6 production from MOs in co-culture with BF-spheroids is a prognostic factor for survival whereas co-culture with MF-spheroids is a prognostic factor for recurrence of disease.

- F-spheroids do not secrete TNF-α supporting the hypothesis that TAMs function as a tumour-supporting cell.

- LLME can be used to deplete MNPs in tumour tissue with no significant effect on vitality of the developed F-spheroids.

- TAM is a main producer of IL-6 and MCP-1 in F-spheroids.

12. REFERENCES

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. CA Cancer J Clin 2006;56(2):106-30.
2. Marur S, Forastiere AA. Head and neck cancer: changing epidemiology, diagnosis, and treatment. Mayo Clin Proc 2008;83(4):489-501.
3. Wei WI. Commentary: Head and neck carcinomas in the developing world. Bmj 2002;325(7368):827.
4. Johnson N. Tobacco use and oral cancer: a global perspective. J Dent Educ 2001;65(4):328-39.
5. McMahon S, Chen AY. Head and neck cancer. Cancer Metastasis Rev 2003;22(1):21-4.
6. Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. N Engl J Med 2001;345(26):1890-900.
7. Brockstein B, Haraf DJ, Rademaker AW, et al. Patterns of failure, prognostic factors and survival in locoregionally advanced head and neck cancer treated with concomitant chemoradiotherapy: a 9-year, 337-patient, multi-institutional experience. Ann Oncol 2004;15(8):1179-86.
8. Pignon JP, Bourhis J, Domenge C, Designe L. Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. MACH-NC Collaborative Group. Meta-Analysis of Chemotherapy on Head and Neck Cancer. Lancet 2000;355(9208):949-55.
9. Cooper JS, Pajak TF, Forastiere AA, et al. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. N Engl J Med 2004;350(19):1937-44.
10. Mork J, Glattre E. Squamous cell carcinomas of the head and neck in Norway, 1953-92: an epidemiologic study of a low-risk population. Cancer Causes Control 1998;9(1):37-48.
11. Kim ES, Kies M, Herbst RS. Novel therapeutics for head and neck cancer. Curr Opin Oncol 2002;14(3):334-42.
12. Seiwert TY, Cohen EE. State-of-the-art management of locally advanced head and neck cancer. Br J Cancer 2005;92(8):1341-8.
13. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med 2006;354(6):567-78.
14. Choong NW, Cohen EE. Epidermal growth factor receptor directed therapy in head and neck cancer. Crit Rev Oncol Hematol 2006;57(1):25-43.
15. de Visser KE, Korets LV, Coussens LM. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. Cancer Cell 2005;7(5):411-23.
16. Van Ginderachter JA, Movahedi K, Hassanzadeh Ghassabeh G, et al. Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. Immunobiology 2006;211(6-8):487-501.

17. Okamoto M, Ohe G, Oshikawa T, et al. Induction of Th1-type cytokines by lipoteichoic acid-related preparation isolated from OK-432, a penicillin-killed streptococcal agent. Immunopharmacology 2000;49(3):363-76.
18. Baran J, Baj-Krzyworzeka M, Weglarczyk K, Ruggiero I, Zembala M. Modulation of monocyte-tumour cell interactions by Mycobacterium vaccae. Cancer Immunol Immunother 2004;53(12):1127-34.
19. Ooi VE, Liu F. Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. Curr Med Chem 2000;7(7):715-29.
20. Moschos SJ, Edington HD, Land SR, et al. Neoadjuvant treatment of regional stage IIIB melanoma with high-dose interferon alfa-2b induces objective tumor regression in association with modulation of tumor infiltrating host cellular immune responses. J Clin Oncol 2006;24(19):3164-71.
21. Knecht R, Peters S, Solbach C, Baghi M, Gstottner W, Hambek M. EGFR antibody-supplemented TPE-chemotherapy. Preclinical investigations to a novel approach for head and neck cancer induction treatment. Anticancer Res 2003;23(6C):4789-95.
22. Tanaka S, Tatsuguchi A, Futagami S, et al. Monocyte chemoattractant protein 1 and macrophage cyclooxygenase 2 expression in colonic adenoma. Gut 2006;55(1):54-61.
23. Oba K, Teramukai S, Kobayashi M, Matsui T, Kodera Y, Sakamoto J. Efficacy of adjuvant immunochemotherapy with polysaccharide K for patients with curative resections of gastric cancer. Cancer Immunol Immunother 2007;56(6):905-11.
24. Sakamoto J, Teramukai S, Watanabe Y, Okayasu T, Nakazato H, Ohashi Y. Meta-analysis of adjuvant immunochemotherapy using OK-432 in patients with resected non-small-cell lung cancer. J immunother 2001: 24(3):250-6
25. Naito K, Ueda Y, Itoh T, et al. Mature dendritic cells generated from patient-derived peripheral blood monocytes in one-step culture using streptococcal preparation OK-432 exert an enhanced antigen-presenting capacity. Int J Oncol 2006;28(6):1481-9.
26. Elojeimy S, McKillop JC, El-Zawahry AM, et al. FasL gene therapy: a new therapeutic modality for head and neck cancer. Cancer Gene Ther 2006.
27. Ward RC, Kaufman HL. Targeting costimulatory pathways for tumor immunotherapy. Int Rev Immunol 2007;26(3-4):161-96.
28. Ferris RL. Progress in head and neck cancer immunotherapy: can tolerance and immune suppression be reversed? ORL J Otorhinolaryngol Relat Spec 2004;66(6):332-40.
29. Janeway CT, P; Walport, M; Shlomchik, M. Immunobiology. The immune system in health and disease. . 6th edition ed: Garland Science Publishing; 2005. 823 p.
30. Johnston RB, Jr. Current concepts: immunology. Monocytes and macrophages. N Engl J Med 1988;318(12):747-52.
31. Seljelid R. Effector functions of macrophages. Acta Med Scand Suppl 1987;715:131-8.
32. Seljelid R, Eskeland T. The biology of macrophages: I. General principles and properties. Eur J Haematol 1993;51(5):267-75.

33. Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. Science 1996;272(5258):50-3.
34. Mackaness GB. The Immunological Basis of Acquired Cellular Resistance. J Exp Med 1964;120:105-20.
35. Nathan CF, Murray HW, Wiebe ME, Rubin BY. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J Exp Med 1983;158(3):670-89.
36. Reed SG, Nathan CF, Pihl DL, et al. Recombinant granulocyte/macrophage colony-stimulating factor activates macrophages to inhibit Trypanosoma cruzi and release hydrogen peroxide. Comparison with interferon gamma. J Exp Med 1987;166(6):1734-46.
37. Hayes MP, Zoon KC. Priming of human monocytes for enhanced lipopolysaccharide responses: expression of alpha interferon, interferon regulatory factors, and tumor necrosis factor. Infect Immun 1993;61(8):3222-7.
38. Scheibenbogen C, Andreesen R. Developmental regulation of the cytokine repertoire in human macrophages: IL-1, IL-6, TNF-alpha, and M-CSF. J Leukoc Biol 1991;50(1):35-42.
39. Chow CW, Grinstein S, Rotstein OD. Signaling events in monocytes and macrophages. New Horiz 1995;3(2):342-51.
40. Ljunggren HG, Malmberg KJ. Prospects for the use of NK cells in immunotherapy of human cancer. Nat Rev Immunol 2007;7(5):329-39.
41. Zamai L, Ponti C, Mirandola P, et al. NK cells and cancer. J Immunol 2007;178(7):4011-6.
42. Moretta L, Bottino C, Pende D, Vitale M, Mingari MC, Moretta A. Human natural killer cells: Molecular mechanisms controlling NK cell activation and tumor cell lysis. Immunol Lett 2005;100(1):7-13.
43. Abbas AL, A and Pober J. Cellular and Molecular Immunology. 4Rev Ed edition (Jul 2000) ed: Saunders (W.B.) Co Ltd; 2000.
44. Kerrebijn JD, Balm AJ, Freeman JL, Dosch HM, Drexhage HA. Who is in control of the immune system in head and neck cancer? Crit Rev Oncol Hematol 1999;31(1):31-53.
45. Burnet FM. The concept of immunological surveillance. Prog Exp Tumor Res 1970;13:1-27.
46. De Boer RJ, Hogeweg P. Tumor escape from immune elimination: simplified precursor bound cytotoxicity models. J Theor Biol 1985;113(4):719-36.
47. Hellstrom I, Hellstrom KE. Cell-mediated reactivity to human tumor-type associated antigens: does it exist? J Biol Response Mod 1983;2(4):310-20.
48. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 2004;4(1):71-8.
49. Gorelik E, Gunji Y, Goldfarb RH. Interaction of tumor cells and immune system in the metastatic process. Biochem Cell Biol 1988;66(6):617-25.
50. Miller FR. Immune mechanisms in the sequential steps of metastasis. Crit Rev Oncog 1993;4(3):293-311.

51. Malmberg KJ, Ljunggren HG. Escape from immune- and nonimmune-mediated tumor surveillance. Semin Cancer Biol 2006;16(1):16-31.
52. Lamagna C, Aurrand-Lions M, Imhof BA. Dual role of macrophages in tumor growth and angiogenesis. J Leukoc Biol 2006;80(4):705-13.
53. Rakoff-Nahoum S. Why cancer and inflammation? Yale J Biol Med 2006;79(3-4):123-30.
54. Bunt SK, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. Cancer Res 2007;67(20):10019-26.
55. Hafkamp HC, Manni JJ, Speel EJ. Role of human papillomavirus in the development of head and neck squamous cell carcinomas. Acta Otolaryngol 2004;124(4):520-6.
56. Bunt SK, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. J Immunol 2006;176(1):284-90.
57. Burstein E, Fearon ER. Colitis and cancer: a tale of inflammatory cells and their cytokines. J Clin Invest 2008;118(2):464-7.
58. Hayden MS, West AP, Ghosh S. NF-kappaB and the immune response. Oncogene 2006;25(51):6758-80.
59. Whiteside TL. Immunobiology of head and neck cancer. Cancer Metastasis Rev 2005;24(1):95-105.
60. Vuk-Pavlovic S. Rebuilding immunity in cancer patients. Blood Cells Mol Dis 2007.
61. Kusmartsev S, Gabrilovich DI. Role of immature myeloid cells in mechanisms of immune evasion in cancer. Cancer Immunol Immunother 2006;55(3):237-45.
62. Gallo O, Gori AM, Attanasio M, et al. Interleukin-1 beta and interleukin-6 release by peripheral blood monocytes in head and neck cancer. Br J Cancer 1993;68(3):465-8.
63. Heimdal JH, Aarstad HJ, Klementsen B, Olofsson J. Ex vivo interleukin (IL)-1 beta, IL-6, IL-12 and tumor necrosis factor-alpha responsiveness with monocytes from patients with head and neck carcinoma. Eur Arch Otorhinolaryngol 1999;256(5):250-6.
64. Mytar B, Woloszyn M, Szatanek R, et al. Tumor cell-induced deactivation of human monocytes. J Leukoc Biol 2003;74(6):1094-101.
65. Mantovani A, Allavena P, Sica A. Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. Eur J Cancer 2004;40(11):1660-7.
66. Ueno T, Toi M, Saji H, et al. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. Clin Cancer Res 2000;6(8):3282-9.
67. Nakayama Y, Nagashima N, Minagawa N, et al. Relationships between tumor-associated macrophages and clinicopathological factors in patients with colorectal cancer. Anticancer Res 2002;22(6C):4291-6.
68. Li C, Shintani S, Terakado N, Nakashiro K, Hamakawa H. Infiltration of tumor-associated macrophages in human oral squamous cell carcinoma. Oncol Rep 2002;9(6):1219-23.

69. Marcus B, Arenberg D, Lee J, et al. Prognostic factors in oral cavity and oropharyngeal squamous cell carcinoma. Cancer 2004;101(12):2779-87.
70. Kleine-Lowinski K, Gillitzer R, Kuhne-Heid R, Rosl F. Monocyte-chemo-attractant-protein-1 (MCP-1)-gene expression in cervical intra-epithelial neoplasias and cervical carcinomas. Int J Cancer 1999;82(1):6-11.
71. Leek RD, Harris AL. Tumor-associated macrophages in breast cancer. J Mammary Gland Biol Neoplasia 2002;7(2):177-89.
72. Valkovic T, Fuckar D, Stifter S, et al. Macrophage level is not affected by monocyte chemotactic protein-1 in invasive ductal breast carcinoma. J Cancer Res Clin Oncol 2005;131(7):453-8.
73. Varney ML, Johansson SL, Singh RK. Tumour-associated macrophage infiltration, neovascularization and aggressiveness in malignant melanoma: role of monocyte chemotactic protein-1 and vascular endothelial growth factor-A. Melanoma Res 2005;15(5):417-25.
74. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. J Leukoc Biol 2004;76(3):509-13.
75. Watkins SK, Egilmez NK, Suttles J, Stout RD. IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages in vitro and in vivo. J Immunol 2007;178(3):1357-62.
76. Almand B, Resser JR, Lindman B, et al. Clinical significance of defective dendritic cell differentiation in cancer. Clin Cancer Res 2000;6(5):1755-66.
77. Shurin GV, Ferris RL, Tourkova IL, et al. Loss of new chemokine CXCL14 in tumor tissue is associated with low infiltration by dendritic cells (DC), while restoration of human CXCL14 expression in tumor cells causes attraction of DC both in vitro and in vivo. J Immunol 2005;174(9):5490-8.
78. Molling JW, Langius JA, Langendijk JA, et al. Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. J Clin Oncol 2007;25(7):862-8.
79. Yoon SR, Chung JW, Choi I. Development of natural killer cells from hematopoietic stem cells. Mol Cells 2007;24(1):1-8.
80. Heimdal JH, Aarstad HJ, Klementsen B, Olofsson J. Disease stage related in vitro responsiveness of peripheral blood T-lymphocytes in patients with head and neck carcinoma. Acta Otolaryngol 1998;118(6):887-91.
81. Vlock DR. Immunobiologic aspects of head and neck cancer. Clinical and laboratory correlates. Hematol Oncol Clin North Am 1991;5(4):797-820.
82. Chikamatsu K, Sakakura K, Whiteside TL, Furuya N. Relationships between regulatory T cells and CD8+ effector populations in patients with squamous cell carcinoma of the head and neck. Head Neck 2007;29(2):120-7.
83. Aarstad HJ, Heimdal JH, Klementsen B, Olofsson J, Ulvestad E. Presence of activated T lymphocytes in peripheral blood of head and

neck squamous cell carcinoma patients predicts impaired prognosis. Acta Otolaryngol 2006;126(12):1326-33.
84. Kontny E, Ziolkowska M, Ryzewska A, Maslinski W. Protein kinase c-dependent pathway is critical for the production of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6). Cytokine 1999;11(11):839-48.
85. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. Eur J Cancer 2005;41(16):2502-12.
86. Kamimura D, Ishihara K, Hirano T. IL-6 signal transduction and its physiological roles: the signal orchestration model. Rev Physiol Biochem Pharmacol 2003;149:1-38.
87. Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gutkind JS. Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system. Cancer Res 2003;63(11):2948-56.
88. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000;19(21):2474-88.
89. Grandis JR, Drenning SD, Zeng Q, et al. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo. Proc Natl Acad Sci U S A 2000;97(8):4227-32.
90. Kanazawa T, Nishino H, Hasegawa M, et al. Interleukin-6 directly influences proliferation and invasion potential of head and neck cancer cells. Eur Arch Otorhinolaryngol 2007;264(7):815-21.
91. Nishino H, Miyata M, Kitamura K. The effect of interleukin-6 on enhancing the invasiveness of head and neck cancer cells in vitro. Eur Arch Otorhinolaryngol 1998;255(9):468-72.
92. Taub DD, Proost P, Murphy WJ, et al. Monocyte chemotactic protein-1 (MCP-1), -2, and -3 are chemotactic for human T lymphocytes. J Clin Invest 1995;95(3):1370-6.
93. Allavena P, Bianchi G, Zhou D, et al. Induction of natural killer cell migration by monocyte chemotactic protein-1, -2 and -3. Eur J Immunol 1994;24(12):3233-6.
94. Leonard EJ, Skeel A, Yoshimura T, Rankin J. Secretion of monocyte chemoattractant protein-1 (MCP-1) by human mononuclear phagocytes. Adv Exp Med Biol 1993;351:55-64.
95. Yoshimura T, Robinson EA, Tanaka S, Appella E, Kuratsu J, Leonard EJ. Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. J Exp Med 1989;169(4):1449-59.
96. Conti I, Rollins BJ. CCL2 (monocyte chemoattractant protein-1) and cancer. Semin Cancer Biol 2004;14(3):149-54.
97. Liss C, Fekete MJ, Hasina R, Lam CD, Lingen MW. Paracrine angiogenic loop between head-and-neck squamous-cell carcinomas and macrophages. Int J Cancer 2001;93(6):781-5.
98. Huang B, Lei Z, Zhao J, et al. CCL2/CCR2 pathway mediates recruitment of myeloid suppressor cells to cancers. Cancer Lett 2007;252(1):86-92.
99. Bailey C, Negus R, Morris A, et al. Chemokine expression is associated with the accumulation of tumour associated macrophages (TAMs) and progression in human colorectal cancer. Clin Exp Metastasis 2007;24(2):121-30.

100. Bemelmans MH, van Tits LJ, Buurman WA. Tumor necrosis factor: function, release and clearance. Crit Rev Immunol 1996;16(1):1-11.
101. Bradley JR. TNF-mediated inflammatory disease. J Pathol 2008;214(2):149-60.
102. Kumar R, Herbert PE, Warrens AN. An introduction to death receptors in apoptosis. Int J Surg 2005;3(4):268-77.
103. Yao J, Mackman N, Edgington TS, Fan ST. Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF-kappaB transcription factors. J Biol Chem 1997;272(28):17795-801.
104. Chen Z, Malhotra PS, Thomas GR, et al. Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer. Clin Cancer Res 1999;5(6):1369-79.
105. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. Cancer Res 2007;67(7):2912-5.
106. Johansson M, Tan T, de Visser KE, Coussens LM. Immune cells as anti-cancer therapeutic targets and tools. J Cell Biochem 2007.
107. Boyum A. Isolation of human blood monocytes with Nycodenz, a new non-ionic iodinated gradient medium. Scand J Immunol 1983;17(5):429-36.
108. Yamanaka N, Himi T, Harabuchi Y, Hoki K, Kataura A. Soluble immune complexes and squamous cell carcinoma-related antigens in patients with head and neck cancer. Cancer 1988;62(9):1932-8.
109. Yentis SM, Gooding RP, Riches PG. The effects of IgG and immune complexes on the endotoxin-induced cytokine response. Cytokine 1994;6(3):247-54.
110. Steinsvag SK, Strand M, Berg O, Miaguchi M, Olofsson J. Human respiratory mucosa in a nonadhesive stationary organ culture system. Laryngoscope 1991;101(12 Pt 1):1323-31.
111. Heimdal J, Aarstad HJ, Olofsson J. Monocytes secrete interleukin-6 when co-cultured in vitro with benign or malignant autologous fragment spheroids from squamous cell carcinoma patients. Scand J Immunol 2000;51(3):271-8.
112. Bijman JT, Wagener DJ, Wessels JM, Elprana D, van den Broek P. In vitro proliferation of primary human head and neck squamous cell carcinomas evaluated by flow cytometry. Cancer Lett 1987;36(1):71-81.
113. Thiele DL, Kurosaka M, Lipsky PE. Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotropic agent, L-leucine methyl ester. J Immunol 1983;131(5):2282-90.
114. Warnke RA, Pulford KA, Pallesen G, et al. Diagnosis of myelomonocytic and macrophage neoplasms in routinely processed tissue biopsies with monoclonal antibody KP1. Am J Pathol 1989;135(6):1089-95.
115. Krause SW, Kreutz M, Andreesen R. Differential effects of cell adherence on LPS-stimulated cytokine production by human monocytes and macrophages. Immunobiology 1996;196(5):522-34.

116. Cabie A, Fitting C, Farkas JC, et al. Influence of surgery on in-vitro cytokine production by human monocytes. Cytokine 1992;4(6):576-80.
117. Ledur A, Fitting C, David B, Hamberger C, Cavaillon JM. Variable estimates of cytokine levels produced by commercial ELISA kits: results using international cytokine standards. J Immunol Methods 1995;186(2):171-9.
118. Silzle T, Kreutz M, Dobler MA, Brockhoff G, Knuechel R, Kunz-Schughart LA. Tumor-associated fibroblasts recruit blood monocytes into tumor tissue. Eur J Immunol 2003;33(5):1311-20.
119. Olsnes C, Heimdal JH, Kross K, Olofsson J, Aarstad HJ. Mechanisms for monocyte activation in co-culture with autologous tumor spheroids. Cell Immunol 2002;219(1):11-21.
120. Olsnes C, Heimdal JH, Kross KW, Olofsson J, Aarstad HJ. Viable head and neck tumor spheroids stimulate in vitro autologous monocyte MCP-1 secretion through soluble substances and CD14/lectin-like receptors. Eur Arch Otorhinolaryngol 2005;262(12):953-60.
121. Ohta M, Kitadai Y, Tanaka S, et al. Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas. Int J Cancer 2002;102(3):220-4.
122. Nakayama M, Kayagaki N, Yamaguchi N, Okumura K, Yagita H. Involvement of TWEAK in interferon gamma-stimulated monocyte cytotoxicity. J Exp Med 2000;192(9):1373-80.
123. Van Ophoven A, Ng CP, Patel B, Bonavida B, Belldegrun A. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) for treatment of prostate cancer: first results and review of the literature. Prostate Cancer Prostatic Dis 1999;2(5/6):227-233.
124. Pechhold K, Kabelitz D. Human peripheral blood gamma delta T cells are uniformly sensitive to destruction by the lysosomotropic agents leucine methyl ester and leucyl leucine methyl ester. Eur J Immunol 1993;23(2):562-5.
125. Thiele DL, Lipsky PE. Modulation of human natural killer cell function by L-leucine methyl ester: monocyte-dependent depletion from human peripheral blood mononuclear cells. J Immunol 1985;134(2):786-93.
126. Dysvik B, Vasstrand EN, Lovlie R, et al. Gene expression profiles of head and neck carcinomas from Sudanese and Norwegian patients reveal common biological pathways regardless of race and lifestyle. Clin Cancer Res 2006;12(4):1109-20.
127. Ha PK, Califano JA. The molecular biology of mucosal field cancerization of the head and neck. Crit Rev Oral Biol Med 2003;14(5):363-9.
128. De Schutter H, Landuyt W, Verbeken E, Goethals L, Hermans R, Nuyts S. The prognostic value of the hypoxia markers CA IX and GLUT 1 and the cytokines VEGF and IL 6 in head and neck squamous cell carcinoma treated by radiotherapy +/- chemotherapy. BMC Cancer 2005;5(1):42.
129. Pries R, Wollenberg B. Cytokines in head and neck cancer. Cytokine Growth Factor Rev 2006;17(3):141-6.

130. Riedel F, Zaiss I, Herzog D, Gotte K, Naim R, Hormann K. Serum levels of interleukin-6 in patients with primary head and neck squamous cell carcinoma. Anticancer Res 2005;25(4):2761-5.
131. Whiteside TL, Vujanovic NL, Herberman RB. Natural killer cells and tumor therapy. Curr Top Microbiol Immunol 1998;230:221-44.
132. Leibowitz MS, Nayak JV, Ferris RL. Head and neck cancer immunotherapy: clinical evaluation. Curr Oncol Rep 2008;10(2):162-9.
133. Hong DS, Angelo LS, Kurzrock R. Interleukin-6 and its receptor in cancer: implications for Translational Therapeutics. Cancer 2007;110(9):1911-28.
134. Crittenden M, Gough M, Harrington K, Olivier K, Thompson J, Vile RG. Expression of inflammatory chemokines combined with local tumor destruction enhances tumor regression and long-term immunity. Cancer Res 2003;63(17):5505-12.
135. Cittera E, Leidi M, Buracchi C, et al. The CCL3 family of chemokines and innate immunity cooperate in vivo in the eradication of an established lymphoma xenograft by rituximab. J Immunol 2007;178(10):6616-23.

13. CURRICULUM VITAE

The author of this thesis was born on the 21st of June 1971 in The Hague, the Netherlands. After finishing secondary school, he enrolled in 1989 his Medical Studies at the University of Leiden. After finishing his studies in 1997, he worked as a resident in Surgery and Orthopaedics and as a communal doctor at the Municipal Health Service (G.G.D) in Zoetermeer before starting his residency in Otolaryngology in Bergen, Norway. He became certified in Otolaryngology in 2005.

In September 2006, he enrolled in a fellowship at the Head and Neck Surgery department at Maastricht University Medical Centre in Maastricht under the supervision of Prof. Dr. B. Kremer. During this fellowship he worked 6 months at the Netherlands Cancer Institute in Amsterdam under supervision of Prof. Dr. F.J.M. Hilgers.

He currently lives in Maastricht, the Netherlands, where he is employed as Head and Neck surgeon at the Maastricht University Medical Centre.

Paper I

Acta Oto-Laryngologica, 2005; 125: 1097–1104

Taylor & Francis

ORIGINAL ARTICLE

Head and neck squamous cell carcinoma spheroid- and monocyte spheroid-stimulated IL-6 and monocyte chemotactic protein-1 secretion are related to TNM stage, inflammatory state and tumor macrophage density*

KENNETH W. KROSS, JOHN-HELGE HEIMDAL, CARLA OLSNES, JAN OLOFSSON & HANS JØRGEN AARSTAD

Department of Otolaryngology/Head and Neck Surgery, Haukeland University Hospital and Broegelmann Research Laboratory, University of Bergen, Bergen, Norway

Abstract

Conclusion. Monocyte fragment (F)-spheroid-stimulated and F-spheroid IL-6 and monocyte chemotactic protein (MCP)-1 secretion are related to inflammatory state, macrophage density and the TNM stage of patients with head and neck squamous cell carcinoma (HNSCC). *Objective.* Fragment (F)-spheroids from HNSCC patients in vitro secrete and stimulate autologous monocytes to secrete IL-6 and MCP-1. The aim of this investigation was to study this cytokine secretion in relation to other cytokines, spheroid composition and host factors. *Material and methods.* In series I ($n = 14$) the densities of epithelial cells, fibroblasts and macrophages were determined in sections from F-spheroids and donor tissue. In series II ($n = 17$) the TNM stage, donor inflammatory state, macrophage density and the secretion of F-spheroid- and monocyte F-spheroid-stimulated IL-6, MCP-1 and tumor necrosis factor (TNF)-α were determined. *Results.* Epithelial cells were partly replaced by interstitial tissue during spheroid formation. Malignant (M) F-spheroids secreted more MCP-1 than benign (B) F-spheroids. No F-spheroid secreted measurable amounts of TNF-α. Monocytes secreted more IL-6 when co-cultured with MF- compared to BF-spheroids. Monocyte IL-6 MF- and MCP-1 MB-spheroid-stimulated secretion correlated with macrophage density. In addition, there was an association between MF- and BF-spheroid-stimulated monocyte cytokine secretion, as well as between BF- and MF-spheroid-stimulated MCP-1 secretion. An inverse relation was also noted between the erythrocyte sedimentation rate at monocyte harvest and the monocyte MCP-1 F-spheroid responses.

Keywords: *Co-culture, macrophage, monocyte, neoplasms, spheroids*

Introduction

There has been growing interest concerning the interaction between the cellular immune system, i.e. natural killer (NK) cells [1] and mononuclear phagocytes (MNPs) [2], and cancer cells [3–5]. MNPs originate from bone marrow as monoblasts, which are released into the general circulation as monocytes. Monocytes then migrate into tissue-forming tissue macrophages, of which dendritic, Langerhans, microglial and Kupffer cells are examples.

Both monocytes and NK cells act as defenses against distant metastases [6]. Such a relatively efficient system may in part be responsible for the low incidence of distant metastasis in head and neck squamous cell carcinoma (HNSCC) patients, despite a substantial percentage of HNSCC patients carrying tumor cells in the general circulation [7].

One of the major functions of MNPs is to secrete cytokines [8], of which IL-6 [9,10], tumor necrosis factor (TNF)-α [11] and monocyte chemoattractant protein (MCP)-1 [12] are examples. These cytokines have diverse functions. IL-6 stimulates, for example, HNSCC tumor cell growth [13] and invasion into adjacent tissue [14]. It was originally

Correspondence: Hans J. Aarstad, Department of Otorhinolaryngology/Head and Neck Surgery, Haukeland University Hospital, NO-5021 Bergen, Norway. Tel: +47 55 972669. Fax: +47 55 972643. E-mail: hans.aarstad@ore.uib.no

* Part of this work has been presented at the 6th International Conference on Head and Neck Cancer, 7–11 August 2004, Washington D.C., USA.

(Received 27 August 2004; accepted 23 March 2005)

ISSN 0001-6489 print/ISSN 1651-2251 online © 2005 Taylor & Francis
DOI: 10.1080/00016480510038031

determined that MCP-1 recruited monocytes [15], but it has also been found, for example, to augment IL-6 secretion in monocytes [16]. TNF-α not only induces apoptosis in tumor cells but also regulates inflammation and repair [17].

MNPs have the ability to bind and, if activated, kill tumor cells [6] by secretion of, for example, TNF-α [18] when studied in vitro. Monocytes may thus destroy tumor cells in peripheral blood [19] but this interaction remains, however, largely unexplored [8]. We have studied [16] monocyte cytokine secretion upon HNSCC tumor stimulation using a model in which autologous HNSCC cells and monocytes are co-cultured in vitro. In this model, fragment (F)-spheroids are used as vectors for live autologous tumor cells. Benign tissue harvested adjacent to the tumor may be used to establish benign F-spheroids as controls in parallel cultures. The exact cellular composition of the F-spheroid, compared to the original tumor it is derived from, has not been determined so far, and nor has the composition of the malignant compared to the benign counterparts. The first aim of this investigation was to study the transition of an original tumor to a malignant F-spheroid.

Several authors [20] have shown that the general inflammatory state is associated with the monocyte function in an individual. To what extent this applies to the MNP tumor tissue compartment is not known. We therefore also aimed to investigate this by measuring the erythrocyte sedimentation rate (ESR) of patients and correlating this value with the various determined characteristics of MNP function. Furthermore, it is not known to what extent MNPs of the human body are compartmentalized, and to what extent MNPs in each compartment communicate with each other. The presence of a correlation between the various MNP compartment functions would support a functional relationship between each MNP compartment. Such analyses have therefore been performed in the present investigation.

We have previously shown [16] that monocytes secrete IL-6 and MCP-1, but not IL-1β or TNF-α, when stimulated in vitro by autologous HNSCC F-spheroids. F-spheroids themselves also secrete the same cytokines [16,21]. The actual secreting cell(s) of F-spheroids have not been determined. However, our hypothesis is that tumor-associated macrophages (TAMs) are major contributors. This view is strengthened by our previous studies [16,21], in which monocytes allowed to differentiate in vitro in co-culture with F-spheroids retained both MCP-1 and IL-6 secretion upon becoming macrophages. To gain a better insight into the role of TAMs in F-spheroids, we aimed to correlate the spheroid secretion rate of IL-6 and MCP-1 with the F-spheroid-stimulated monocyte secretion rate of these cytokines. The presence of such a correlation supports the role of TAMs in the production of IL-6 and MCP-1 by F-spheroids.

TNM stage plays an important role in the prognosis of HNSCC. We therefore correlated TAM density in F-spheroids with TNM stage.

Whether TAMs actually support or inhibit tumor cell growth is a matter of debate [19], and the role of the apoptosis-inducing cytokine TNF-α [22] in F-spheroids is unknown. Thus, we investigated whether F-spheroids secreted TNF-α.

We found it pertinent to study a series of HNSCC patients based on our co-culture model, investigate the cellular composition of both tissue and F-spheroids and determine the TNM stage and ESR of the patients. Correlation studies show that to some extent all these parameters are associated with each other.

Material and methods

Patients

The study population comprised patients hospitalized at the Department of Otolaryngology/Head & Neck Surgery, Haukeland University Hospital. All patients had HNSCC. Patients with autoimmune disease and those receiving corticosteroid medications were excluded. The patients were consecutively included into the series. The site and TNM stage of HNSCC for the series II patients are shown in Table I. The study was approved by Regional Committee for Medical Ethics of Haukeland University Hospital, with each patient giving their written consent before participating in the study. The TNM stage was determined in each of the included patients. Series I consisted of 14 patients. Series II consisted of 17 patients.

Monocyte preparation

Monocytes were isolated from peripheral blood by gradient centrifugation followed by separation based on adherence to plastic according to slight modifications of previously described methods [17,23]. Briefly, peripheral blood mononuclear cells (PBMC) were separated by gradient centrifugation using Lymphoprep® (Nycomed, Oslo, Norway) as the density gradient medium. The PBMC yield of 8.5 ml of blood was allocated to all wells in a 24-well plate (Nunc A/S, Roskilde, Denmark) containing Roswell Park Memorial Institute (RPMI)-1640 medium (BioWhittaker, Walkersville, MD) supplemented with amphotericin B (2.5 μg/ml) and glucose (both from Sigma, St. Louis, MO),

Table I. Site and TNM stage of HNSCC for the patients in series II.

Patient No.	Gender	Site	TNM stage
1	F	Gingiva	T2N0M0
2	M	Tonsil	T3N1M0
3	F	Gingiva	T4N0M0
4	M	Tongue	T2N1M0
5	M	Floor of the mouth	T4N0M0
6	M	Tonsil	T3N2M0
7	M	Gingiva	T4N1M0
8	M	Tonsil	T3N0M0
9	M	Tongue	T2N1M0
10	M	Tongue	T3N0M0
11	M	Tongue	T3N0M0
12	F	Soft palatum	T3N1M0
13	F	Tongue	T1N1M0
14	M	Larynx supraglottic	T3N1M0
15	M	Mandibula	NA
16	M	Tonsil	T3N2M0
17	M	Tongue	T2N2M0

NA = not applicable.

4-(2-hydroxyethyl)-1-piperazinethansulfonic acid, L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), sodium bicarbonate, sodium pyruvate (all from BioWhittaker) and 20% autologous serum to a total volume of 0.5 ml/well. After 40 min of pre-incubation, the adherent monocytes were purified by washing, and then cultured in complete RPMI/20% autologous serum (0.5 ml/well). Such cells were >95% monocyte-positive as determined by non-specific esterase staining and their viability was >95% as tested by tryphan blue staining.

F-spheroid generation

Biopsies were obtained at surgery from tumor or benign control mucosa and maintained viable in an organ culture as F-spheroids according to previously described procedures [16]. In brief, macroscopically vital tissue was randomly chopped from malignant tissue. Benign tissue was harvested from the epithelial part of the mucosal biopsies. The mucosal biopsies were taken at sites microscopically proven to be tumor-free. Cubes were transferred to agar-coated tissue culture flasks (Nunc A/S). The F-spheroids were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 15% heat-inactivated newborn calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and non-essential amino acid mixture (1%) (all from BioWhittaker), amphotericin (2.5 μg/ml; Sigma) and metronidazole (Norcox Pharma AS, Oslo, Norway). The cultures were maintained at 37°C in 5% CO_2 and 95% air with a relative humidity of 100%. Fragments, which had rounded after 7 days in vitro, were selected for experiments. Such F-spheroids were transferred (4–7 spheroids in each well) to agar-coated 24-well plates (Nunc A/S) and maintained until co-cultured in DMEM medium supplemented as described above. In series II, a sample from each well, containing F-spheroid-conditioned media accumulated in the last 72 h before co-culture initiation, was drawn and stored until further analysis.

Co-culture

Co-cultures were initiated when the contents of each well containing malignant (M) or benign (B) F-spheroids were transferred to freshly isolated monocyte cultures in 24-well plates. The co-cultures were incubated 24 h before the supernatant was removed and stored until later analysis. The monocytes did not detach from the plastic surface nor adhere to or infiltrate the F-spheroids during co-culture. Monocytes cultured in wells with the indicated medium and with or without the addition of 1 μg/ml lipopolysaccharide (LPS) from Escherichia coli (Sigma) served as controls. The spheroids' own cytokine secretion, as estimated by the measured cytokine secretion of F-spheroids within the last 72 h before co-culture initiation, as well as the background monocyte secretion, were subtracted from the co-culture response.

The individual IL-6 background and response level to LPS varied substantially between patients. Therefore, the IL-6 response rate (RR) was calculated according to the following formula (all secretions in picomoles/milliliter):

[co-culture secretion−background secretion (monocyte/F-spheroid)]/[LPS-stimulated secretion−background secretion (monocyte)]

From all successfully completed co-cultures, a supernatant sample was placed on Sabouraud glucose agar or blood agar plates and further incubated for 1 week at 37°C. No bacterial or fungal growth was detected.

IL-6, MCP-1 and TNF-α ELISA

The amounts of IL-6, MCP-1 and TNF-α were determined by ELISA using MCP-1, IL-6 and TNF-α capture and detection antibody pairs, compared to recombinant human (r-hu) MCP-1, IL-6 and TNF-α as standards (R&D Systems Europe Ltd, Abingdon, UK). All procedures were performed according to the manufacturer's specifications. In brief, 96-well microtiter plates (Costar Corning, Corning, NY) were coated overnight at room temperature with monoclonal mouse anti-MCP-1, IL-6 or TNF-α capture antibodies. After blocking, diluted samples and respective r-hu standards were added and incubated for 2 h at room temperature, followed by the addition of biotinylated polyclonal goat anti-human MCP-1, IL-6 or TNF-α. The plates were incubated for 20 min at room temperature with streptavidin-conjugated horseradish peroxidase. Tetramethyl-benzidine (Sigma) and H_2O_2 were used as substrates. Absorbance values were measured at 450 nm using Softmax Pro version 4.0 on an Emax Precision microtiter plate reader (Molecular Devices, Sunnyvale, CA). The sensitivity level was 15 pg/ml.

Immunohistochemistry of tumor tissue and spheroids

In series I, tumor samples and F-spheroids were formalin-fixed, paraffin-embedded, cut into 4.0-μm sections, mounted on 3-aminopropyltriethoxy-silane-coated glass (Fluka, Buchs, Switzerland) overnight at 37°C, and then re-hydrated in toluene followed by a graded alcohol series. De-paraffinized sections were either treated with 0.1% protease for 10 min at 37°C, followed by 30 min of incubation with 1% H_2O_2 in methanol, or incubated for 30 min with 1% H_2O_2 in methanol, followed by treatment in a microwave oven for 5 min at 700 W after covering them with 10 mM citrate buffer (pH 6.0). The sections were subsequently incubated with rabbit serum for 30 min, followed by 60 min of incubation with monoclonal mouse anti-human cytokeratin (DAKO, Copenhagen, Denmark), anti-CD68 (DAKO) or anti-vimentin (DAKO) antibodies. The sections were then covered with biotinylated rabbit anti-mouse IgG (DAKO) and incubated for 30 min at room temperature, followed by 30 min of incubation with avidin–biotinylated horseradish peroxidase (DAKO) and 5 min of incubation with 3,3-diaminobenzidine-tetrahydrochloride (Sigma) in PBS/0.02% H_2O_2. The sections were finally counterstained with Mayer's hematoxylin. Sections from tonsil biopsies served as positive controls. Visualization was performed on a mono-ocular microscope (Leica, Germany) where all stained cells were manually counted throughout the whole section and the section size was measured using the Qwin-link program (Leica). The cell density was calculated by dividing the number of counted cells by the measured area.

Statistics

The SPSS statistical program package was used (Version 11; SPSS, Chicago, IL). Student's *t*-test or Kendall's τ was utilized. Differences were considered significant at $p < 0.05$.

Results

Series I (n = 14)

Epithelial cells. In MF-spheroids, the proportion of epithelial tissue decreased during spheroid formation from 28% ± 6.1% to 12.9% ± 3.6% ($p < 0.05$) (Table II). The proportion of epithelial tissue was higher in MF- compared to BF-spheroids (12.9% ± 3.6% vs 4.9% ± 1.0%; $p < 0.05$; Table II). In contrast, the proportion of epithelial cell coverage was higher in BF- compared to MF-spheroids (64.1% ± 4.0% vs 36.3% ± 4.2%; $p < 0.001$; Table II).

Fibroblasts. The percentage of fibroblast tissue was not significantly reduced during spheroid formation, and nor did it differ between BF- and MF-spheroids (Table II).

TAMs. The densities of macrophages (CD68-positive cells) were determined in the primary tumor and in both BF- and MF-spheroids. The density of macrophages in tumor tissue did not change throughout formation of the MF-spheroids, and nor was there any significant difference in the percentage of macrophages between BF- and MF-spheroids (Table II). The macrophage density on the spheroid surface was similar for both BF- and MF-spheroids.

Table II. Spheroid content and surface level of epithelial (cytokeratin-positive) cells and content level of fibroblasts (vimentin-positive) and macrophages (CD68-positive cells) for the patients in series I.

Sample	Epithelial content (%)	Epithelial coverage (%)	Fibroblast content (%)	Macrophage content[a]
Tumor biopsy	28.0 ± 6.1	NA	15.9 ± 3.9	1.8 ± 0.46
Malign spheroid	12.9 ± 3.6*	36.3 ± 4.2	10.6 ± 2.2	2.0 ± 0.46
Benign spheroid	4.9 ± 1.0**	64.1 ± 4.0***	10.5 ± 1.7	1.5 ± 0.26

[a]Number of CD68-stained cells per section area (μm²).
*$p < 0.05$ vs tumor biopsy.
**$p < 0.05$ vs malign spheroid.
***$p < 0.001$ vs malign spheroid.
NA = not applicable.

Series II (n = 17)

Macrophage density. The density of macrophages was estimated only in MF-spheroids in series II. An association was determined between macrophage density and monocyte BF-spheroid MCP-1-stimulated secretion ($\tau = 0.43$; $p < 0.05$; Table III), as well as between monocyte MF-spheroid IL-6 stimulation and macrophage density ($\tau = 0.41$; $p < 0.05$; Table III). The macrophage density was inversely associated with the ESR at diagnosis ($\tau = -0.46$; $p < 0.05$; Table III). There was a trend towards a greater density of macrophages in the MF-spheroids in more advanced TNM stage tumors ($\tau = 0.34$; $p < 0.1$; Table III). Analysis suggested that this was mainly due to increased macrophage infiltration in HNSCC tumors with multiple nodal spreading.

F-spheroid IL-6, MCP-1 and TNF-α secretion. No TNF-α secretion was detected upon analysis of the supernatants from spheroid culture in vitro (data not shown).

MCP-1 was determined in 4/10 tested cases with BF-spheroids and in 10/14 tested cases with MF-spheroids (Table IV). Furthermore, Table V shows that there was an association between the BF- and MF-spheroids and which patients they originated from ($\tau = 0.66$; $p < 0.01$). In addition, a trend was determined showing a correlation between the secretion rates of IL-6 and MCP-1 from BF-spheroids ($\tau = 0.34$; $p < 0.1$; Table V).

IL-6 was secreted from BF-spheroids in 13/14 tested patients and from MF-spheroids in all 13 tested patients. IL-6 secretion did not, however, differ between BF- and MF-spheroids (Table IV).

Table IV. Correlations (Kendall's τ) between density of macrophages and various other parameters.

Parameter	F-spheroid macrophage density	*p*
pN stage	0.34	<0.1
BF-spheroid		
MCP-1	0.23	
IL-6	0.27	
MF-spheroid		
MCP-1	0.28	
IL-6	−0.10	
Monocyte benign co-culture		
MCP-1	0.43	<0.05
IL-6	0.12	
Monocyte malign co-culture		
MCP-1	0.16	
IL-6	0.41	<0.05
Pre-treatment ESR	−0.46	<0.05
Co-culture ESR	−0.21	

Monocyte co-culture IL-6 and MCP-1 responses

Monocytes stimulated by MF-spheroids secreted more IL-6, but not more MCP-1, than those stimulated by BF-spheroids (Table V). There was a positive correlation between the amount of monocyte IL-6 or MCP-1 secreted depending on whether it originated from BF- or MF-spheroid stimulation ($\tau = 0.46$; $p < 0.05$; $\tau = 0.43$; $p < 0.05$; Table VI). A positive correlation was also determined between the monocyte MF-spheroid MCP-1 response and BF-spheroid IL-6 secretion ($\tau = 0.45$; $p < 0.05$; Table VI).

Table III. Spheroid or monocyte co-culture-stimulated secretion of IL-6 and MCP-1.

	BF-spheroids	MF-spheroids	*p*[a]
Co-culture			
MCP-1 (pg/ml)	5264 ± 3210	8082 ± 3214	
IL-6 (RR)	0.18 ± 0.08	0.34 ± 0.08	<0.01
Spheroid			
MCP-1 (pg/ml)	169 ± 95	826 ± 353	<0.05
IL-6 (RR)	905 ± 326	1374 ± 316	

[a]Repeated measures *t*-test.

Table V. Correlations (Kendall's τ) between F-spheroid MCP-1 and IL-6 secretion.

	Benign		Malignant
	MCP-1	IL-6	MCP-1
Benign			
IL-6	0.34*		
Malignant			
MCP-1	0.66**	0.23	
IL-6	0.03	−0.01	0.06

*$p < 0.1$.
**$p < 0.01$.

Inflammatory state

The mean ESRs at diagnosis and co-culture were 24±4 and 50±7 mm/h, respectively. However, the C-reactive protein (CRP) level in serum was 17±5 g/l at diagnosis and 13±4 g/l when the monocytes used in co-culture experiments were harvested.

Inverse relationships were demonstrated between the ESR at diagnosis as well as at monocyte harvest and both the BF- and MF-spheroid-monocyte MCP-1 responses ($\tau = 0.45–0.49$; $p < 0.05$; Table VI).

Discussion

F-spheroids harbor mainly three types of cell: macrophages, fibroblasts and epithelial or tumor cells. The monocyte IL-6 and MCP-1 responses in co-culture are dependent on both direct contact with and products secreted from the F-spheroid [24]. In this study we have shown that epithelial tissue is replaced by interstitial tissue during F-spheroid formation, but that a higher percentage of epithelial cells are present in MF- compared to BF-spheroids. In contrast, epithelial coverage was more extensive on BF- than MF-spheroids. Furthermore, the monocyte IL-6, but not the MCP-1, response observed with MF-spheroid stimulation was higher than that with BF-spheroid stimulation. Thus, the monocyte co-culture IL-6 response does not in general reflect the percentage of epithelial cells on the surface, but in this case is more likely to reflect the total epithelial content of the F-spheroids.

Fibroblasts from benign tissue originating from HNSCC patients may be the sole stimulators for the production of IL-6 in co-culture, but we have previously ruled this out [16]. It has, however, been suggested that tumor-associated fibroblasts, but not regular fibroblasts, may stimulate monocytes to aggregate in breast cancer tumors [25]. The fact that cytokines are secreted in parallel from BF- and MF-spheroids argues against this in HNSCC.

The macrophage density was not significantly different between MF-spheroids and parental tumor tissue, and nor was there a significant difference between MF- and BF-spheroids. Therefore, in series II, the TAM content of MF-spheroids was determined.

The monocytes employed in the co-culture experiments were harvested at least 2 weeks after surgical removal of the tumor and control benign tissues. Fourteen days following surgery, monocyte function has usually recovered from the surgical trauma [26]. A general restoration of monocyte function following surgery is also supported in the present investigation by the return to baseline of CRP serum levels upon monocyte harvest. Thus, the presence of a co-culture response is not solely a consequence of surgery-induced inflammation. On the contrary, F-spheroid monocyte-stimulated MCP-1 secretion, as well as TAM density, was inversely associated with the ESR level at both diagnosis and co-culture. Thus, the presence of

Table VI. Correlations (Kendall's τ) between IL-6 and MCP-1 monocyte F-spheroid stimulation and various other parameters.

	Monocyte F-spheroid stimulation			
Parameter	MCP-1 ben	IL-6 ben	MCP-1 mal	IL-6 mal
Monocyte F-spheroid stimulation				
IL-6 ben	0.01			
MCP-1 mal	0.43*	−0.07		
IL-6 mal	0.23	0.46*	0.13	
Spheroid secretion				
MCP-1 ben	−0.02	0.23	0.02	0.29
IL-6 ben	0.31	0.23	0.45*	0.23
MCP-1 mal	−0.14	0.12	0.20	0.46*
IL-6 mal	0.16	0.03	−0.10	−0.01
Pre-treatment ESR	−0.43*	0.13	−0.21	−0.34
Co-culture ESR	−0.44*	0.32	−0.49*	−0.36

*$p < 0.05$.
ben = benign; mal = malignant.

general inflammation may actually suppress some MNP functions in HNSCC patients, the monocyte MCP-1 co-culture response being one example of this.

The fact that the macrophage density of F-spheroids is associated with the monocyte co-culture response, the similar TAM percentages of BF- and MF-spheroids and the association between MCP-1 secretion and BF- and MF-spheroids all suggest that a TAM equilibrium with the other compartments of the MNP system is found in HNSCC patients. This is also supported by evidence that the monocytes of HNSCC patients are primed compared to control conditions, as shown previously [27]. The presence of HNSCC thus seems to induce changes within the host beyond the actual site of the tumor.

An association was determined between MF-spheroid MCP-1 secretion and the monocyte co-culture IL-6-stimulated secretion. Otherwise, the magnitudes of cytokine secretion from F-spheroids and monocyte co-culture-stimulated secretion of the same cytokine did not support a strong simple linear relation between TAM density and co-culture-stimulated secretion of the same cytokine. In contrast, we showed a correlation between monocyte BF- and MF-spheroid-stimulated secretion of MCP-1/IL-6. This supports the fact that monocyte properties adjust both the BF- and MF-spheroid stimulatory capacities. This is in line with the parallel ligand–receptor interaction that was determined to be present during monocyte BF- versus MF-spheroid co-culture [28].

There was a trend towards a positive correlation between TNM stage and TAM density. This suggestion corresponds to other reports showing that TAM density and function may be related to prognosis in cancer patients [29], as well as to a recently published strong association between macrophage content and N stage and lymph node metastasis in oral SCC tumors.

The fact that F-spheroids did not secrete detectable amounts of TNF-α strengthens the view that TAMs function as support cells within an established tumor, as suggested previously. We did not, however, investigate the secretion of several other potential cytotoxic monokines, e.g. TNF-related apoptosis-inducing ligand (TRAIL) and TNF-like weak inducer of apoptosis (TWEAK).

The associations shown in this study between the macrophage density, spheroid spontaneous cytokine secretion, monocyte co-culture response, host inflammatory state and TNM stage can merely suggest a causal relationship. As the number of patients was limited, the importance of a lack of a significant correlation should not be overestimated. Furthermore, the associations found need to be confirmed by additional studies.

The findings of this study support associations between TAM density and monocyte co-culture stimulation, BF- and MF-spheroid cytokine secretion and the inflammatory state of the organism and TAM density. Furthermore, monocyte function was related to the inflammatory state of the organism. Thus, MNP function in HNSCC patients is more a functional entity that is related to the inflammatory state of the organism than previously recognized. Studies on the molecular counterparts of the shown interactions, such as the density of lectins/lectin receptors [24,28], the density of integrins/integrin receptors [24,28] and additional receptors, should shed further light on mononuclear function in HNSCC patients. If a thorough understanding of these mechanisms can be achieved it may lead to a future cancer therapy based on a modulated interaction between tumor cells and the MNP system.

Acknowledgements

We thank Dagny Ann Sandnes and Turid Tynning for excellent technical assistance, and the Medical Research Center, University of Bergen for providing some of the laboratory equipment used in this study. The work was supported by The Norwegian Cancer Society.

References

[1] Whiteside TL, Vujanovic NL, Herberman RB. Natural killer cells and tumor therapy. Curr Top Microbiol Immunol 1998; 230:221–44.
[2] Andreesen R, Hennemann B, Krause S. Adoptive immunotherapy of cancer using monocyte-derived macrophages: rationale, current status and perspectives. J Leukoc Biol 1998;64:419–26.
[3] Alexandroff AB, Robins RA, Murray A, James K. Tumour immunology: false hopes new horizons? Immunol Today 1998;19:247–50.
[4] Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420:860 7.
[5] Kerrebijn JD, Balm AJ, Freeman JL, Dosch HM, Drexhage HA. Who is in control of the immune system in head and neck cancer? Crit Rev Oncol Hematol 1999;31:31–53.
[6] Fidler IJ. Therapy of cancer metastasis by systemic activation of macrophages. Adv Pharmacol 1994;30:271–326.
[7] Wollenberg B, Ollesch A, Maag K, Funke I, Wilmes E. Micrometastases in bone marrow of patients with cancers in the head and neck area. Laryngorhinootologie 1994;73:88 93 (in German).
[8] Seljelid R. Tumour immunology: alternative perspectives. Scand J Immunol 1997;46:437–44.
[9] Barton BE. IL-6: insights into novel biological activities. Clin Immunol Immunopathol 1997;85:16–20.
[10] Horn F, Henze C, Heidrich K. Interleukin-6 signal transduction and lymphocyte function. Immunobiology 2000; 202:151–67.

[11] Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003;10:45–65.
[12] Gu L, Rutledge B, Fiorillo J, Ernst C, Grewal I, Flavell R, et al. In vivo properties of monocyte chemoattractant protein-1. J Leukoc Biol 1997;62:577–80.
[13] Hong SH, Ondrey FG, Avis IM, Chen Z, Loukinova E, Cavanaugh PF, et al. Cyclooxygenase regulates human oropharyngeal carcinomas via the proinflammatory cytokine IL-6: a general role for inflammation? FASEB J 2000;14: 1499–507.
[14] Nishino H, Miyata M, Kitamura K. The effect of interleukin-6 on enhancing the invasiveness of head and neck cancer cells in vitro. Eur Arch Otorhinolaryngol 1998;255: 468 72.
[15] Martinet N, Beck G, Bernard V, Plenat F, Vaillant P, Schooneman F, et al. Mechanism for the recruitment of macrophages to cancer site. In vivo concentration gradient of monocyte chemotactic activity. Cancer 1992;70:854–60.
[16] Heimdal J-H, Aarstad HJ, Olofsson J. Monocytes secrete interleukin-6 when co-cultured in vitro with benign or malignant autologous fragment spheroids from squamous cell carcinoma patients. Scand J Immunol 2000;51:271–8.
[17] Heimdal JH, Aarstad HJ, Aakvaag A, Olofsson J. T-lymphocyte function in head and neck cancer patients. Eur Arch Otorhinolaryngol 1997;254:318–22.
[18] Williams MA, Rhoades CJ, Newland AC, Kelsey SM. The potential for monocyte-mediated immunotherapy during infection and malignancy. Part II. In vivo activation by exogenous cytokines and clinical applications. Leuk Lymphoma 1999;34:207–30.
[19] Sica A, Saccani A, Mantovani A. Tumor-associated macrophages: a molecular perspective. Int Immunopharmacol 2002;2:1045–54.
[20] Ramadori G, Christ B. Cytokines and the hepatic acute phase response. Semin Liver Dis 1999;19(2):141–55.
[21] Heimdal J-H, Olsnes C, Olofsson J, Aarstad HJ. Macrophage secretion of mcp-1 in co-culture with autologous malignant and benign control fragment spheroids. Cancer Immunol Immunother 2001;50:300–6.
[22] Varfolomeev EE, Ashkenazi A. Tumor Necrosis factor: an apoptotic JuNKie? Cell 2004;116(49):491–7.
[23] Boyum A. Isolation of human blood monocytes with Nycodenz, a new non-ionic iodinated gradient medium. Scand J Immunol 1983;17:429–36.
[24] Olsnes C, Heimdal JH, Kross K, Olofsson J, Aarstad HJ. Mechanisms for monocyte activation in co-culture with autologous tumor spheroids. Cell Immunol 2002;219:11–21.
[25] Silzie T, Kreut M, Dobler MA, Brockhoff G, Kneuchel R, Kunz-Schughart LA. Tumor-associated fibroblasts recruit blood monocytes into tumor tissue. Eur J Immunolog 2003; 33(5):1311 20.
[26] Hensler T, Hecker H, Heeg K, Heidecke CD, Bartels H, Barthlen W, et al. Distinct mechanisms of immunosuppression as a consequence of major surgery. Infect Immun 1997; 65:2283–91.
[27] Heimdal J-H, Aarstad HJ, Klementsen B, Olofsson J. Ex vivo interleukin (IL)-1 beta, IL-6, IL-12 and tumor necrosis factor-alpha responsiveness with monocytes from patients with head and neck carcinoma. Eur Arch Otorhinolaryngol 1999;256:250–6.
[28] Olsnes C, Heimdal JH, Kross K, Olofsson J, Aarstad HJ. Autologous HNSCC tumors stimulate monocyte mcp-1-generated secretion via lectin-like receptors and CD14 epitope. Eur Arch Otorhinolaryngol 2005; In press.
[29] Ohta M, Kitadai Y, Tanaka S, Yoshihara M, Yasui W, Mukadai N, et al. Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas. Int J Cancer 2002;102:220–2.

Paper II

Acta Oto-Laryngologica, 2007; 127: 532–539

informa
healthcare

ORIGINAL ARTICLE

Tumour-associated macrophages secrete IL-6 and MCP-1 in head and neck squamous cell carcinoma tissue

KENNETH W. KROSS[1], JOHN-HELGE HEIMDAL[2], CARLA OLSNES[1], JAN OLOFSON[2], & HANS JØRGEN AARSTAD[1]

[1]*Section for Otorhinolaryngology and Head and Neck Surgery, Institute of Surgical Science, Bergen and* [2]*Department of Otolaryngology/Head and Neck Surgery, Haukeland University Hospital, Bergen, Norway*

Abstract

Conclusion. Tumour-associated macrophages (TAMs) in head and neck squamous cell carcinomas (HNSCCs) secrete interleukin 6 (IL-6) and monocyte chemotactic protein (MCP-1) that can be down-regulated by L-leucine-methylester (LLME); however, there is no qualitative difference between function of TAMs and tissue macrophages in mucosa as measured by IL-6 and MCP-1 secretion. *Objectives.* TAMs play an important role in the interaction with tumour cells in malignant tumours. The cells in the tumours that are the main sources of the various signal substances need to be further elucidated. The aim of this investigation was to reveal whether TAMs in HNSCCs secrete IL-6 and MCP-1. These cytokines influence tumour cell growth and macrophage influx in tumours, respectively. *Materials and methods.* In order to inhibit macrophage function in F-spheroids, in some experiments the tissue fragments were initially incubated with LLME, a substance that selectively inhibits function of phagocytes. IL-6 and MCP-1 secretion from untreated F-spheroids was compared to cytokine secretion from LLME-treated F-spheroids as measured by ELISA. *Results.* LLME did not affect the viability of F-spheroids and reduced IL-6 and MCP-1 secretion from monocyte-derived macrophages in vitro. F-spheroids from LLME-treated tissue fragments showed lower IL-6 and MCP-1 secretion compared with F-spheroids from tissue fragment untreated with LLME.

Keywords: *Squamous cell carcinoma, tumour-associated macrophages, cytokines*

Introduction

Monocytes are recruited by cytokine and chemokine gradients to migrate from circulation into tissues where further differentiation to macrophages is regulated by environmental signals [1]. In neoplasms, tumour-associated macrophages (TAMs) represent a major component of the leukocyte infiltrate. TAMs may play a dual role in the interaction with tumour cells [1]. A symbiotic relationship may exist between TAMs and tumour cells, thereby giving support to the tumour in its growth and metastasizing capacity. For example, it has been shown that the amount of TAMs in tumours can be associated with increased neo-angiogenesis and a worsened survival rate [2]. TAMs also have potential for cytotoxicity towards tumour cells and some reports state an improvement in prognosis in relation to high numbers of TAMs in tumours [3].

It is generally accepted that TAMs may play a regulatory role in growth of malignant tumours including head and neck squamous cell carcinomas (HNSCCs) [4]. The potential for using mononuclear phagocytes (MNPs) as therapeutic tools in HNSCC has also been discussed [5] with, in recent years, particular attention to dendritic cells (DCs) [6]. It has proved difficult to study TAMs function in vivo. TAMs could be isolated from tumours (e.g. by mechanical and enzymatic dissociation techniques), or TAMs could be studied in the tumour tissues (e.g. by in situ hybridization or immunohistochemical staining). However, these methods are encumbered with sources of error-like disturbance of cell functionality and provide only a depicted moment of a

Correspondence: Kenneth W. Kross, Department of Otolaryngology/Head and Neck Surgery, University Hospital Maastricht, PO Box 5800 Maastricht, The Netherlands. E-mail: kenneth.kross@kir.uib.no

(Received 2 May 2006; accepted 30 July 2006)

ISSN 0001-6489 print/ISSN 1651-2251 online
DOI: 10.1080/00016480600951384

From all successfully completed co-cultures, a supernatant sample was placed on Sabouraud glucose agar or blood agar plates and further incubated for 1 week at 37°C. No bacterial or fungal growth was detected.

IL-6, MCP-1 and TNF-α ELISA

The amounts of IL-6, MCP-1 and TNF-α were determined by ELISA using MCP-1, IL-6 and TNF-α capture and detection antibody pairs, compared to recombinant human (r-hu) MCP-1, IL-6 and TNF-α as standards (R&D Systems Europe Ltd, Abingdon, UK). All procedures were performed according to the manufacturer's specifications. In brief, 96-well microtiter plates (Costar Corning, Corning, NY) were coated overnight at room temperature with monoclonal mouse anti-MCP-1, IL-6 or TNF-α capture antibodies. After blocking, diluted samples and respective r-hu standards were added and incubated for 2 h at room temperature, followed by the addition of biotinylated polyclonal goat anti-human MCP-1, IL-6 or TNF-α. The plates were incubated for 20 min at room temperature with streptavidin-conjugated horseradish peroxidase. Tetramethyl-benzidine (Sigma) and H_2O_2 were used as substrates. Absorbance values were measured at 450 nm using Softmax Pro version 4.0 on an Emax Precision microtiter plate reader (Molecular Devices, Sunnyvale, CA). The sensitivity level was 15 pg/ml.

Immunohistochemistry of tumor tissue and spheroids

In series I, tumor samples and F-spheroids were formalin-fixed, paraffin-embedded, cut into 4.0-μm sections, mounted on 3-aminopropyltriethoxy-silane-coated glass (Fluka, Buchs, Switzerland) overnight at 37°C, and then re-hydrated in toluene followed by a graded alcohol series. De-paraffinized sections were either treated with 0.1% protease for 10 min at 37°C, followed by 30 min of incubation with 1% H_2O_2 in methanol, or incubated for 30 min with 1% H_2O_2 in methanol, followed by treatment in a microwave oven for 5 min at 700 W after covering them with 10 mM citrate buffer (pH 6.0). The sections were subsequently incubated with rabbit serum for 30 min, followed by 60 min of incubation with monoclonal mouse anti-human cytokeratin (DAKO, Copenhagen, Denmark), anti-CD68 (DAKO) or anti-vimentin (DAKO) antibodies. The sections were then covered with biotinylated rabbit anti-mouse IgG (DAKO) and incubated for 30 min at room temperature, followed by 30 min of incubation with avidin–biotinylated horseradish peroxidase (DAKO) and 5 min of incubation with 3,3-diaminobenzidine-tetrahydrochloride (Sigma) in PBS/0.02% H_2O_2. The sections were finally counterstained with Mayer's hematoxylin. Sections from tonsil biopsies served as positive controls. Visualization was performed on a mono-ocular microscope (Leica, Germany) where all stained cells were manually counted throughout the whole section and the section size was measured using the Qwin-link program (Leica). The cell density was calculated by dividing the number of counted cells by the measured area.

Statistics

The SPSS statistical program package was used (Version 11; SPSS, Chicago, IL). Student's t-test or Kendall's τ was utilized. Differences were considered significant at $p < 0.05$.

Results

Series I (n = 14)

Epithelial cells. In MF-spheroids, the proportion of epithelial tissue decreased during spheroid formation from 28% ± 6.1% to 12.9% ± 3.6% ($p < 0.05$) (Table II). The proportion of epithelial tissue was higher in MF- compared to BF-spheroids (12.9% ± 3.6% vs 4.9% ± 1.0%; $p < 0.05$; Table II). In contrast, the proportion of epithelial cell coverage was higher in BF- compared to MF-spheroids (64.1% ± 4.0% vs 36.3% ± 4.2%; $p < 0.001$; Table II).

Fibroblasts. The percentage of fibroblast tissue was not significantly reduced during spheroid formation, and nor did it differ between BF- and MF-spheroids (Table II).

TAMs. The densities of macrophages (CD68-positive cells) were determined in the primary tumor and in both BF- and MF-spheroids. The density of macrophages in tumor tissue did not change throughout formation of the MF-spheroids, and nor was there any significant difference in the percentage of macrophages between BF- and MF-spheroids (Table II). The macrophage density on the spheroid surface was similar for both BF- and MF-spheroids.

Table II. Spheroid content and surface level of epithelial (cytokeratin-positive) cells and content level of fibroblasts (vimentin-positive) and macrophages (CD68-positive cells) for the patients in series I.

Sample	Epithelial content (%)	Epithelial coverage (%)	Fibroblast content (%)	Macrophage content[a]
Tumor biopsy	28.0 ± 6.1	NA	15.9 ± 3.9	1.8 ± 0.46
Malign spheroid	12.9 ± 3.6*	36.3 ± 4.2	10.6 ± 2.2	2.0 ± 0.46
Benign spheroid	4.9 ± 1.0**	64.1 ± 4.0***	10.5 ± 1.7	1.5 ± 0.26

[a]Number of CD68-stained cells per section area (μm²).
*$p < 0.05$ vs tumor biopsy.
**$p < 0.05$ vs malign spheroid.
***$p < 0.001$ vs malign spheroid.
NA = not applicable.

Table IV. Correlations (Kendall's τ) between density of macrophages and various other parameters.

Parameter	F-spheroid macrophage density	p
pN stage	0.34	<0.1
BF-spheroid		
MCP-1	0.23	
IL-6	0.27	
MF-spheroid		
MCP-1	0.28	
IL-6	−0.10	
Monocyte benign co-culture		
MCP-1	0.43	<0.05
IL-6	0.12	
Monocyte malign co-culture		
MCP-1	0.16	
IL-6	0.41	<0.05
Pre-treatment ESR	−0.46	<0.05
Co-culture ESR	−0.21	

Series II (n = 17)

Macrophage density. The density of macrophages was estimated only in MF-spheroids in series II. An association was determined between macrophage density and monocyte BF-spheroid MCP-1-stimulated secretion ($\tau = 0.43$; $p < 0.05$; Table III), as well as between monocyte MF-spheroid IL-6 stimulation and macrophage density ($\tau = 0.41$; $p < 0.05$; Table III). The macrophage density was inversely associated with the ESR at diagnosis ($\tau = -0.46$; $p < 0.05$; Table III). There was a trend towards a greater density of macrophages in the MF-spheroids in more advanced TNM stage tumors ($\tau = 0.34$; $p < 0.1$; Table III). Analysis suggested that this was mainly due to increased macrophage infiltration in HNSCC tumors with multiple nodal spreading.

F-spheroid IL-6, MCP-1 and TNF-α secretion. No TNF-α secretion was detected upon analysis of the supernatants from spheroid culture in vitro (data not shown).

MCP-1 was determined in 4/10 tested cases with BF-spheroids and in 10/14 tested cases with MF-spheroids (Table IV). Furthermore, Table V shows that there was an association between the BF- and MF-spheroids and which patients they originated from ($\tau = 0.66$; $p < 0.01$). In addition, a trend was determined showing a correlation between the secretion rates of IL-6 and MCP-1 from BF-spheroids ($\tau = 0.34$; $p < 0.1$; Table V).

IL-6 was secreted from BF-spheroids in 13/14 tested patients and from MF-spheroids in all 13 tested patients. IL-6 secretion did not, however, differ between BF- and MF-spheroids (Table IV).

Monocyte co-culture IL-6 and MCP-1 responses

Monocytes stimulated by MF-spheroids secreted more IL-6, but not more MCP-1, than those stimulated by BF-spheroids (Table V). There was a positive correlation between the amount of monocyte IL-6 or MCP-1 secreted depending on whether it originated from BF- or MF-spheroid stimulation ($\tau = 0.46$; $p < 0.05$; $\tau = 0.43$; $p < 0.05$; Table VI). A positive correlation was also determined between the monocyte MF-spheroid MCP-1 response and BF-spheroid IL-6 secretion ($\tau = 0.45$; $p < 0.05$; Table VI).

Table III. Spheroid or monocyte co-culture-stimulated secretion of IL-6 and MCP-1.

	BF-spheroids	MF-spheroids	p[a]
Co-culture			
MCP-1 (pg/ml)	5264 ± 3210	8082 ± 3214	
IL-6 (RR)	0.18 ± 0.08	0.34 ± 0.08	<0.01
Spheroid			
MCP-1 (pg/ml)	169 ± 95	826 ± 353	<0.05
IL-6 (RR)	905 ± 326	1374 ± 316	

[a]Repeated measures t-test.

Table V. Correlations (Kendall's τ) between F-spheroid MCP-1 and IL-6 secretion.

	Benign		Malignant
	MCP-1	IL-6	MCP-1
Benign			
IL-6	0.34*		
Malignant			
MCP-1	0.66**	0.23	
IL-6	0.03	−0.01	0.06

*$p < 0.1$.
**$p < 0.01$.

Inflammatory state

The mean ESRs at diagnosis and co-culture were 24±4 and 50±7 mm/h, respectively. However, the C-reactive protein (CRP) level in serum was 17±5 g/l at diagnosis and 13±4 g/l when the monocytes used in co-culture experiments were harvested.

Inverse relationships were demonstrated between the ESR at diagnosis as well as at monocyte harvest and both the BF- and MF-spheroid-monocyte MCP-1 responses ($\tau = 0.45–0.49$; $p < 0.05$; Table VI).

Discussion

F-spheroids harbor mainly three types of cell: macrophages, fibroblasts and epithelial or tumor cells. The monocyte IL-6 and MCP-1 responses in co-culture are dependent on both direct contact with and products secreted from the F-spheroid [24]. In this study we have shown that epithelial tissue is replaced by interstitial tissue during F-spheroid formation, but that a higher percentage of epithelial cells are present in MF- compared to BF-spheroids. In contrast, epithelial coverage was more extensive on BF- than MF-spheroids. Furthermore, the monocyte IL-6, but not the MCP-1, response observed with MF-spheroid stimulation was higher than that with BF-spheroid stimulation. Thus, the monocyte co-culture IL-6 response does not in general reflect the percentage of epithelial cells on the surface, but in this case is more likely to reflect the total epithelial content of the F-spheroids.

Fibroblasts from benign tissue originating from HNSCC patients may be the sole stimulators for the production of IL-6 in co-culture, but we have previously ruled this out [16]. It has, however, been suggested that tumor-associated fibroblasts, but not regular fibroblasts, may stimulate monocytes to aggregate in breast cancer tumors [25]. The fact that cytokines are secreted in parallel from BF- and MF-spheroids argues against this in HNSCC.

The macrophage density was not significantly different between MF-spheroids and parental tumor tissue, and nor was there a significant difference between MF- and BF-spheroids. Therefore, in series II, the TAM content of MF-spheroids was determined.

The monocytes employed in the co-culture experiments were harvested at least 2 weeks after surgical removal of the tumor and control benign tissues. Fourteen days following surgery, monocyte function has usually recovered from the surgical trauma [26]. A general restoration of monocyte function following surgery is also supported in the present investigation by the return to baseline of CRP serum levels upon monocyte harvest. Thus, the presence of a co-culture response is not solely a consequence of surgery-induced inflammation. On the contrary, F-spheroid monocyte-stimulated MCP-1 secretion, as well as TAM density, was inversely associated with the ESR level at both diagnosis and co-culture. Thus, the presence of

Table VI. Correlations (Kendall's τ) between IL-6 and MCP-1 monocyte F-spheroid stimulation and various other parameters.

	Monocyte F-spheroid stimulation			
Parameter	MCP-1 ben	IL-6 ben	MCP-1 mal	IL-6 mal
Monocyte F-spheroid stimulation				
IL-6 ben	0.01			
MCP-1 mal	0.43*	−0.07		
IL-6 mal	0.23	0.46*	0.13	
Spheroid secretion				
MCP-1 ben	−0.02	0.23	0.02	0.29
IL-6 ben	0.31	0.23	0.45*	0.23
MCP-1 mal	−0.14	0.12	0.20	0.46*
IL-6 mal	0.16	0.03	−0.10	−0.01
Pre-treatment ESR	−0.43*	0.13	−0.21	−0.34
Co-culture ESR	−0.44*	0.32	−0.49*	−0.36

*$p < 0.05$.
ben = benign; mal = malignant.

general inflammation may actually suppress some MNP functions in HNSCC patients, the monocyte MCP-1 co-culture response being one example of this.

The fact that the macrophage density of F-spheroids is associated with the monocyte co-culture response, the similar TAM percentages of BF- and MF-spheroids and the association between MCP-1 secretion and BF- and MF-spheroids all suggest that a TAM equilibrium with the other compartments of the MNP system is found in HNSCC patients. This is also supported by evidence that the monocytes of HNSCC patients are primed compared to control conditions, as shown previously [27]. The presence of HNSCC thus seems to induce changes within the host beyond the actual site of the tumor.

An association was determined between MF-spheroid MCP-1 secretion and the monocyte co-culture IL-6-stimulated secretion. Otherwise, the magnitudes of cytokine secretion from F-spheroids and monocyte co-culture-stimulated secretion of the same cytokine did not support a strong simple linear relation between TAM density and co-culture-stimulated secretion of the same cytokine. In contrast, we showed a correlation between monocyte BF- and MF-spheroid-stimulated secretion of MCP-1/IL-6. This supports the fact that monocyte properties adjust both the BF- and MF-spheroid stimulatory capacities. This is in line with the parallel ligand–receptor interaction that was determined to be present during monocyte BF- versus MF-spheroid co-culture [28].

There was a trend towards a positive correlation between TNM stage and TAM density. This suggestion corresponds to other reports showing that TAM density and function may be related to prognosis in cancer patients [29], as well as to a recently published strong association between macrophage content and N stage and lymph node metastasis in oral SCC tumors.

The fact that F-spheroids did not secrete detectable amounts of TNF-α strengthens the view that TAMs function as support cells within an established tumor, as suggested previously. We did not, however, investigate the secretion of several other potential cytotoxic monokines, e.g. TNF-related apoptosis-inducing ligand (TRAIL) and TNF-like weak inducer of apoptosis (TWEAK).

The associations shown in this study between the macrophage density, spheroid spontaneous cytokine secretion, monocyte co-culture response, host inflammatory state and TNM stage can merely suggest a causal relationship. As the number of patients was limited, the importance of a lack of a significant correlation should not be overestimated. Furthermore, the associations found need to be confirmed by additional studies.

The findings of this study support associations between TAM density and monocyte co-culture stimulation, BF- and MF-spheroid cytokine secretion and the inflammatory state of the organism and TAM density. Furthermore, monocyte function was related to the inflammatory state of the organism. Thus, MNP function in HNSCC patients is more a functional entity that is related to the inflammatory state of the organism than previously recognized. Studies on the molecular counterparts of the shown interactions, such as the density of lectins/lectin receptors [24,28], the density of integrins/integrin receptors [24,28] and additional receptors, should shed further light on mononuclear function in HNSCC patients. If a thorough understanding of these mechanisms can be achieved it may lead to a future cancer therapy based on a modulated interaction between tumor cells and the MNP system.

Acknowledgements

We thank Dagny Ann Sandnes and Turid Tynning for excellent technical assistance, and the Medical Research Center, University of Bergen for providing some of the laboratory equipment used in this study. The work was supported by The Norwegian Cancer Society.

References

[1] Whiteside TL, Vujanovic NL, Herberman RB. Natural killer cells and tumor therapy. Curr Top Microbiol Immunol 1998; 230:221–44.
[2] Andreesen R, Hennemann B, Krause S. Adoptive immunotherapy of cancer using monocyte-derived macrophages: rationale, current status and perspectives. J Leukoc Biol 1998;64:419–26.
[3] Alexandroff AB, Robins RA, Murray A, James K. Tumour immunology: false hopes new horizons? Immunol Today 1998;19:247–50.
[4] Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420:860 7.
[5] Kerrebijn JD, Balm AJ, Freeman JL, Dosch HM, Drexhage HA. Who is in control of the immune system in head and neck cancer? Crit Rev Oncol Hematol 1999;31:31–53.
[6] Fidler IJ. Therapy of cancer metastasis by systemic activation of macrophages. Adv Pharmacol 1994;30:271–326.
[7] Wollenberg B, Ollesch A, Maag K, Funke I, Wilmes E. Micrometastases in bone marrow of patients with cancers in the head and neck area. Laryngorhinootologie 1994;73:88 93 (in German).
[8] Seljelid R. Tumour immunology: alternative perspectives. Scand J Immunol 1997;46:437–44.
[9] Barton BE. IL-6: insights into novel biological activities. Clin Immunol Immunopathol 1997;85:16–20.
[10] Horn F, Henze C, Heidrich K. Interleukin-6 signal transduction and lymphocyte function. Immunobiology 2000; 202:151–67.

[11] Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003;10:45–65.
[12] Gu L, Rutledge B, Fiorillo J, Ernst C, Grewal I, Flavell R, et al. In vivo properties of monocyte chemoattractant protein-1. J Leukoc Biol 1997;62:577–80.
[13] Hong SH, Ondrey FG, Avis IM, Chen Z, Loukinova E, Cavanaugh PF, et al. Cyclooxygenase regulates human oropharyngeal carcinomas via the proinflammatory cytokine IL-6: a general role for inflammation? FASEB J 2000;14: 1499–507.
[14] Nishino H, Miyata M, Kitamura K. The effect of interleukin-6 on enhancing the invasiveness of head and neck cancer cells in vitro. Eur Arch Otorhinolaryngol 1998;255: 468 72.
[15] Martinet N, Beck G, Bernard V, Plenat F, Vaillant P, Schooneman F, et al. Mechanism for the recruitment of macrophages to cancer site. In vivo concentration gradient of monocyte chemotactic activity. Cancer 1992;70:854–60.
[16] Heimdal J-H, Aarstad HJ, Olofsson J. Monocytes secrete interleukin-6 when co-cultured in vitro with benign or malignant autologous fragment spheroids from squamous cell carcinoma patients. Scand J Immunol 2000;51:271–8.
[17] Heimdal JH, Aarstad HJ, Aakvaag A, Olofsson J. T-lymphocyte function in head and neck cancer patients. Eur Arch Otorhinolaryngol 1997;254:318–22.
[18] Williams MA, Rhoades CJ, Newland AC, Kelsey SM. The potential for monocyte-mediated immunotherapy during infection and malignancy. Part II. In vivo activation by exogenous cytokines and clinical applications. Leuk Lymphoma 1999;34:207–30.
[19] Sica A, Saccani A, Mantovani A. Tumor-associated macrophages: a molecular perspective. Int Immunopharmacol 2002;2:1045–54.
[20] Ramadori G, Christ B. Cytokines and the hepatic acute phase response. Semin Liver Dis 1999;19(2):141–55.
[21] Heimdal J-H, Olsnes C, Olofsson J, Aarstad HJ. Macrophage secretion of mcp-1 in co-culture with autologous malignant and benign control fragment spheroids. Cancer Immunol Immunother 2001;50:300–6.
[22] Varfolomeev EE, Ashkenazi A. Tumor Necrosis factor: an apoptotic JuNKie? Cell 2004;116(49):491–7.
[23] Boyum A. Isolation of human blood monocytes with Nycodenz, a new non-ionic iodinated gradient medium. Scand J Immunol 1983;17:429–36.
[24] Olsnes C, Heimdal JH, Kross K, Olofsson J, Aarstad HJ. Mechanisms for monocyte activation in co-culture with autologous tumor spheroids. Cell Immunol 2002;219:11–21.
[25] Silzie T, Kreut M, Dobler MA, Brockhoff G, Kneuchel R, Kunz-Schughart LA. Tumor-associated fibroblasts recruit blood monocytes into tumor tissue. Eur J Immunolog 2003; 33(5):1311 20.
[26] Hensler T, Hecker H, Heeg K, Heidecke CD, Bartels H, Barthlen W, et al. Distinct mechanisms of immunosuppression as a consequence of major surgery. Infect Immun 1997; 65:2283–91.
[27] Heimdal J-H, Aarstad HJ, Klementsen B, Olofsson J. Ex vivo interleukin (IL)-1 beta, IL-6, IL-12 and tumor necrosis factor-alpha responsiveness with monocytes from patients with head and neck carcinoma. Eur Arch Otorhinolaryngol 1999;256:250–6.
[28] Olsnes C, Heimdal JH, Kross K, Olofsson J, Aarstad HJ. Autologous HNSCC tumors stimulate monocyte mcp-1-generated secretion via lectin-like receptors and CD14 epitope. Eur Arch Otorhinolaryngol 2005; In press.
[29] Ohta M, Kitadai Y, Tanaka S, Yoshihara M, Yasui W, Mukadai N, et al. Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas. Int J Cancer 2002;102:220–2.

Paper II

Acta Oto-Laryngologica, 2007; 127: 532–539

informa
healthcare

ORIGINAL ARTICLE

Tumour-associated macrophages secrete IL-6 and MCP-1 in head and neck squamous cell carcinoma tissue

KENNETH W. KROSS[1], JOHN-HELGE HEIMDAL[2], CARLA OLSNES[1], JAN OLOFSON[2], & HANS JØRGEN AARSTAD[1]

[1]*Section for Otorhinolaryngology and Head and Neck Surgery, Institute of Surgical Science, Bergen and* [2]*Department of Otolaryngology/Head and Neck Surgery, Haukeland University Hospital, Bergen, Norway*

Abstract

Conclusion. Tumour-associated macrophages (TAMs) in head and neck squamous cell carcinomas (HNSCCs) secrete interleukin 6 (IL-6) and monocyte chemotactic protein (MCP-1) that can be down-regulated by L-leucine-methylester (LLME); however, there is no qualitative difference between function of TAMs and tissue macrophages in mucosa as measured by IL-6 and MCP-1 secretion. *Objectives.* TAMs play an important role in the interaction with tumour cells in malignant tumours. The cells in the tumours that are the main sources of the various signal substances need to be further elucidated. The aim of this investigation was to reveal whether TAMs in HNSCCs secrete IL-6 and MCP-1. These cytokines influence tumour cell growth and macrophage influx in tumours, respectively. *Materials and methods.* In order to inhibit macrophage function in F-spheroids, in some experiments the tissue fragments were initially incubated with LLME, a substance that selectively inhibits function of phagocytes. IL-6 and MCP-1 secretion from untreated F-spheroids was compared to cytokine secretion from LLME-treated F-spheroids as measured by ELISA. *Results.* LLME did not affect the viability of F-spheroids and reduced IL-6 and MCP-1 secretion from monocyte-derived macrophages in vitro. F-spheroids from LLME-treated tissue fragments showed lower IL-6 and MCP-1 secretion compared with F-spheroids from tissue fragment untreated with LLME.

Keywords: *Squamous cell carcinoma, tumour-associated macrophages, cytokines*

Introduction

Monocytes are recruited by cytokine and chemokine gradients to migrate from circulation into tissues where further differentiation to macrophages is regulated by environmental signals [1]. In neoplasms, tumour-associated macrophages (TAMs) represent a major component of the leukocyte infiltrate. TAMs may play a dual role in the interaction with tumour cells [1]. A symbiotic relationship may exist between TAMs and tumour cells, thereby giving support to the tumour in its growth and metastasizing capacity. For example, it has been shown that the amount of TAMs in tumours can be associated with increased neo-angiogenesis and a worsened survival rate [2]. TAMs also have potential for cytotoxicity towards tumour cells and some reports state an improvement in prognosis in relation to high numbers of TAMs in tumours [3].

It is generally accepted that TAMs may play a regulatory role in growth of malignant tumours including head and neck squamous cell carcinomas (HNSCCs) [4]. The potential for using mononuclear phagocytes (MNPs) as therapeutic tools in HNSCC has also been discussed [5] with, in recent years, particular attention to dendritic cells (DCs) [6]. It has proved difficult to study TAMs function in vivo. TAMs could be isolated from tumours (e.g. by mechanical and enzymatic dissociation techniques), or TAMs could be studied in the tumour tissues (e.g. by in situ hybridization or immunohistochemical staining). However, these methods are encumbered with sources of error-like disturbance of cell functionality and provide only a depicted moment of a

Correspondence: Kenneth W. Kross, Department of Otolaryngology/Head and Neck Surgery, University Hospital Maastricht, PO Box 5800 Maastricht, The Netherlands. E-mail: kenneth.kross@kir.uib.no

(Received 2 May 2006; accepted 30 July 2006)

ISSN 0001-6489 print/ISSN 1651-2251 online
DOI: 10.1080/00016480600951384

constantly changing process by the two last mentioned methods. The function of TAMs should preferably be studied while the cells are still viable in situ. This can be achieved by the use of an organ culture technique. A modified organ culture technique has previously been described in which samples from HNSCC tumours are maintained viably as malignant fragment (F)-spheroids in vitro. The organ culture technique also enables the establishment of benign F-spheroids derived from benign mucosa serving as controls for the malignant F-spheroids.

The malignant F-spheroids consist mainly of epithelial cells, fibroblasts and macrophages [7]. TAM density in the F-spheroid is not significantly changed compared to the tissue that the F-spheroids originate from [8]. Malignant and benign F-spheroids secrete different levels of monocyte chemotactic protein (MCP-1) and interleukin 6 (IL-6), but not IL-1 and tumour necrosis factor (TNF)-α [8]. The production of IL-6 from HNSCCs may be important, since IL-6 co-regulates cell differentiation, survival and apoptosis [9]. The secretion of MCP-1 from HNSCCs is also of importance, as this C-C chemokine recruits MNPs to tumours. Macrophages have potential for secreting numerous products such as cytokines [10], including IL-6 and MCP-1. It is therefore pertinent to study whether TAMs are a main source of MCP-1 and IL-6 secretion within HNSCC tissue.

To address this question we decided to utilize the F-spheroid organ culture model to study whether a down-regulation of TAM activity in F-spheroids correlates with reduced production of IL-6 and MCP-1 by F-spheroids. L-leucine-methylester (LLME) has been shown to cause selective apoptosis of human MNPs and natural killer (NK) cells by lysozomal disruption [11]. LLME kills phagocytes following interaction with intracellular proteases which cleaves the molecules inside the lysosomes. Osmotic force then causes disruption of the lysosomes and subsequent cell necrosis [12,13] The specificity of this effect is based on the content of lysosomal enzymes which differentiates monocytes and macrophages from other cells. Initially we studied the effect of LLME on the overall viability of F-spheroids and on the regulation of MCP-1 and IL-6 secretion from monocytes and macrophages in vitro. Thereafter, the production of MCP-1 and IL-6 from both malignant and benign F-spheroids with and without LLME treatment was investigated. Finally, the effect of LLME treatment of F-spheroids on co-culture-induced monocyte stimulation was studied.

Materials and methods

Patients

Patients who were admitted for uvulopalatoplasty for snoring or surgery for squamous cell carcinomas of the oral cavity and hypopharynx/larynx were included in the study. The Regional Committee for Medical Ethics at Haukeland University Hospital approved the study. Each patient gave written consent before participating in the study.

Fragment spheroid generation

An organ culture model was used, by which free-floating F-spheroids were established from tumour samples or benign mucosa of HNSCC patients as described previously [14]. Biopsies were obtained at surgery. Macroscopically vital tissue was randomly chopped from malignant tissue. Benign tissue was harvested from the epithelial part of the mucosa biopsies. Cubes (0.5–1.0 mm) were transferred to agar-coated tissue culture flasks (Nunc A/S, Roskilde, Denmark).The F-spheroids were cultured in Dulbecco's MEM (BioWhittaker, Walkersville, MD, USA) supplemented with 15% heat-inactivated fetal bovine serum (FBS; Sigma, St Louis, MO, USA), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin (2.5 μg/ml), L-glutamine (2 mM) and non-essential amino acid mixture (1%; BioWhittaker). The cultures were maintained at 37°C in 5% CO_2 and 95% air with 100% relative humidity. Fragments, which had rounded to spheroid-like structures after 10–14 days in vitro, were selected for experiments.

Viability tests

Viability of the F-spheroids treated with LLME was tested by transferring a random selection of F-spheroids into 16 mm multiwells with 0.5 ml of culture medium containing 2 μm Calveni AM and 4 μm ethidium homodimer-1 (LIVE/DEAD Eukolight viability/Cytotoxicity Kit (L-3224); Molecular Probes Europe, Leiden, The Netherlands). After incubation for 30 min at room temperature (RT), F-spheroids were transferred to 16 mm wells containing PBS and examined by a scanning confocal microscope (Biorad MRC-1000, Hemel Hempstead, UK) using a krypton-argon mixed gas laser. Cells with green fluorescence were assessed as viable and cells with red fluorescence as non-viable. This method evaluated the cells on the surface.

Bromodeoxyuridine labelling

Spheroids treated with 10 mM and 30 mM LLME and spheroids from the control group were used for bromodeoxyuridine (BrdU) labelling. F-spheroids were cultured for 4 h in standard growth medium supplemented with 10 μM BrdU and 10 μM 2-deoxycytidine and subsequently fixed in 70% alcohol for 16 h and embedded in paraffin. The specimens were cut into 4 μm sections and mounted on 3-aminopropyltriethoxy-silane-coated glasses overnight at 37°C. After deparaffinization in toluene, followed by rehydration in a graded alcohol series, the sections were incubated in 0.3% H_2O_2/methanol for 20 min and then for 30 min in 2 M HCl. The sections were subsequently incubated for 45 min with horse serum at RT and then for 2 h with a 1:40 dilution of mouse anti-BrdU monoclonal IgG (Dako). Subsequently, the slides were overlaid with biotinylated rabbit anti-mouse IgG (Dako) for 30 min at RT. This was followed by incubation for 30 min with avidin-biotinylated horseradish peroxidase (HRP) and incubation for 8 min with DAB in PSB/0.02% H_2O_2. Mayers' haematoxylin was used as a counterstain.

Monocyte preparation

The peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation with Lymphoprep^R (Nycomed, Oslo, Norway) as density gradient medium as described previously [15,16]. The PBMC yield of 8.5 ml blood was allocated to all wells in a 24 well plate (Nunc) with RPMI-1640 supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin (2.5 μg/ml), L-glutamine (2 mM) and 20% autologous serum to a total volume of 0.5 ml/well. After pre-incubation for 40 min, the monocytes (MOs) were purified by washing, and then cultured in RPMI/20% autologous serum 0.5 ml/well. The cells were shown to be more than 95% MO-positive by non-specific esterase stain.

LLME function was controlled by incubating purified MOs with 30 mM LLME for 90 min followed by stimulation with lipopolysaccharide (LPS; Sigma) at a concentration of 1 μg/ml. Non-treated MOs were used as control. Supernatants were sampled after 24 h and assessed for production of IL-6 and MCP-1 as described below.

Macrophage preparation

MOs were incubated for 7 days in RPMI + 20% AB serum in vitro and showed changes in morphology as measured by size and phagocyte vacuole formation as well as elongation of cytoplasm protrusions, indicating a differentiation towards monocyte-derived macrophages (MDMs).

AFTER 7 days half of the MDM cultures were supplied with 30 mM LLME for 90 min. All MDM cultures were then washed three times with RPMI and incubated with 1 μg/ml LPS (Sigma) from Escherichia coli for 24 h, with some cultures left not stimulated as controls. Supernatants were harvested and stored at −80°C until further analysis.

Co-culture

Malignant or benign F-spheroids were washed and transferred to freshly isolated MO cultures in 24 × 16 mm well plates (Nunc) with 1 ml RPMI/20% autologous serum (BioWhittaker) per well. MOs cultured in wells with the indicated medium with or without addition of 1 μg/ml LPS from E. coli served as controls. Five to six spheroids were allocated to each well.

LLME treatment of F-spheroids

In some experiments half of the freshly chopped tissue cubes, either from mucosa or HNSCCs, were treated with 30 mM LLME in the culture medium described above and incubated for 90 min, concluding with three washes in DEMEM. The tissue cubes were thereafter cultured as described in parallel cultures to tissue cubes that were not treated with LLME. Medium was collected every 24 h and assessed for IL-6 and MCP-1 concentration.

IL-6 and MCP-1 ELISA

The contents of IL-6 and MCP-1 were determined by ELISA using MCP-1/IL-6 capture and detection antibody pairs, compared to r-hu MCP-1/IL-6 as standards (R&D Systems Europe, Abingdon, UK). All procedures were performed according to the specifications of the manufacturer. In short, 96-well microtitration plates (Costar Corning, NY, USA) were coated overnight at RT with monoclonal mouse anti-human MCP-1 or IL-6 capture antibodies, respectively. After blocking, diluted samples and respective recombinant human standards were added and incubated for 2 h at RT followed by addition of biotinylated polyclonal goat anti-human MCP-1 or IL-6, respectively. The plates were incubated for 20 min at RT with streptavidin-conjugated horseradish peroxidase. Tetramethyl-benzidine (TMB; Sigma) and H_2O_2 were used as substrate.

In co-cultures, the background MCP-1/IL-6 levels from unstimulated cultures were substracted from the levels of the parallel stimulated cultures when the MCP-1/IL-6 levels are shown. In some experiments MCP-1/IL-6 responses were calculated as a percentage of appropriate LPS-stimulated MO response.

Statistics

The statistical program package Statistical Package for the Social Sciences was used (version 13; SPSS, Chicago, IL, USA). The Wilcoxon signed ranks test was used and results were considered significant when $p < 0.05$.

Results

Viability of F-spheroids treated with LLME

Uvula mucosa biopsies were obtained from six patients treated with surgery for snoring. Spheroids obtained from mucosa fragments were treated either with 30 mM LLME or not treated. Nearly 100% of the spheroid surface consisted of live cells as determined by the LIVE/DEAD kit, indicating that spheroids are viable after treatment with LLME 30 mM and 14 days of culture in vitro.

To examine viability further, BrdU labelling was used as described above. Five malignant and five benign F-spheroids from a patient with HNSCC were selected for this experiment. BrdU labelling showed that some cells in the benign and several cells in the malignant F-spheroids proliferated, showing that LLME treatment does not impair cell proliferation in spheroids.

Treatment of MNPs in monolayer with LLME

MOs were incubated with 30 mM LLME with non-treated MOs as control. There was no production of IL-6 and MCP-1 as such in MOs treated with LLME (results not shown).

MDMs were treated with 30 mM LLME for 90 min and non-treated MDMs were used as a control. MDMs treated with LLME showed a significant decrease in LPS-stimulated IL-6 (Figure 1a) ($Z = -1.753$; $p = 0.08$) and MCP-1 (Figure 1b) ($Z = -2.023$; $p = 0.043$) production.

IL-6 and MCP-1 production from benign F-spheroids obtained from patients treated for snoring

The production of IL-6 and MCP-1 from F-spheroids after a 24 h period was assessed and F-spheroids treated with LLME were compared to untreated control F-spheroids from six patients. There was a decrease in the production of IL-6 (Figure 2a) ($Z = -2.021$; $p = 0.028$) and MCP-1 (Figure 2b) ($Z = -2.023$; $p = 0.012$) by F-spheroids treated with LLME as compared with the control spheroids. This difference in cytokine production was sustained during 7 days of medium sampling.

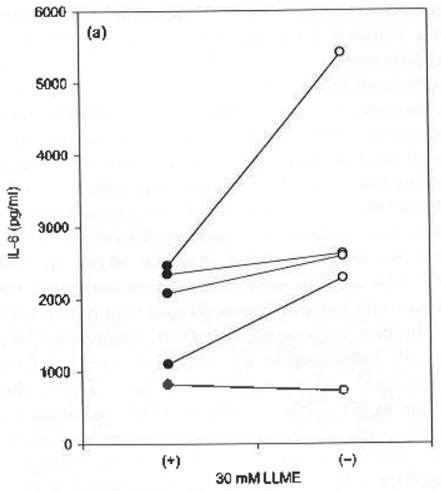


Figure 1. Effect of LLME on the LPS-stimulated production of IL-6 (a) and MCP-1 (b) by cultured macrophages. Macrophages were treated with 30 mM LLME for 90 min (-●-) and thereafter stimulated with LPS at a concentration of 1 μg/ml. Non-treated cultured macrophages were used as control (-○-). Medium was sampled for IL-6 and analysed by ELISA (pg/ml) after 24 h. Mean production during 24 is shown.

II-6 and MCP-1 production from spheroids obtained from patients with HNSCC

The production of IL-6 and MCP-1 from malignant and benign F-spheroids after 24 h was assessed and F-spheroids treated with LLME were compared to untreated F-spheroids. There was a depletion of spontaneous production of IL-6 (Figure 3a)

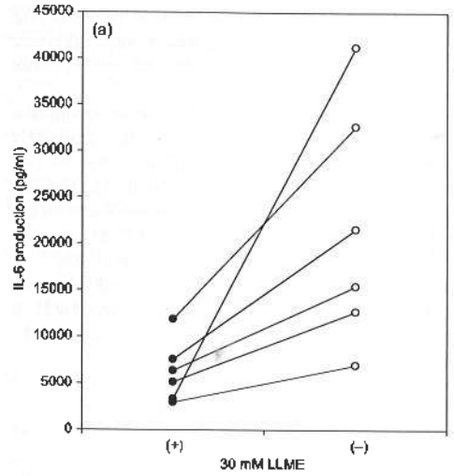


Figure 2. Effect of LLME on the production of IL-6 (a) and MCP-1 (b) by F-spheroids from squamous tissue, derived from patients undergoing elective surgery for snoring, from 7 days after harvesting. Biopsies were chopped into fragments and treated with 30 mM LLME for 90 min. Thereafter fragments were allowed to develop into F-spheroids either treated (-●-) with LLME or as control (-○-). Medium was sampled for IL-6 and analysed by ELISA (pg/ml) every 24 h for a total of 168 hours. Mean production during 48 h is shown.

($Z = -2.521$; $p = 0.012$) and MCP-1 (Figure 3b) ($Z = -2.521$; $p = 0.012$) from both malignant F-spheroids and benign F-spheroids treated with LLME, compared with autologous untreated control F-spheroids. This phenomenon was sustained during the 7 days that medium was sampled, as shown in Figure 4a and b.

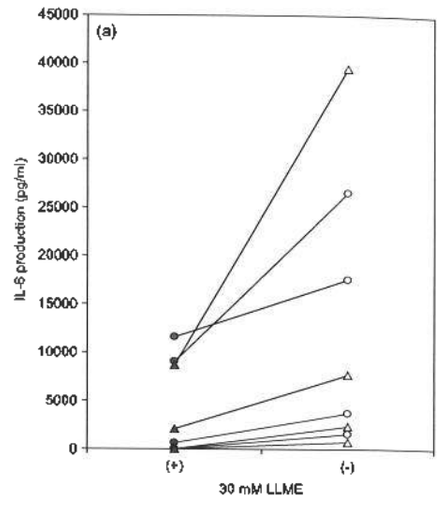


Figure 3. Effect of LLME on the production of IL-6 (a) and MCP-1 (b) by F-spheroids from 7 days after harvesting. Biopsies were chopped into fragments and treated with 30 mM LLME for 90 min. Thereafter fragments were allowed to develop into benign (-●-) and malignant (-▲-) F-spheroids. Autologous, non-treated benign (-○-) and malignant (-△-) F-spheroids were used as controls. Medium was sampled for IL-6 and analysed by ELISA (pg/ml) every 24 h for a total of 168 h. Mean production during 48 h is shown.

Co-culture of F-spheroids with autologous monocytes

Malignant and benign F-spheroids treated with LLME were co-cultured with autologous monocytes as described in Materials and methods. There was no difference in monocyte co-culture-induced

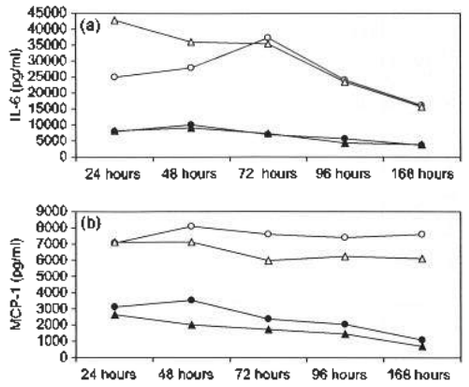


Figure 4. Effect of LLME on the spontaneous production of IL-6 (a) and MCP-1 (b) by F-spheroids, derived from an HNSCC patient, from 7 days after harvesting. Biopsies were chopped into fragments and treated with 30 mM LLME for 90 min. Thereafter fragments were allowed to develop into benign (-●-) and malignant (-▲-) F-spheroids. Autologous, non-treated benign (-○-) and malignant (-△-) F-spheroids were used as controls. Medium was sampled for IL-6 and analysed by ELISA (pg/ml) every 24 h for a total of 168 h. Mean production during 168 h is shown.

MCP-1 and IL-6 secretion when cultures with LLME-treated F-spheroids were compared to non-treated control F-spheroids (results not shown). Monocytes are stimulated by contact as well as by production of cytokines by the spheroids themselves. An unchanged co-culture response suggests that the presence of soluble products from the F-spheroids (besides IL-6 and MCP-1) contributes to the co-culture response. This demonstrates that F-spheroids are viable after treatment with LLME.

Discussion

An organ culture technique with F-spheroids was selected as a study model since malignant F-spheroids represent micro-elements of tumour tissue, simulating the in vivo situation pertaining to tumour-associated cells and stroma. Tissue fragments from benign mucosa taken either from healthy donors or HNSCC patients served as sources for control spheroids. LLME is a substance that selectively inhibits mononuclear phagocyte function by forming free leucin which causes lysis of the lysosomes [11–13,17]. It has been shown that this substance can eliminate monocytes and NK cells [17,18]. However, the present study shows that cytokine secretion of macrophages becomes impaired following LLME exposure, indicating an impairment of functionality. We also incubated tissue fragments from benign mucosa from healthy donors with LLME. The production of IL-6 and MCP-1 was reduced following treatment, suggesting that LLME selectively inhibits the function of tissue macrophages.

Incubation with LLME did not cause significant epithelial cell death of F-spheroids as determined by using the LIVE/DEAD kit and did not eradicate cell proliferation as shown by BrDU staining. On the other hand, both malignant and benign F-spheroids from HNSCC patients treated with LLME showed reduced levels of secretion of IL-6 and MCP-1. These results suggest that LLME can reduce tissue macrophage and TAM function as measured by IL-6 and MCP-1 secretion while the surface cells on F-spheroids remain vital.

We concluded that TAMs are a main producer of IL-6 and MCP-1 in HNSCCs. HNSCC cells [19], as well as other cells such as tumour infiltrating lymphocytes (TILs), are also presumably able to produce these cytokines and thus responsible for the production of IL-6 and MCP-1 by the F-spheroid after treatment with LLME. On the other hand, LLME at the concentration used might not reach the TAM lysozymes in sufficient concentration to exert cell death. Recently, pro-inflammatory cytokines such as IL-6 have been shown to have an important role in cancer development associated with chronic inflammation [20]. It has been postulated that dysregulation of an inflammatory response may cause initiation as well as progression of neoplastic growth [21]. IL-6 may play an important role in this process by serving as an anti-apoptotic agent through activation of NF-κβ [20]. HNSCC cells also have been shown to secrete IL-6 [19] and may thereby serve as a paracrine stimulator for TAMs to secrete IL-6. IL-6 secretion from TAMs in HNSCC tumours may therefore be a part of HNSCC carcinogenesis. This argument is strengthened by the observations that a high monocyte IL-6 secretion following LPS stimulation heralds impaired prognosis for HNSCC patients (unpublished data). MCP-1 could be associated with accumulation of TAMs in squamous cell carcinomas [22]. Liss and co-workers observed that attraction of TAMs producing angiogenic factors in HNSCC was due to secretion of MCP-1 by tumour cells [23]. Our observations in the present study indicate that TAMs also produce MCP-1 and thereby form a self-enhancing circuit of TAM influx in HNSCC tumours.

Co-culture with autologous monocytes showed no significant differences in co-culture-induced monocyte IL-6 and MCP-1 production when LLME-treated spheroid cultures were compared to untreated spheroid cultures. This indicates that IL-6 and MCP-1 secretion from TAMs is not the primary

stimulus of monocyte co-culture-stimulated cytokine secretion. It might be speculated that TAMs and monocytes in co-culture with F-spheroids share the same stimulatory pathway when secreting IL-6 and MCP-1 in vitro. The co-culture response did not change following LLME treatment of the F-spheroids and this further strengthens the claim that F-spheroid epithelial cells are fully viable following LLME exposure.

It has been shown that TAMs are physiologically different to tissue macrophages [1]. Such a difference, expressed by IL-6 and MCP-1 secretion, could not be demonstrated in this study as there was no difference in responsiveness to LLME treatment between malignant and benign F-spheroids. Further studies with additional cytokine measurements will be performed to answer this question more thoroughly.

The function of TAMs is still not fully known. Correlation analyses of the amount of TAMs and prognosis of cancer have shown that high levels of TAMs in HNSCC may be related to worse long-term prognosis [4,24]. However, in other cancer diseases this may be different [2,22,25–28].

One of the reported experiments in the present series was actually performed with tissue from a patient with sinonasal carcinoma. The results of this study showed almost identical results to those for HNSCC patients. This argues that MNP responses to tumours are similar beyond squamous cell carcinomas.

For HNSCC, as for most other studies concerning TAM accumulation in malignant tumours, expression and secretion of cytokines in tumour tissues have been determined by the use of immunohistochemical methods and/or cell lines. This report shows the dynamics of cytokine secretion during the interactions between tumour cells and tumour stroma, thus offering a possible strategy for performing such studies. Incubation of tumour tissue with LLME before forming F-spheroids might be an important TAM research tool, especially since TAM density in the F-spheroid is not changed significantly compared to the original tumour tissue [8].

In recent years, there has been much focus on the study of tumour biology by micro-array technology [29]; however, such studies use whole tumour tissue. Thus it cannot be determined if the differences shown are actually attributable to tumours cells or to the various support cells – of which TAMs might be an example. Treating tissue with LLME before processing could help to show which of the changes in mRNA construct levels actually reflects tumour cell changes.

We have shown that LLME incubation of HNSCC tissue can decrease the secretion of IL-6 and MCP-1 from F-spheroids in vitro. This indicates that TAMs are a main source of these cytokines in HNSCCs. The observations in this study and recent work by others are in accordance with the proposition that TAM-secreted IL-6 contributes to the carcinogenesis of HNSCC.

References

[1] Mantovani A, Allavena P, Sica A. Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. Eur J Cancer 2004; 40:1660–7.

[2] Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, et al. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. Clin Cancer Res 2000;6:3282–9.

[3] Nakayama Y, Nagashima N, Minagawa N, Inoue Y, Katsuki T, Onitsuka K, et al. Relationships between tumor-associated macrophages and clinicopathological factors in patients with colorectal cancer. Anticancer Res 2002;22: 4291–6.

[4] Marcus B, Arenberg D, Lee J, Kleer C, Chepeha DB, Schmalbach CE, et al. Prognostic factors in oral cavity and oropharyngeal squamous cell carcinoma. Cancer 2004;101: 2779–87.

[5] Andreesen R, Hennemann B, Krause SW. Adoptive immunotherapy of cancer using monocyte-derived macrophages: rationale, current status, and perspectives. J Leukoc Biol 1998;64:419–26.

[6] Whiteside TL. Immunobiology of head and neck cancer. Cancer Metastasis Rev 2005;24:95–105.

[7] Heimdal JH, Aarstad HJ, Olsnes C, Olofsson J. Human autologous monocytes and monocyte-derived macrophages in co-culture with carcinoma F-spheroids secrete IL-6 by a non-CD14-dependent pathway. Scand J Immunol 2001;53: 162–70.

[8] Kross KW, Heimdal JH, Olsnes C, Olofsson J, Aarstad HJ. Head and neck squamous cell carcinoma spheroid- and monocyte spheroid-stimulated IL-6 and monocyte chemotactic protein-1 secretion are related to TNM stage, inflammatory state and tumor macrophage density. Acta Otolaryngol (Stockh) 2005;125:1097–104.

[9] Kamimura D, Ishihara K, Hirano T. IL-6 signal transduction and its physiological roles: the signal orchestration model. Rev Physiol Biochem Pharmacol 2003;149:1–38.

[10] Ohno S, Suzuki N, Ohno Y, Inagawa H, Soma G, Inoue M. Tumor-associated macrophages: foe or accomplice of tumors? Anticancer Res 2003;23:4395–409.

[11] Thiele DL, Lipsky PE. Modulation of human natural killer cell function by L-leucine methyl ester: monocyte-dependent depletion from human peripheral blood mononuclear cells. J Immunol 1985;134:786–93.

[12] Sawada T, Hashimoto S, Tohma S, Nishioka Y, Nagai T, Sato T, et al. Inhibition of L-leucine methyl ester mediated killing of THP-1, a human monocytic cell line, by a new anti-inflammatory drug, T614. Immunopharmacology 2000;49:285–94.

[13] Thiele DL, Lipsky PE. The immunosuppressive activity of L-leucyl-L-leucine methyl ester: selective ablation of cytotoxic lymphocytes and monocytes. J Immunol 1986;136: 1038–48.

[14] Heimdal J, Aarstad HJ, Olofsson J. Monocytes secrete interleukin-6 when co-cultured in vitro with benign or

malignant autologous fragment spheroids from squamous cell carcinoma patients. Scand J Immunol 2000;51:271–8.
[15] Boyum A. Isolation of human blood monocytes with Nycodenz, a new non-ionic iodinated gradient medium. Scand J Immunol 1983;17:429–36.
[16] Heimdal JH, Aarstad HJ, Aakvaag A, Olofsson J. In vitro T-lymphocyte function in head and neck cancer patients. Eur Arch Otorhinolaryngol 1997;254:318–22.
[17] Thiele DL, Kurosaka M, Lipsky PE. Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotropic agent, L-leucine methyl ester. J Immunol 1983;131:2282–90.
[18] Pechhold K, Kabelitz D. Human peripheral blood gamma delta T cells are uniformly sensitive to destruction by the lysosomotropic agents leucine methyl ester and leucyl leucine methyl ester. Eur J Immunol 1993;23:562–5.
[19] Chen Z, Malhotra PS, Thomas GR, Ondrey FG, Duffey DC, Smith CW, et al. Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer. Clin Cancer Res 1999;5:1369–79.
[20] Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 2005;5:749–59.
[21] Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. Eur J Cancer 2005;41:2502–12.
[22] Kleine-Lowinski K, Gillitzer R, Kuhne-Heid R, Rosl F. Monocyte-chemo-attractant-protein-1 (MCP-1)-gene expression in cervical intra-epithelial neoplasias and cervical carcinomas. Int J Cancer 1999;82:6–11.
[23] Lissa C, Fekete MJ, Hasina R, Lam CD, Lingen MW. Paracrine angiogenic loop between head-and-neck squamous-cell carcinomas and macrophages. Int J Cancer 2001;93:781–5.
[24] Li C, Shintani S, Terakado N, Nakashiro K, Hamakawa H. Infiltration of tumor-associated macrophages in human oral squamous cell carcinoma. Oncol Rep 2002;9:1219–23.
[25] Leek RD, Harris AL. Tumor-associated macrophages in breast cancer. J Mammary Gland Biol Neoplasia 2002;7:177–89.
[26] Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 2004;4:71–8.
[27] Valkovic T, Fuckar D, Stifter S, Matusan K, Hasan M, Dobrila F, et al. Macrophage level is not affected by monocyte chemotactic protein-1 in invasive ductal breast carcinoma. J Cancer Res Clin Oncol 2005;131:453–8.
[28] Varney ML, Johansson SL, Singh RK. Tumour-associated macrophage infiltration, neovascularization and aggressiveness in malignant melanoma: role of monocyte chemotactic protein-1 and vascular endothelial growth factor-A. Melanoma Res 2005;15:417–25.
[29] Dysvik B, Vasstrand EN, Lovlie R, Elgindi OA, Kross KW, Aarstad HJ, et al. Gene expression profiles of head and neck carcinomas from Sudanese and Norwegian patients reveal common biological pathways regardless of race and lifestyle. Clin Cancer Res 2006;12:1109–20.

Paper III

BMC Cancer

BioMed Central

Research article

Open Access

Stimulated monocyte IL-6 secretion predicts survival of patients with head and neck squamous cell carcinoma

John-Helge Heimdal*[1], Kenneth Kross[1], Beate Klementsen[1], Jan Olofsson[1] and Hans Jørgen Aarstad[1,2]

Address: [1]Department of Otolaryngology/Head & Neck Surgery, Haukeland University Hospital, Bergen, Norway and [2]Broegelmann Research Laboratory, University of Bergen, Bergen, Norway

Email: John-Helge Heimdal* - john.heimdal@helse-bergen.no; Kenneth Kross - kenneth.kross@kir.uib.no; Beate Klementsen - beate.klementsen@abm.uib.no; Jan Olofsson - jan.olofsson@helse-bergen.no; Hans Jørgen Aarstad - hans.jorgen.aarstad@helse-bergen.no

* Corresponding author

Published: 30 January 2008

BMC Cancer 2008, 8:34 doi:10.1186/1471-2407-8-34

Received: 7 June 2007
Accepted: 30 January 2008

This article is available from: http://www.biomedcentral.com/1471-2407/8/34

Abstract

Background: This study was performed in order to determine whether monocyte *in vitro* function is associated with presence, stage and prognosis of head and neck squamous cell carcinoma (HNSCC) disease.

Methods: Prospective study describing outcome, after at least five years observation, of patients treated for HNSCC disease in relation to their monocyte function. Sixty-five patients with newly diagnosed HNSCC and eighteen control patients were studied. Monocyte responsiveness was assessed by measuring levels of monocyte *in vitro* interleukin (IL)-6 and monocyte chemotactic peptide (MCP)-1 secretion after 24 hours of endotoxin stimulation in cultures supplied either with 20% autologous serum (AS) or serum free medium (SFM). Survival, and if relevant, cause of death, was determined at least 5 years following primary diagnosis.

Results: All patients, as a group, had higher *in vitro* monocyte responsiveness in terms of IL-6 (AS) ($t = 2.03$; $p < 0.05$) and MCP-1 (SFM) ($t = 2.49$; $p < 0.05$) compared to controls. Increased *in vitro* monocyte IL-6 endotoxin responsiveness under the SFM condition was associated with decreased survival rate (Hazard ratio (HR) = 2.27; Confidence interval (CI) = 1.05–4.88; $p < 0.05$). The predictive value of monocyte responsiveness, as measured by IL-6, was also retained when adjusted for age, gender and disease stage of patients (HR = 2.67; CI = 1.03–6.92; $p < 0.05$). With respect to MCP-1, low endotoxin-stimulated responsiveness (AS), analysed by Kaplan-Meier method, predicted decreased survival ($\chi = 4.0$; $p < 0.05$).

Conclusion: In HNSCC patients, changed monocyte *in vitro* response to endotoxin, as measured by increased IL-6 (SFM) and decreased MCP-1 (AS) responsiveness, are negative prognostic factors.

Background

Knowledge about the interactions between tumour cells and the immune system has increased in the last decades. Yet, many basic issues concerning tumour immunology remain unanswered. An intriguing question is why the immune system, capable of eliminating malignant cells under experimental conditions, fails to eliminate tumour cells in patients with progressive cancer disease. Thus, it remains relevant to study functional changes in various immune cells during cancer disease [1,2].

Head and Neck squamous cell carcinoma (HNSCC) is one example of diseases where functional changes in immune cells have been demonstrated [3,4]. Alterations in immune cell functions in HNSCC patients are shown to be both directly disease dependent as well as indirectly related to disease as, e.g., when correlating to impaired general status of patients [5]. Furthermore, it has been shown that *in vitro*-stimulated lymphocyte proliferation, as well as *in vivo* expression of lymphocyte activation epitopes, may be associated with prognosis in HNSCC patients [6,7].

Mononuclear phagocytes (MNPs) are also determined to be functionally changed in patients with HNSCC [8]. Monocytes and macrophages may be separated into type-I and type-II cells according to their cytokine repertoire upon activation [9]. Interleukin (IL)-6 and other pro-inflammatory cytokines are secreted from type-I cells, whereas chemotactic substances such as monocyte chemotactic peptide (MCP)-1 are secreted mainly from type-II cells [9]. When monocytes are stimulated in co-culture with HNSCC tumour cells, high levels of both IL-6 and MCP-1 can be detected in supernatants [10].

IL-6 is a pluripotent cytokine with mostly stimulatory functions. IL-6 may, e.g., act as an autocrine or paracrine growth factor, but also as an anti-apoptotic agent on cancer cells, as is the case in oral cancer [11-13]. MCP-1 was originally determined to recruit macrophages into malignant lesions [14]. MCP-1 receptor expression on tumour cells may be important in the context of tumour cell proliferation and invasion, e.g., in prostate cancer [15].

An increased influx of tumour-associated macrophages (TAMs) in HNSCC tumours is mirrored by a worsened prognosis, but so far no association between monocyte function and survival of HNSCC patients has been published [16]. We have in a previous study observed a correlation between monocyte and TAM IL-6 secretion in HNSCC, suggesting that monocyte function indeed reflects TAM function in HNSCC patients [10]. We therefore suggest that monocyte function may be related to prognosis in HNSCC patients. The aim of the present investigation was to study this hypothesis.

Monocyte function may be assessed by measuring cytokine secretion after *in vitro* stimulation of purified monocytes with endotoxin. We have studied whether monocyte function in HNSCC patients, as measured by *in vitro* endotoxin-stimulated monocyte IL-6 and MCP-1 secretion, was different from monocyte function in control patients and dependent on stage of HNSCC disease as well as prognosis.

Methods

Patients and controls

The study comprised patients hospitalised at the Department of Otolaryngology and Head & Neck Surgery, Haukeland University Hospital, Bergen, Norway. Patients had either squamous cell carcinoma (SCC) (N = 65) or benign diseases of the head and neck (HN) (N = 18). Patients with autoimmune disease or patients on corticosteroid medications were excluded from the study. The study was approved by the Regional Committee for Medical Ethics. Each patient gave written consent before participating in the study. Primary sites of carcinomas were: oral cavity (28), pharynx (22), larynx (13), maxilla (1) and unknown primary (1). TNM stages of patients are shown in Table 1. The diagnoses of the patients with benign disease were: dysplastic lesions in the oral cavity or larynx (8), benign tumours in the larynx or middle ear (3), obstructive sleep apnoea (3), benign oesophageal conditions like stenosis or diverticulum (3) as well as tympanic membrane defect (1). Variables such as age, tobacco smoking and alcohol consumption are known to affect monocyte function [17-19]. In order to evaluate the effect of malignant disease per se on immune function, healthy controls, matching cancer patients to these possible confounding variables, were selected. Ages of HNSCC patients (62.2 ± 1.3) and controls (64.4 ± 2.5) were similar. Neither tobacco smoking (33.8 ± 2.5 versus 29.8 ± 5.1 years) nor alcohol consumption history (2.3 ± 0.16 versus 2.1 ± 0.13) differed significantly between the two groups. After at least 5 years post-inclusion, survival was determined from the Norwegian population registry and cause of death from a continuous follow-up registration of HNSCC patients at our department. We found that 30 of the 65 cancer patients were still alive, 27 had succumbed from HNSCC disease and eight from other causes.

Table 1: TNM stages of cancer patients evaluated for *in vitro* monocyte function. (Figures represent the final cTNM score of patients before treatment or pTNM scores based on histology from surgery, if such information was present)

		N stage				
		0	1	2	3	Sum
T stage	is	2				2
	1	10		6	3	19
	2	12	2	4	1	19
	3	5	2	4		11
	4	8	3	3		14
	Sum	37	7	17	4	65

is = in situ

Alcohol consumption assessment

Each patient was interviewed at time of inclusion in the study in order to assess smoking and alcohol habits. History of alcohol consumption was scored as follows [20]: 1, no alcohol consumed; 2, modest alcohol consumption (less than once per week); 3, middle level alcohol consumption (1–2 times weekly); 4, alcohol consumed twice weekly; 5, alcohol consumed more than twice weekly.

Monocyte preparation

Patients were included in the study upon their arrival to the department before any treatment started. All blood samples were drawn at 7.30 a.m. as a bedside procedure and each patient was asked to stay in bed until the blood was drawn. Monocytes were isolated from blood by gradient centrifugation followed by adherence to plastic as previously described. [8] In short, peripheral blood mononuclear cells (PBMC) were separated by gradient centrifugation with Lymphoprep® (Nycomed, Oslo, Norway) as density gradient medium. The PBMC yield of 8.5 ml blood was allocated to a 24-well plate (Nunc A/S, Roskilde, Denmark) with RPMI-1640 (BioWhittaker) supplemented with amphotericin B (2.5 μg/ml) and glucose (both Sigma), HEPES, L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), sodium bicarbonate, sodium pyruvate (all from BioWhittaker) and 20% autologous serum (AS) to a total volume of 0.5 ml/well. After 40 minutes pre-incubation, adherent monocytes were purified by washing, and then cultured in complete RPMI (BioWhittaker)/20% AS with 0.5 ml/well. This method yields more than 95% monocytes positive by non-specific esterase stain with more than 95% viable cells as tested by tryphan blue stain.

Culture conditions and stimulation

After monocytes were isolated from each donor, cells were without delay further cultured in 0.5 ml/well, either supplied with 20% (AS) or with serum free medium (SFM)(UltraCulture, BioWhittaker). Stimulation was provided for 24 hours by 1 μg/ml lipopolysaccharide (LPS) derived from *Escherichia coli* (Sigma, St. Louis, Mo., USA) before sample collection. Cultures without LPS as stimulant were used as background control conditions.

IL-6 and MCP-1 analysis

The contents of IL-6 and MCP-1 analysis in supernatants were determined by enzyme-linked immunosorbant assay kit (ELISA) manufactured by R&D Systems (R&D Systems Europe Ltd., Abingdon, Great Britain). All procedures were performed according to the specifications of the manufacturer. Briefly, 96-well microtiter plates (Costar Corning, Lowell, MA, USA) were coated overnight at room temperature (RT) with monoclonal mouse α-human IL-6 or monoclonal mouse α-human MCP-1 capture antibodies. Diluted samples or recombinant human IL-6 or MCP-1 standard were added and incubated for 2 h at RT followed by addition of biotinylated polyclonal goat α-human IL-6 or goat α-human MCP-1. The plates were then incubated for 20 minutes at RT with streptavidin-conjugated horseradish peroxidase. Tetra-methyl-benzidine (TMB) (Sigma) and H_2O_2 were used as substrate. Absorbency values were measured at 450 nm using Softmax Pro version 4.0 on an Emax Precision microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA). The lower detection level was 9 pg/ml for IL-6 and 16 pg/ml for MCP-1. The median LPS-stimulated (stimulated – background) IL-6 SFM and AS was 44251 (range 0 – 133014) pg/ml and 40551 (range -8273 – 149411) pg/ml, respectively. The median LPS-stimulated MCP-1 SFM (LPS-stimulated – background) and AS (background – LPS-stimulated) was 7207 (range from -20025 to 66147) pg/ml and -163 (range from -25718 to 13908) pg/ml, respectively.

Statistical analysis

The statistical program package SPSS (Ver. 14; Inc Chicago, IL, USA) was employed. Figures are given as mean ± standard error of the mean. The groups were compared by the student *t*-test. Survival analyses were performed by Cox regression analysis or Kaplan-Meier (Log Rank (Mantel Cox) test) analyses. When limited versus extended disease patients were compared, the patients were dichotomised by the median of the numeric sum of T and N stage, i.e. the T and N integer score (TANIS) [21] Scores between 0 and 3 were considered as limited disease and scores between 4 and 7 as extended disease. With Kaplan-Meier analyses, monocyte responsiveness was dichotomised into high or low responders by the median value, as measured by the positive account of difference in IL-6

and MCP-1 levels in monocyte cultures when stimulated compared to not stimulated cultures. The median value calculation was based on all included cytokine values. Statistical significance was considered if $P < 0.05$.

Results

Monocyte IL-6 and MCP-1 secretion in cancer patients compared to controls

When monocytes were cultured with AS and stimulated with LPS, supernatant IL-6 levels in cultures from HNSCC patients were higher compared to those from control patients (53459 ± 4789 pg/ml versus 39165 ± 8179 pg/ml. ($t = 2.03$; $p < 0.05$)). No significant difference could be proven when SFM was utilised (Fig. 1A). A significantly changed endotoxin-induced response compared to background release of MCP-1 secretion from monocytes in cancer patients compared to controls was observed (1232 ± 588 pg/ml versus -3860 ± 1958 pg/ml. ($t = 2.49$; $p < 0.05$)) when cultures were supplied with SFM, but not with AS (Fig. 1B).

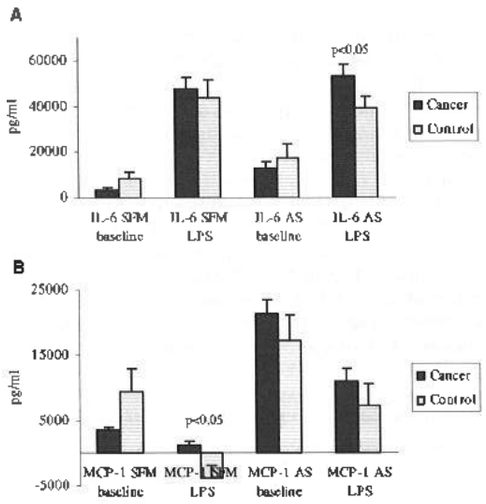


Figure 1
Levels of Interleukin (IL)-6 and Monocyte chemotactic protein (MCP)-1 in supernatants of 24-hours *in vitro* endotoxin (1 μg/ml lipo-poly saccharide (LPS))-stimulated purified monocytes from HNSCC patients and control patients. Cultures were either supplied with autologous serum (AS) or serum free medium (SFM). Bars represent means ± SEM of supernatant levels. (LPS-stimulated – baseline levels: IL-6 SFM/AS & MCP-1 SFM. Baseline – LPS-stimulated: MCP-1 AS). Statistics by students' t-test.

Monocyte IL-6 and MCP-1 secretion as related to disease stage

There were no differences in monocyte endotoxin response, as measured by increased IL-6 secretion (Fig. 2A) or decreased MCP-1 secretion (Fig. 2B), when monocyte cultures from HNSCC patients with extended (TANIS = 4–7) tumour burden were compared to patients with limited (TANIS = 0–3) tumour burden. This held true both with autologous and serum- free medium.

Prognostic value of monocyte function

With all patients included, endotoxin-stimulated monocyte IL-6 secretion was found to be significantly higher in monocyte cultures from patients that had died after 5 years follow-up compared to that of living patients, both at SFM ($t = 2.03$; $p < 0.05$) and AS ($t = 2.17$; $p < 0.05$) conditions (Fig. 3A). Endotoxin-induced compared to background monocyte MCP-1 secretion was not different in patients alive after five years compared to those that had

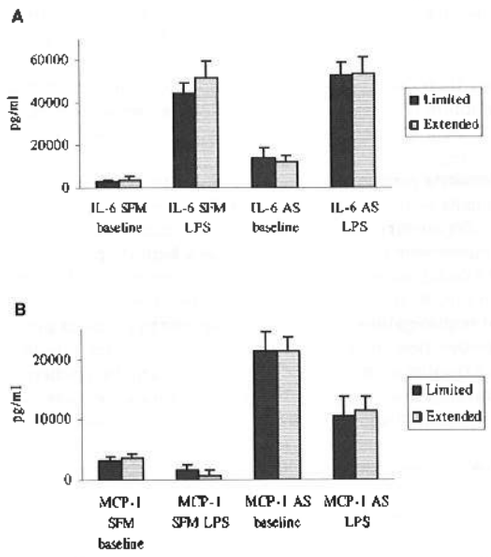


Figure 2
Levels of IL-6 and MCP-1 in the supernatants of 24 hours, *in vitro* endotoxin (1 μg/ml LPS)- stimulated purified monocytes from cancer patients with low (T- + N-stage < 3) (limited) versus high (T- + N-stage > 3) (extended) tumour burden. Cultures were either supplied with 20% autologous serum (AS) or serum free medium (SFM). The bars represent means ± SEM of supernatant levels. (LPS-stimulated – baseline levels: IL-6 SFM/AS & MCP-1 SFM. Baseline – LPS-stimulated: MCP-1 AS).

died during the same period at any of the two culture conditions (Fig. 3B).

Including HNSCC patients only, Kaplan-Meier analysis showed that patients with high monocyte responsiveness to endotoxin, as measured by high IL-6 secretion (SFM), had decreased total (Fig. 4A) ($\chi = 4.3$; $p < 0.05$) as well as disease-specific (Fig. 4B) ($\chi = 4.4$; $p < 0.05$) survival compared to patients with low monocyte responsiveness. When MCP-1 values from AS conditions were analysed by Kaplan-Meier analysis, a low responsiveness predicted decreased total survival ($\chi = 4.0$; $p < 0.05$) (Fig. 5A), and with a trend toward the same with disease-specific survival ($\chi = 3.6$; $p = 0.06$) (Fig. 5B).

With only HNSCC patients included, IL-6 secretion at SFM predicted survival as follows: Cox regression survival analyses, showed that LPS-stimulated monocyte IL-6 secretion, adjusted for gender and age of patients, predicted both total as well as disease-specific survival ($p < 0.05$) when analysed with original results (Table 2, upper

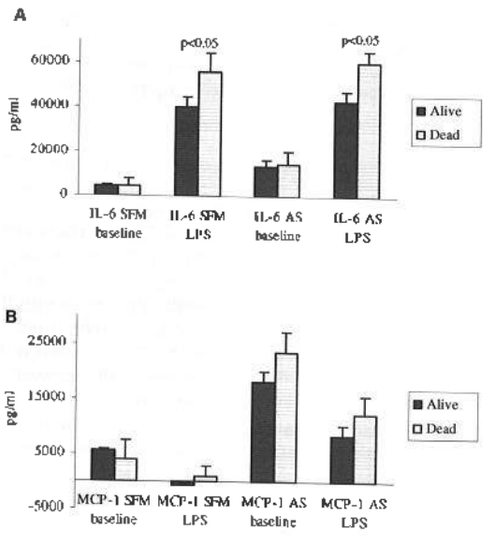


Figure 3
Levels of IL-6 and MCP-1 in the supernatants of 24 hours, *in vitro* endotoxin (1 μg/ml LPS)- stimulated purified monocytes cultures from dead versus live patients with observations at least 5 years following inclusion. Cultures were either supplied with autologous serum (AS) or serum free medium (SFM). The bars represent means ± SEM of supernatant level. (LPS-stimulated – baseline levels: IL-6 SFM/AS & MCP-1 SFM. Baseline – LPS-stimulated: MCP-1 AS). Statistics by students' *t*-test.

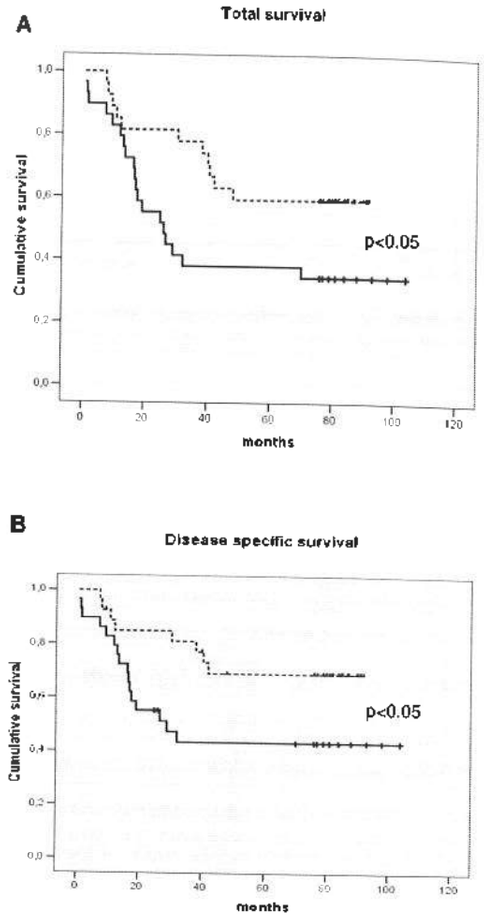


Figure 4
Kaplan-Meier plot survival dependent on total survival (A) or disease-specific survival (B) according to IL-6 *in vitro* secretion from purified monocytes following 24 hours, endotoxin (1 μg/ml LPS) stimulation dichotomised by median value to low (hatched line) or high (continuous line) response with serum free medium (SFM) applied (background subtracted). Statistics by Log Rank (Mantel Cox) test.

panel) and when analysed dichotomised (Hazard ratio (HR) = 2.27; Confidence interval (CI) = 1.05–4.88; $p < 0.05$ and HR = 2.68; CI = 1.11–6.45; $p < 0.05$, respectively) (Table 3, upper panel).

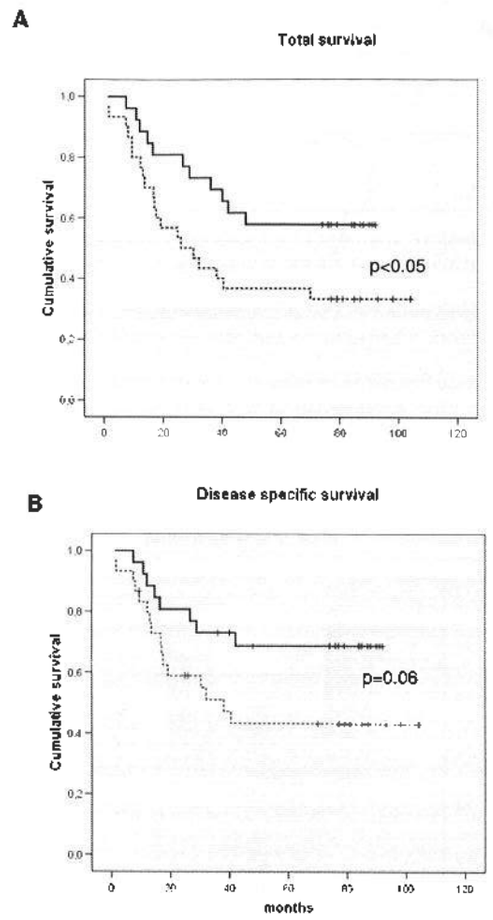


Figure 5
Kaplan-Meier plot survival dependent on total survival (A) or disease-specific survival (B) according to MCP-1 *in vitro* secretion of purified monocytes following 24 hours endotoxin (1 μg/ml LPS) stimulation dichotomised by median value to low (hatched line) or high (continuous line) responsiveness with 20% autologous serum (AS) added to the medium applied (background – LPS stimulated response). Statistics by Log Rank (Mantel Cox) test.

When adjusting for age, gender as well as TNM stage, a prediction for disease-specific survival was determined when IL-6 levels were analysed dichotomised (HR = 2.44; CI = 1.01–5.92; $p < 0.05$) (Table 3, lower panel). A trend towards disease-specific survival prediction was also observed when IL-6 levels were analysed with original results (p = 0.066), (Table 2, lower panel). Likewise, a trend towards survival prediction was observed as to total survival when IL-6 levels were analysed dichotomised (HR = 2.08; CI = 0.97–4.48; p = 0.061), (Table 3, lower panel). Furthermore, when monocyte LPS-stimulated IL-6 secretion values were analysed dichotomised, survival prediction was also present with disease-specific survival (HR = 2.62; CI = 1.14–6.08; $p < 0.05$) and total survival (HR = 3.10; CI = 1.15–8.39; $p < 0.05$), when adjusted for gender and age as well as serum albumin and erythrocyte sedimentation rate (ESR) values of patients (analyses not shown). When adjustment for TNM stage was additionally introduced, predictions were still observed, both as measured by disease-specific survival (HR = 2.31; CI = 1.02–5.21; $p < 0.05$) and total survival (HR = 2.67; CI = 1.03–6.92; $p < 0.05$) (analyses not shown).

When dichotomised MCP-1 values were introduced in a Cox regression survival analysis, adjusted for age and gender of patients, we determined a trend as to prediction of total survival at the AS condition (HR = 1.99; CI = 0.95–4.18; p = 0.069) (Table 4, upper panel). Adjusting for age, gender as well as TNM stage of patients, we determined prediction of survival at SFM conditions (HR = 2.42; CI = 1.03–5.69; $p < 0.05$), (Table 4, lower panel).

Discussion

In this study, we have examined monocyte responsiveness, as measured by *in vitro* endotoxin responsiveness, by monocyte IL-6 and MCP-1 secretion. Monocyte responsiveness was increased in monocytes from HNSCC patients compared to control conditions. On the other hand, no difference in monocyte responsiveness was found when HNSCC patients with limited versus extended disease were compared. Patients with high monocyte responsiveness as measured by IL-6 secretion at serum-free conditions had lower disease-specific survival than patients with low such monocyte responsiveness. Predictions for survival based on monocyte IL-6 secretion, were still valid after adjusting for gender, age, TNM stage, albumin and ESR levels. Furthermore, we determined to some extent that MCP-1 secretion following endotoxin stimulation was related to prognosis. We have, however, determined a much more thorough correlation to prognosis with IL-6 levels than with MCP-1 levels. We therefore suggest that MCP-1 level survival prediction should be more closely studied before any firm conclusions can be drawn. Our observations are in line with results from a recent study by Clinchy and co-workers showing that increased IL-6 secretion, in short-duration *in vitro* cultures of peripheral blood mononuclear cells stimulated with LPS, was associated with impaired prognosis in patients radically operated for colon cancer [22]. Monocyte IL-6 and MCP-1 secreted from endotoxin- stimulated monocytes may be linked to an altered inflammatory state as previously shown in HNSCC patients. Examples are increased ESR, lowered albumin values in serum, increased levels of acute-phase proteins and pro-inflammatory cytokines [23,24]. This has been studied by adding serum albumin and ESR level information to the Cox regression analyses. We determined only minor explanatory power upon adjusting for ESR and albumin in serum. On the contrary, to some extent the IL-6 secretion level, serum albumin levels and ESR independently predicted survival.

Table 2: Multivariate Cox Regression Survival Analysis with Monocyte IL-6 secretion in pg/ml (serum-free medium) Adjusted for Age, Gender and TN stage of HNSCC Patients

	Total survival			Disease-specific survival		
	B	SE	*P* value	B	SE	*P* value
Gender	-.365	.531	.492	-.707	.654	.280
Age	.062	.021	.003	.068	.024	.004
IL-6	**.000**	**.000**	**.029**	**.000**	**.000**	**.024**
Gender	-.451	.510	0.779	-.726	.632	0.250
Age	.057	.022	0.022	.055	.025	0.028
T stage	.496	.167	0.003	.647	.195	0.001
N stage	.384	.199	0.054	.420	.230	0.068
IL-6	**.000**	**.000**	**0.110**	**.000**	**.000**	**0.066**

B = regression coefficient, SE = Standard error of B

There is evidence to claim that nuclear factor -κB (NF-κB), which regulates expression of multiple genes in cells, may act as a link between infection, inflammation and carcinogens in development of cancer [25]. The pro-inflammatory cytokine IL-6 may have an important role in this process by serving as an anti-apoptotic agent through activation of NF-κB [26]. Our present finding, that increased IL-6 secretion from monocytes predicts prognosis of HNSCC disease, further supports the notion that inflammatory responses may cause both initiation and progression of neoplastic growth [12].

We found no association between tumour burden and monocyte function in the present study. This argues against monocyte function being linearly regulated by HNSCC disease-related factors, such as cytokines secreted from tumour-associated cells. The findings in the present study indicate that monocyte changes are generally present in malignant disease and to a lesser extent influenced by tumour burden.

Another effect of IL-6 is its increased promotion of monocyte differentiation towards macrophages at the expense of dendritic cell (DC) differentiation [27]. Monocytes are recruited by chemokine gradients to migrate from circulation into tumour tissues where a further differentiation to TAMs or DCs takes place under the regulation of environmental signals of such as IL-6 [27]. There is currently an increasing agreement that TAMs in carcinoma disease may

Table 3: Multivariate Cox Regression Survival Analysis with Dichotomised Scored Monocyte IL-6 Secretion (serum-free medium) Adjusted for Gender and Age (upper panel), or adjusted for Gender, Age and TN stage (lower panel) of HNSCC Patients

	Total survival			Disease-specific survival		
	HR	95% CI (HR)	*P* value	HR	95% CI (HR)	*P* value
Gender	0.52	0.18–1.52	0.236	0.35	0.10–1.31	0.119
Age	1.07	1.02–1.11	0.002	1.08	1.03–1.28	0.002
IL-6	**2.27**	**1.05–4.88**	**0.036**	**2.68**	**1.11–6.45**	**0.028**
Gender	0.54	0.20–1.50	0.237	0.40	0.11–1.41	0.153
Age	1.06	1.02–1.11	0.008	1.06	1.01–1.11	0.025
T stage	1.64	1.18–2.29	0.003	1.89	1.29–2.79	0.001
N stage	1.55	1.06–2.28	0.029	1.61	1.03–2.51	0.035
IL-6	**2.08**	**0.97–4.48**	**0.061**	**2.44**	**1.01–5.92**	**0.049**

HR = Hazard ratio 95% CI(HR) = 95% confidence interval for HR

Table 4: Multivariate Cox Regression Total Survival Analysis with Dichotomised Scored Monocyte MCP-1 Secretion adjusted for Gender, Age, and TNM stage of HNSCC Patients

	Serum-free medium			Autologous serum medium		
	HR	95% CI (HR)	*P* value	HR	95% CI (HR)	*P* value
Gender	0.73	0.26–2.03	0.546	0.61	0.24–1.59	0.311
Age	1.05	1.01–1.09	0.014	1.04	1.00–1.08	0.027
MCP-1	1.20	0.58–2.48	0.617	1.99	0.95–4.18	0.069
Gender	0.41	0.12–1.41	0.166	0.55	0.22–1.41	0.216
Age	1.05	1.00–1.10	0.043	1.04	1.00–1.09	0.036
T stage	2.01	1.41–2.87	0.000	1.57	1.12–2.20	0.008
N stage	1.84	1.16–2.90	0.009	1.50	1.02–2.22	0.041
MCP-1	2.42	1.03–5.69	**0.043**	1.28	0.57–2.89	0.554

HR = Hazard ratio 95% CI(HR) = 95% confidence interval for HR

support tumour growth by virtue of their differentiation into type II macrophages [28]. Compared to TAMs, DCs apparently have a contrary effect within HNSCC tumours, whereby a high number of DCs correlates with better prognosis [29,30]. Furthermore, it has previously been demonstrated that monocytes maintain IL-6 secretion throughout their differentiation to macrophages when continuously stimulated with HNSCC tumour spheroids *in vitro* [31]. We therefore suggest that the malignancy potential of HNSCC relies to some extent on IL-6 stimulation by TAMs.

MCP-1 regulates TAM influx into tumours and may also be secreted by TAMs [10]. It is therefore possible that MCP-1 mediates a self-enhancing effect driven by TAMs within tumours. Increased expression of MCP-1 in squamous cell carcinomas of the oesophagus has been associated with increased influx of TAMs and an impaired prognosis [32]. *In vitro* experiments indicate that these findings may be relevant for HNSCC as well [16]. To what extent the shown lowered monocyte MCP-1 responsiveness association to increased prognosis can be linked to TAM influx in HNSCC tumours needs to be further elucidated.

The observations in the present study add weight to the arguments that activated MNPs may in fact increase rather than reduce tumour cell aggressiveness in HNSCC. Still, TAMs may in some cytokine environments have tumour suppressive potentials, which probably explains observations of improved prognosis associated with high numbers of TAMs in some other types of malignancies [33,34]. The observed reductions in HNSCC tumour mass when injected with biological response modifiers such as OK-432, may be in part be explained by such macrophage activation [35].

Previously, it has been shown that monocytes in HNSCC patients compared to control patients are primed for an increased sensitivity to endotoxin stimulation as measured by cytokine secretion [8]. The present study confirms these observations and further shows that monocyte function actually may provide information as to prognosis of HNSCC disease. We have previously determined in another patient sample that IL-6 secretion from monocytes did not predict survival. It should, however, be noted that patients with more affected capability, as measured by Karnofsky scores below 75, were included in the present study as opposed to a previous study [20]. Furthermore, when both of these samples were combined, prediction relying on monocyte IL-6 secretion was similar to this study (manuscript in preparation).

Both alcohol consumption and tobacco smoking are expected to be higher among HNSCC patients than in the general population because consumption of these substances has been linked to an increased risk of HNSCC [36]. Smoking and alcohol use may influence monocyte function [18,19,37]. In the present investigation, however, differences between HNSCC patients and control patients, as well as differences between the two HNSCC patient groups, and prognosis were present still after adjusting for tobacco and alcohol consumption. The observed changed monocyte function in HNSCC patients can therefore not be explained by alcohol consumption or tobacco smoking.

Monocyte sensitivity to endotoxin reflects prognosis when adjusted for TNM stage. Therefore it may be possible to identify patients having a better prognosis despite extended HNSCC disease. This might justify the use of a more extensive therapy regime in some selected patients with otherwise very extended TNM stage.

Observations from *in vitro* studies suggest that IL-6 promotes cell proliferation and prevents apoptosis in HNSCC cell lines via activation of signal-transducers-and-activators-of-transcription-3 (STAT3) via a common β-chain of the epidermal growth factor receptor (EGFR) [38]. STAT3 plays a critical role in the oncogenesis of several malignancies and has been shown to be activated in tumour tissue and in normal mucosa of HNSCC patients [39]. The activation of STAT3 is, however, shown to be complexly regulated via different kinases and supressors of cytokine signalling genes, which may explain the failure of treatment protocols based on EGFR- tyrosine kinase inhibitors [40]. Lee and co-workers therefore suggest that multiple pathways to stimulate STAT3 should be targeted in patients with HNSCC in order to achieve maximal clinical efficacy [40]. Thus, one possible additional therapeutic pathway could be an inhibition of the IL-6 stimulation of the tumour cells through therapeutic use of anti-IL-6 antibodies [41].

Conclusion

We have shown that monocyte function, as measured by endotoxin-induced *in vitro* monocyte regulation of IL-6 secretion at AS conditions was higher, whereas, MCP-1 secretion at SFM condition was less inhibited in HNSCC patients compared to controls. Monocyte function also predicted outcome in HNSCC patients. A high LPS-induced monocyte IL-6 responsiveness, and to some extent decreased MCP-1 responsiveness, predicted worsened prognosis independent of TNM stage. Thus, monocyte function is directly associated with the biology of HNSCC. We suggest that future studies should take into account the possible use of α-IL-6 antibodies as an adjuvant treatment for HNSCC disease.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

JHH planned and designed the study together with HJA who also performed the statistical analysis, helped draft the manuscript and critically revised the manuscript. JHH included each patient in the study and wrote the manuscript together with KK, who also took a major part when the manuscript was drafted. BK performed most of the laboratory work and carried out the immunoassays. JO revised the manuscript critically. All authors read and approved the final manuscript.

Acknowledgements

This work has been supported by The Norwegian Cancer Society. We are indebted to T. Tynning, D.A. Sandnes, for excellent technical assistance and C. Olsnes for proofreading the manuscript. We thank statistician Stein Atle Lie, University of Bergen, for valuable discussions about methods of analysis of the present work.

References

1. Seliger B: **Strategies of tumor immune evasion.** *BioDrugs* 2005, **19**:347-354.
2. Whiteside TL: **Immune responses to malignancies.** *J Allergy Clin Immunol* 2003, **111**:677-686.
3. Whiteside TL: **Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention.** *Semin Cancer Biol* 2006, **16**:3-15.
4. McKechnie A, Robins RA, Eremin O: **Immunological aspects of head and neck cancer: biology, pathophysiology and therapeutic mechanisms.** *Surgeon* 2004, **2**:187-207.
5. Hadden JW: **Immunodeficiency and cancer: prospects for correction.** *Int Immunopharmacol* 2003, **3**:1061-1071.
6. Heimdal J-H, Aarstad HJ, Olofsson J: **Peripheral blood T-lymphocyte and monocyte function and survival in patients with head and neck carcinoma.** *Laryngoscope* 2000, **110**:402-407.
7. Aarstad HJ, Heimdal J-H, Klementsen B, Olofsson J, Ulvestad E: **Presence of activated T-lymphocytes in peripheral blood of head and neck squamous cell carcinoma patients predict impaired prognosis.** *Acta Oto-Laryngologica* 2006, **126**:1326-1333.
8. Heimdal J-H, Aarstad HJ, Klementsen B, Olofsson J: **Ex vivo interleukin (IL)-1 beta, IL-6, IL-12 and tumor necrosis factor-alpha responsiveness with monocytes from patients with head and neck carcinoma.** *Eur Arch Otorhinolaryngol* 1999, **256**:250-256.
9. Verreck FA, de Boer T, Langenberg DM, van der Zanden L, Ottenhoff TH: **Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation.** *J Leukoc Biol* 2006, **79**:285-293.
10. Kross KW, Heimdal J-H, Olsnes C, Olofsson J, Aarstad HJ: **Head and neck squamous cell carcinoma spheroid- and monocyte spheroid-stimulated IL-6 and monocyte chemotactic protein-1 secretion are related to TNM stage, inflammatory state and tumor macrophage density.** *Acta Otolaryngol* 2005, **125**:1097-1104.
11. Kamimura D, Ishihara K, Hirano T: **IL-6 signal transduction and its physiological roles: the signal orchestration model.** *Rev Physiol Biochem Pharmaco* 2003, **149**:1-38.
12. Hodge DR, Hurt EM, Farrar WL: **The role of IL-6 and STAT3 in inflammation and cancer.** *Eur J Cancer* 2005, **41**:2502-2512.
13. Hong SH, Ondrey FG, Avis IM, Chen Z, Loukinova E, Cavanaugh PF Jr, Van Waes C, Mulshine JL: **Cyclooxygenase regulates human oropharyngeal carcinomas via the proinflammatory cytokine IL-6: a general role for inflammation?** *FASEB J* 2000, **14**:1499-1507.
14. Bottazzi B, Polentarutti N, Acero R, Balsari A, Boraschi D, Ghezzi P, Salmona M, Mantovani A: **Regulation of the macrophage content of neoplasms by chemoattractants.** *Science* 1983, **220**:210-202.
15. Lu Y, Cai Z, Galson DL, Xiao G, Liu Y, George DE, Melhem MF, Yao Z, Zhang J: **Monocyte chemotactic protein-1 (MCP-1) acts as a paracrine and autocrine factor for prostate cancer growth and invasion.** *The Prostate* 2006, **66**:1311-1318.
16. Liss C, Fekete MJ, Hasina R, Lam CD, Lingen MW: **Paracrine angiogenic loop between head-and-neck squamous-cell carcinomas and macrophages.** *Int J Cancer* 2001, **93**:781-785.
17. Sadeghi HM, Schnelle JF, Thoma JK, Nishanian P, Fahey JL: **Phenotypic and functional characteristics of circulating monocytes of elderly persons.** *Exp Gerontol* 1999, **34**:959-970.
18. Nielsen H: **A quantitative and qualitative study of blood monocytes in smokers.** *Eur J Respir Dis* 1985, **66**:327-332.
19. Szabo G: **Monocytes, alcohol use, and altered immunity.** *Alcohol Clin Exp Res* 1998, **22**:216-219.
20. Heimdal J-H, Aarstad HJ, Aakvaag A, Olofsson J: ***In vitro* T-lymphocyte function in head and neck cancer patients.** *Eur Arch Otorhinolaryngol* 1997, **254**:318-322.
21. Jones GW, Browman G, Goodyear M, Marcellus D, Hodson DI: **Comparison of the addition of T and N integer scores with TNM stage groups in head and neck cancer.** *Head Neck* 1993, **15**:497-503.
22. Clinchy B, Fransson A, Druvefors B, Hellsten A, Håkansson A, Gustafsson B, Sjödahl R, Håkansson L: **Preoperative interleukin-6 production by mononuclear blood cells predicts survival after radical surgery for colorectal carcinoma.** *Cancer* 2007, **109**:1742-1749.

23. Riedel F, Zaiss I, Herzog D, Götte K, Naim R, Hörmann K: **Serum levels of interleukin-6 in patients with primary head and neck squamous cell carcinoma.** *Anticancer Res* 2005, **25**:2761-2765.
24. Chen Z, Malhotra PS, Thomas GR, Ondrey FG, Duffey DC, Smith CW, Enamorado I, Yeh NT, Kroog GS, Rudy S, McCullagh L, Mousa S, Quezado M, Herscher LL, Van Waes C: **Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer.** *Clin Cancer Res* 1999, **5**:1369-1379.
25. Van Waes C: **Nuclear factor-kappaB in development, prevention, and therapy of cancer.** *Clin Cancer Res* 2007, **13**:1076-1082.
26. Schottelius AJ, Dinter H: **Cytokines, NF-kappaB, microenvironment, intestinal inflammation and cancer.** *Cancer Treat Res* 2006, **130**:67-87.
27. Chomarat P, Banchereau J, Davoust J, Palucka AK: **IL-6 switches the differentiation of monocytes from dendritic cells to macrophages.** *Nat Immunol* 2000, **1**:510-514.
28. Sica A, Schioppa T, Mantovani A, Allavena P: **Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy.** *Eur J Cancer* 2006, **42**:717-727.
29. Jecker P, Vogl K, Tietze L, Westhofen M: **Dendritic cells, T- and B-lymphocytes and macrophages in supraglottic and glottic squamous epithelial carcinoma. Location and correlation with prognosis of the illness.** *HNO* 1999, **47**:466-471.
30. Reichert TE, Scheuer C, Day R, Wagner W, Whiteside TL: **The number of intratumoral dendritic cells and zeta-chain expression in T cells as prognostic and survival biomarkers in patients with oral carcinoma.** *Cancer* 2001, **91**:2136-2147.
31. Heimdal J-H, Aarstad HJ, Olsnes C, Olofsson J: **Human autologous monocytes and monocyte-derived macrophages in co-culture with carcinoma F-spheroids secrete IL-6 by a non-CD14-dependent pathway.** *Scand J Immunol* 2001, **53**:162-170.
32. Koide N, Nishio A, Sato T, Sugiyama A, Miyagawa S: **Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus.** *Am J Gastroenterol* 2004, **99**:1667-1674.
33. Nakayama Y, Nagashima N, Minagawa N, Inoue Y, Katsuki T, Onitsuka K, Sako T, Hirata K, Nagata N, Itoh H: **Relationships between tumor-associated macrophages and clinicopathological factors in patients with colorectal cancer.** *Anticancer res* 2002, **22**:4291-4296.
34. Ohno S, Suzuki N, Ohno Y, Inagawa H, Soma G, Inoue M: **Tumor-associated macrophages: foe or accomplice to tumor?** *Anticancer res* 2003, **23**:4395-4410.
35. Kumazawa H, Yamashita T, Tachikawa T, Minamino M, Nakata Y: **Local injection of OK-432/fibrinogen gel into head and neck carcinomas.** *Eur J Cancer* 1994, **30A(12)**:1741-1744.
36. Schlecht NF, Franco EL, Pintos J, Negassa A, Kowalski LP, Oliveira BV, Curado MP: **Interaction between tobacco and alcohol consumption and the risk of cancers of the upper aero-digestive tract in Brazil.** *Am J Epidemiol* 1999, **150**:1129-1137.
37. Walters MJ, Paul-Clark MJ, McMaster SK, Ito K, Adcock IM, Mitchell JA: **Cigarette smoke activates human monocytes by an oxidant-AP-1 signaling pathway: implications for steroid resistance.** *Mol Pharmacol* 2005, **68**:1343-1353.
38. Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gutkind JS: **Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system.** *Cancer Res* 2003, **63**:2948-2956.
39. Grandis JR, Drenning SD, Zeng Q, Watkins SC, Melhem MF, Endo S, Johnson DE, Huang L, He Y, Kim JD: **Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo.** *Proc Natl Acad Sci USA* 2000, **97**:4227-4232.
40. Lee TL, Yeh J, Van Waes C, Chen Z: **Epigenetic modification of SOCS-1 differentially regulates STAT3 activation in response to interleukin-6 receptor and epidermal growth factor receptor signaling through JAK and/or MEK in head and neck squamous cell carcinomas.** *Mol Cancer Ther* 2006, **5**:8-19.
41. Kishimoto T: **IL-6: from laboratory to bedside.** *Clin Rev Allergy Immunol* 2005, **28**:177-186.

Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2407/8/34/prepub

Paper IV

doi: 10.1111/j.1365-3083.2008.02072.x

Co-culture of Head and Neck Squamous Cell Carcinoma Spheroids with Autologous Monocytes Predicts Prognosis

K. W. Kross, J. H. Heimdal, C. Olsnes, J. Olofsson & H. J. Aarstad

Section for Otorhinolaryngology/Head and Neck Surgery, Department of Surgical Sciences, Broegelmann Research Laboratory, Haukeland University Hospital, Bergen, Norway

Received 13 June 2007; Accepted in revised form 4 December 2007

Correspondence to: K. W. Kross, ENT-Department, University Hospital Maastricht, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands. E-mail: kkro@skno.azm.nl

Abstract

Co-culture of monocytes with autologous fragment (F) spheroids originating from malignant (M) tumour or benign (B) control mucosa of head and neck squamous cell carcinoma (HNSCC) yields interleukin (IL)-6 and monocyte chemo-attractant protein (MCP)-1 secretion. This study investigates the association between this cytokine co-culture response and prognosis. Analysis of IL-6 and MCP-1 content of supernatants from monocytes *in vitro* co-culture with autologous MF- or BF-spheroids was investigated in a cohort of HNSCC patients (n = 65) diagnosed between 1998 and 2005, all of whom were treated with curative intent by primary surgery. The IL-6 response was expressed as a fraction of the lipopolysaccharid response of the same batch of monocytes. Recurrence, survival and causes of death were then established following the second part of 2005. MCP-1 levels did not predict prognosis. We found that increased levels of IL-6 from autologous monocytes in co-culture with MF-spheroids predicted recurrence with a hazard ratio (HR) of 1.5 [confidence interval (CI): 1.01–2.60; P = 0.05] and co-culture with BF-spheroids and monocytes predicted recurrence (HR = 4.17; CI: 1.54–11.29; P = 0.005). The same results where obtained in addition with TNM stage of the patients. Simultaneous analysis of BF- and MF-spheroid co-culture IL-6 responses as well as adjustment for age and TNM stage of the patients allowed prediction of total survival (HR = 3.1; CI: 1.11–8.56; P = 0.03) based on BF co-culture levels. IL-6 secreted upon *in vitro* co-culture with monocytes and BF-spheroids predicts recurrence and prognosis, whereas co-culture with monocytes and MF-spheroids predicts recurrence.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a cancer disease arising from a microenvironment rich in immune cells [1]. Profound immune activation has been demonstrated in patients with established HNSCC despite the obvious failure of the immune system to eradicate HNSCC tumours upon established clinical disease. Mechanisms responsible for immune system evasion by tumour cells have not been fully elucidated and indeed evidence supports the notion that the immune system in some cases supports tumour growth [2].

The mononuclear phagocyte system (MNP) consists of monoblasts, monocytes (MO), macrophages (MØ) and dendritic cells (DC) forming an important part of both the innate and specific immune system. Cells of the MNP, e.g. present antigens to B- and T lymphocytes as well as co-regulate lymphocytes via direct interaction of CD80/86 to CD28 on lymphocytes [3]. Furthermore, these cells secrete cytokines and other substances, e.g. interleukin (IL)-6, tumour necrosis factor (TNF)-α and monocyte chemo-attractant protein (MCP)-1 [4]. IL-6 is an important pro-inflammatory cytokine [5] but may also stimulate cancer cell proliferation [6] through activation of STAT3 which is associated with deregulated cell growth and neoplasia [7]. IL-6 also stimulates tumour cell invasion into adjacent tissue [8, 9], while TNF-α may promote apoptosis of tumour cells [10]. MCP-1 was originally determined to recruit MO [11], as well as to, e.g. augment IL-6 secretion in MO [12]. MCP-1 may also act as an immune response-limiting chemokine [13]. Thus, evidence suggests that MNP can induce both

apoptosis of tumour cells [14] as well as secrete tumour-stimulating cytokines such as IL-6.

Several investigators have shown that cytokines mainly produced by MNP may be associated with HNSCC biology as measured by prognosis [15]. High serum levels of IL-6 have been shown to herald impaired prognosis in HNSCC patients [15]. We have recently shown that a high level of IL-6 secreted from MO isolated from HNSCC patients, stimulated with endotoxin (lipopolysaccharid, LPS) *in vitro*, is associated with decreased survival [16]. Secretion of MCP-1 from MNP is of potential importance as this C-C chemokine is associated with tumour-associated macrophages (TAM) infiltration in HNSCC [17], which in turn has been associated with HNSCC prognosis [18].

We have studied MO cytokine secretion upon HNSCC tumour stimulation by a modified organ culture model coupling autologous HNSCC tissue and MO in an *in vitro* co-culture system [19]. Samples from HNSCC tumours may be maintained viable as malignant (M) fragment (F)-spheroids *in vitro*. This organ culture technique also enables the establishment of benign (B) F-spheroids originating from adjacent benign mucosa. F-spheroids mainly consist of epithelial (tumour) cells, fibroblasts and macrophages [19]. *In vitro*, MO secrete IL-6 and MCP-1, but not IL-1β or TNF-α following co-culture both with MF- and BF-spheroids [12].The spheroids alone also secrete the same cytokines [20], mainly originating from TAM [21].

The aim of the present study was to study to what extent the magnitude of this co-culture response, as measured by production of IL-6 and MCP-1, is related to prognosis in a cohort of HNSCC patients treated by surgery with curative intent. Obtaining a better understanding of cancer immune responses may establish treatment of HNSCC based on manipulation of the immune system, allowing for improvement in disease-free survival [22].

Materials and methods

Patients. Patients that were admitted for surgery for squamous cell carcinomas of the head and neck were included in the study. Tumour sites are given in Table 1 and TNM stage in Table 2. The Regional Committee for Medical Ethics at the Haukeland University Hospital approved the study. Each patient gave written consent before participating in the study. The study comprised 65 consecutively included patients treated in our clinic between 1998 and 2005. Follow-up was 30 ± 5 months in the survival group and was terminated fall 2005. At the end of the follow-up, 17 of the patients had recurrence of which 15 subsequently died from the disease. In total, 30 patients died throughout the follow-up.

Clinical parameters. Blood levels of haemoglobin, C-reactive protein (CRP), erythrocyte sedimentation level (ESR) and albumin were determined 1 day preoperative and on the day of MO harvest (co-culture), i.e. the 14th postoperative day. Levels were determined according to the standard procedures and performed as part of standard blood sampling tests.

Table 1 Primary tumour sites.

Localization	No.
Lingua	14
Basis lingua	3
Gingiva	10
Mandibula cyst	1
Bucca	2
Palatum molle	1
Floor of the mouth	4
Trigonum retromolare	3
Tonsille	11
Oropharynx	3
Hypopharynx	3
Sinus/nasal cavity	3
Larynx	7
Total	65

Table 2 TNM srage of the included patients.

	N0	N1	N2	N3	Total
T1	6	1	0	0	7
T2	12	1	6	1	20
T3	8	2	6	2	18
T4	13	3	4	0	20
Total	39	7	16	3	65

Fragment spheroid generation. An organ culture model was used by which free floating fragment (F-) spheroids were established as previously described [12]. Biopsies were obtained at surgery from tumour or benign mucosa distally from the frozen sections biopsies. Macroscopically vital tissue was randomly chopped from malignant tissue. Benign tissue was harvested from the epithelial part of the mucosa biopsies. Cubes, with a size of 0.5–1.0 mm, were transferred to agar coated tissue culture flasks (Nunc A/S, Roskilde, Denmark). The fragment spheroids were cultured in Dulbecco's MEM (BioWhittaker, Walkersville, MD, USA) supplemented with 15% heat inactivated fetal bovine serum (FBS) (Sigma, St Louis, MO, USA), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin (2.5 μg/ml), L-glutamine (2 mM) and non-essential amino acid mixture (1%) (BioWhittaker). The cultures were maintained at 37 °C in 5% CO_2 and 95% air with 100% relative humidity. Fragments, which had rounded to spheroid-like structures after 10–14 days *in vitro*, were selected for experiments.

Monocyte and serum preparation. Two weeks postoperatively, peripheral blood mononuclear cells (PBMC) drawn at 7.30 AM, were separated by gradient centrifugation with Lymphoprep^R (Nycomed, Oslo, Norway) as density gradient medium as described previously [12]. The PBMC yield of 8.5 ml blood was allocated to all wells in a 24-well plate (Nunc) with RPMI-1640 supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin (2.5 μg/ml), L-glutamine (2 mM) and 20% autologous serum to a total volume of 0.5 ml per well. After 40-min pre-incubation, the MO were purified by washing, and then cultured in RPMI/20% autologous serum; 0.5 ml per well. The cells were shown to be more than 95% MO positive by non-specific esterase stain. Viability was by this method more than 95% as tested by the tryphan blue stain.

Co-culture. Fourteen days postoperatively, malignant (M) or benign (B) F-spheroids were washed and transferred to freshly isolated MO cultures in 24 × 16 mm well plates (Nunc) with 0.5 ml RPMI/20% autologous serum per well. Four to six spheroids per well were employed. MO cultured in wells with the indicated medium with or without addition of 1 μg/ml LPS from *Escherichia coli* (Sigma) served as controls. The IL-6 secretion rate (RR) was calculated according to the following formula:

$$RR = \frac{\text{pg/ml co-culture secretion} - \text{pg/ml background secretion}}{\text{pg/ml LPS stimulated secretion} - \text{pg/ml background secretion}}$$

IL-6 and MCP-1 ELISA. The contents of IL-6 and MCP-1 were determined by ELISA using MCP-1/IL-6 capture and detection antibody pairs, compared to r-hu MCP-1/IL-6 as standards (R&D Systems Europe Ltd, Abingdon, UK). All procedures were performed according to the specifications of the manufacturer. In short, 96-well microtitreplates (Costar, Corning, NY, USA) were coated overnight at room temperature (RT) with monoclonal mouse anti-human MCP-1 or IL-6 capture antibodies respectively. After blocking, diluted samples and recombinant human respective standards were added and incubated for 2 h at RT followed by the addition of biotinylated polyclonal goat anti-human MCP-1 or IL-6 respectively. The plates were then incubated for 20 min at RT with streptavidin-conjugated horseradish peroxidase. Tetramethyl-benzidine (TMB) (Sigma) and H_2O_2 were used as substrate. Absorbency values were measured at 450 nm using Softmax Pro version 4.0 on an Emax Precision microtitre plate reader (Molecular Devices, Sunnyvale, CA, USA). The lower detection level was 9.4 pg/ml for IL-6 and 15.6 pg/ml for MCP-1.

Statistics. The statistical program package SPSS (Ver. 14; SPSS, Chicago, IL, USA) was used. Figures are given as mean ± standard error of the mean. The groups were compared by the analysis of Student's *t*-test. Survival analyses were performed by Cox regression analysis or Kaplan–Meier [Log Rank (Mantel Cox) test] analyses. With respect to IL-6, analysis was performed both with the results kept as original RR values and dichotomized with RR = 0.50 as cut-off value.

Results

Co-culture IL-6/MCP-1 secretion internal relation and relation to clinical inflammation parameters

We determined that the IL-6 co-culture RR values as well as the MCP-1 co-culture values from the benign versus malignant condition correlated (malignant: $\tau = 0.28$; $P < 0.001$) (Benign: $\tau = 0.53$; $P < 0.001$). We found an inverse correlation between the ESR at diagnosis and the production of MCP-1 by MO in co-culture with MF-spheroids ($\tau = -0.29$; $P < 0.01$). There was a similar trend for co-cultures with BF-spheroids ($P < 0.1$). No correlation was found as to cytokine secretion level and serum CRP levels. There was a significant correlation between the serum albumin level at diagnosis and MCP-1 secretion by MO in co-culture at both BF-spheroid ($\tau = 0.24$; $P < 0.05$) and MF-spheroid ($\tau = 0.32$; $P < 001$) condition.

Recurrence as related to IL-6/MCP-1 co-culture responses

Seventeen patients developed recurrence of disease during follow-up. Comparing co-culture IL-6 secretion levels of these patients to that of patients without recurrence by Student's *t*-test, we found an increased IL-6 supernatant level for BF-spheroids (RR = 0.97 ± 0.39 versus 0.19 ± 0.04 $t = -3.15$; $P = 0.003$) and MF-spheroids (RR = 0.99 ± 0.26 versus 0.45 ± 0.07; $t = -2.75$; $P = 0.008$) (Fig. 1). This was confirmed by Cox regression analysis where, in addition adjusting by age of the patients, we determined a prediction with the IL-6 BF-spheroid (dichotomized with RR = 0.5 as cut-off values) [hazard ratio (HR) = 4.17; confidence interval (CI) = 1.54–11.29; $P = 0.005$] and with the MF-spheroid (HR = 1.5; CI: 1.01–2.60; $P = 0.046$) co-culture response respectively (analyses not shown).

We also performed Cox multivariate regression analysis with RR-transformed MO IL-6 production values from co-culture with MF- or BF-spheroids adjusting for both TNM stage and age of the patients. These analyses confirmed the association between recurrence and MF spheroid secretion with HR = 1.76 (CI: 1.00–3.12; $P = 0.05$) (Table 3). With dichotomized values, BF-spheroid conditions predicted recurrence upon adjusting for age and TNM stage of the patient (HR = 4.0; CI: 1.38–11.4; $P = 0.01$) (Table 3). Kaplan–Meier analysis also showed that a high value for BF-spheroid IL-6 secretion predicted recurrence ($P = 0.003$) (Fig. 2).

Analyses were also performed Cox multivariate regression analyses with age of the patients, TNM stage as well as both benign and malignant RR IL-6 values included.

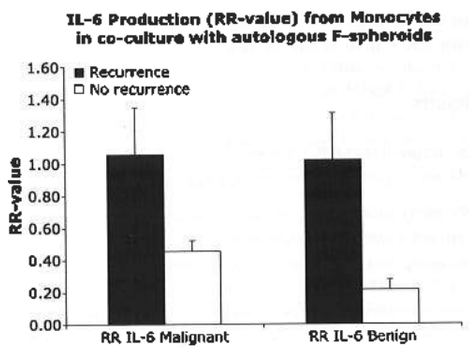


Figure 1 HNSCC patient monocytes were isolated from peripheral blood mononuclear cells 14 days after surgery and co-cultured 24 h *in vitro* with MF- or BF-spheroids. IL-6 levels were determined from supernatants by ELISA analysis and 'Relative to endotoxin/background Response' (RR) values were calculated. Statistics: MF-spheroid co-culture: (t = −2.75) P = 0.008. BF-co-culture: (t = −3.15) P = 0.003.

Theses analyses showed that the unique variance attributed to the benign IL-6 RR co-culture results predicted recurrence (HR = 3.36; CI: 1.03–11.0; P = 0.04) (Table 3).

No association was found between MO co-culture MCP-1 levels and recurrence.

Survival as related to IL-6/MCP-1 co-culture responses

Thirty patients died during follow-up. Their co-culture IL-6 production was compared to that of living patients. An increased IL-6 supernatant level was found for BF spheroids by Student's t-test (RR = 0.68 ± 0.25 versus 0.18 ± 0.05; t = −2.13; P = 0.037) compared such patients with the patients still alive by fall 2005.

Cox multivariate regression analysis was performed with dichotomized RR IL-6 values from BF-spheroid and MF-spheroid co-cultures adjusting for TNM stage and age of patients. This analysis showed a trend towards survival prediction based on BF-spheroid level (HR = 2.2; CI: 0.91–5.10; P = 0.08) (Table 4). This trend was confirmed in a Kaplan–Meier plot (Fig. 3). When benign and malignant IL-6 results were simultaneously included in the Cox multivariate regression analyses, BF-spheroid levels predicted survival (HR = 3.1; CI: 1.11–8.56; P = 0.03) (Table 4). This is also shown in a Kaplan–Meier plot (Fig. 4). MCP-1 co-culture levels were not found to predict survival.

Summary survival & recurrence as related to IL-6/MCP-1 co-culture responses

Figure 5 shows a depiction of the results. The areas of the two ellipses depict the total variance of the benign and malignant RR IL-6 co-culture responses respectively.

Table 3 Recurrence by Multivariate Cox Regression Analyses with co-culture secretion of IL-6 (RR values) adjusted by age, and TN stage of the HNSCC Patients.

	RR values directly scored 95% CI for HR				RR values binomially scored 95% CI for HR			
	HR	Lower	Upper	Sig.	HR	Lower	Upper	Sig.
Age	1	1	1	0.08	1	1	1	0.10
T stage	0.96	0.53	1.73	0.88	0.91	0.54	1.52	0.71
N stage	1.19	0.62	2.28	0.61	1.19	0.62	2.28	0.60
CC Ben. F-spheroid	1.34	0.91	1.99	0.13	4.00	1.38	11.4	0.01
Age	1	1	1	0.10	1	1	1	0.17
T stage	0.83	0.50	1.40	0.48	0.72	0.44	1.20	0.21
N stage	1.25	0.65	2.37	0.50	1.11	0.60	2.05	0.17
CC Mal. F-spheroid	1.76	1.00	3.12	0.05	2.29	0.82	6.41	0.11
Age	1	1	1	0.06	1	1	1	0.10
T stage	0.95	0.52	1.74	0.87	0.77	0.52	1.58	0.77
N stage	1.23	0.64	2.36	0.53	1.18	0.62	2.26	0.61
CC Ben. F-spheroid	0.97	0.53	1.18	0.92	3.36	1.03	11.0	0.04
CC Mal. F-spheroid	1.88	0.75	4.72	0.18	1.43	0.44	4.61	0.55

HR, hazard ratio; 95% CI for HR, 95% confidence interval for HR; Sig., statistical significance; CC, co-culture.

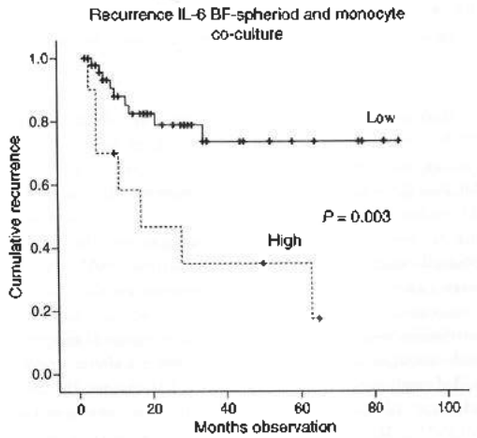


Figure 2 Kaplan–Meier recurrence analysis with the same results as in Fig. 1. The benign IL-6 co-culture RR values were analysed with RR = 0.5 as cut-off value.

The variance of the benign RR IL-6 co-culture response is depicted in dark grey and the malignant RR IL-6 co-culture response in light grey. The unique benign RR IL-6 co-culture variance (dark grey) predicted recurrence and survival, whereas the common area of the two IL-6 co-culture responses predicted recurrence.

Discussion

In this study, we investigated the relations to recurrence and prognosis of *in vitro* IL-6 and MCP-1 secretion

during co-culture of autologous MO with MF- or BF-spheroids 2 weeks following primary surgery in a cohort of HNSCC patients. Associations were found between the amount of IL-6, but not MCP-1, produced in co-culture of MO with F-spheroids and recurrence of disease as well as survival of disease. We determined that MF-spheroid co-culture IL-6 level predicted recurrence, whereas BF-spheroid co-culture IL-6 level predicted both recurrence and survival.

Table 4 Survival by Multivariate Cox Regression Analyses (binomially analysed) with co-culture IL-6 secretion (RR values) adjusted by age, T- and N-stage of the HNSCC patients.

	95% CI for HR			
	HR	Lower	Upper	Sig.
Age	2.96	1.21	7.20	0.02
T stage	1.46	0.64	3.32	0.37
N stage	1.55	0.67	3.57	0.31
CC Ben. F-spheroid	2.10	0.88	5.04	0.09
Age	1.89	0.82	4.33	0.14
T stage	0.97	0.46	2.10	0.97
N stage	1.54	0.69	3.43	0.29
CC Mal. F-spheroid	0.69	0.32	1.49	0.34
Age	2.82	1.15	6.91	0.02
T stage	1.37	0.60	3.15	0.45
N stage	1.55	0.67	3.56	0.31
CC Ben. F-spheroid	2.90	1.05	8.04	0.04
CC Mal. F-spheroid	0.54	0.22	1.34	0.19

HR, hazard ratio; 95% CI for HR, 95% confidence interval for HR; Sig., statistical significance; CC, co-culture.

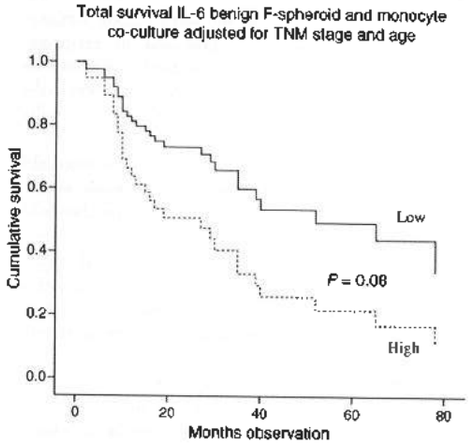


Figure 3 HNSCC patients Kaplan–Meier survival analysis with results obtained as described in legend to Fig. 1. This figure shows a Kaplan–Meier plot as calculated by a Cox multiple regression analysis also including age and TNM stage of the patients. The age and benign IL-6 co-culture RR values were entered into analysis dichotomized with co-culture RR = 0.5 as cut-off value.

Furthermore, we failed to find a significant correlation between cytokine secretion and tumour (T) or node (N) stage in this study (data not shown). This might indicate that MNP function is changed at the early stages of HNSCC disease, although this should be interpreted with limitations as all included patients were treatable with surgery and the majority of the patients had at least a T2 stage disease.

Monocyte chemo-attractant protein-1 is important for MNP aggregation in tumours [23, 24] and levels

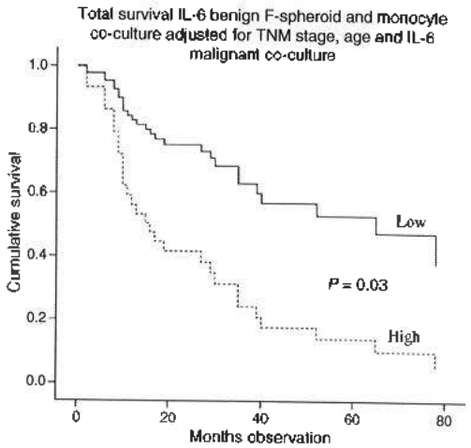


Figure 4 Kaplan–Meier survival analysis with results obtained as described in legend to Fig. 1. This figure shows a Kaplan–Meier plot as calculated by a Cox multiple regression analysis also including age, TNM stage and the MF-IL-6 RR co-culture responses of the patients. The age and IL-6 co-culture RR values entered into analysis dichotomized with co-culture RR = 0.5 as cut-off value.

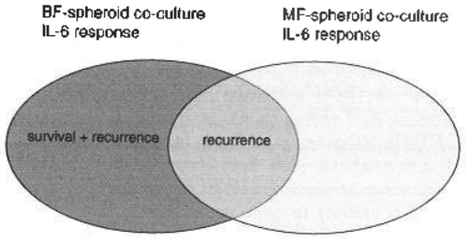


Figure 5 This figure depicts the results of the recurrence and survival analyses as measured by Cox Multiple Regression analyses. The areas of the two ellipses depict the total variance of the benign and malign RR IL-6 co-culture responses respectively. The variance of the benign RR IL-6 co-culture response is depicted with dark grey color and the malignant RR IL-6 co-culture response with light grey colour. The unique benign RR IL-6 co-culture variance (dark grey) predicted recurrence and survival, whereas the common area predicted recurrence.

present upon aggregation may carry prognostic information [18]. MCP-1 may subsequently stimulate IL-6 secretion of MO co-cultured with F-spheroids [25]. Despite this observation, we have not been able to correlate MCP-1 secretion levels to recurrence or prognosis. Other culture conditions may, however, unravel such an association.

Benign as well as malignant co-culture IL-6 responses were associated with recurrence. Statistical analysis suggests that the MF-spheroid co-culture IL-6 prediction is derived from a common variance of the two responses. We also studied Cox regression total survival analyses with both the BF-spheroid and MF-spheroid co-culture responses included together with TN stage and age of the patients and this analysis showed that the unique BF-spheroid co-culture IL-6 variance predicted survival. We were, however, not able to link the MF-spheroid co-culture IL-6 response to survival. This may rely on the experimental conditions as several other studies have shown that IL-6 metabolism is broadly related to survival [26]. We [16] and Clinchy *et al.* [26] have, for example, shown that increased IL-6 secretion from PBMC stimulated with LPS is associated with impaired prognosis in HNSCC patients and patients radically operated for colon cancer respectively.

Both F-spheroids and MO substantially contribute to the co-culture IL-6 response as previously shown, but co-culture MCP-1 secretion is mostly derived from MO [12, 25]. Furthermore, the F-spheroid generated MCP-1 and IL-6 secretions originate to some extent from macrophages residing in the tissue of the F-spheroids [21]. Thus, the present IL-6-based prognosis BF-spheroid associations may be assigned to the serum, the MO, the fibroblasts, the benign mucosa cells and mucosa-associated macrophages (BF-spheroids).

The serum component was studied in more detail by determining serum values of albumin, CRP and ESR values upon MO isolation. None of these variables was associated with the IL-6 co-culture responses arguing against a general inflammatory property of serum influencing the magnitude of the IL-6 co-culture responses.

Lipopolysaccharid stimulates MO through Toll-like receptor (TLR)-4 ending in, e.g. activating IL-6 synthesis via NF-κB activation [5, 27]. A high LPS stimulated MO IL-6 production has been shown to be correlated with a worse prognosis in HNSCC patients [16]. This MO role is unlikely to cause the co-culture IL-6 prediction as co-culture responses were adjusted for MO LPS-stimulated IL-6 production. Other MO functional changes in HNSCC patients may, however, cause the shown prediction.

The shown co-culture IL-6 secretion BF-spheroid secretion prognostic information may be caused by 'field cancerization' as one of several explanations. The concept of 'field cancerization' states that in some patients changed dysplastic epithelia is found throughout mucosa of both the upper and lower respiratory tracts as well as to some extent in the oesophagus, subsequently gives rise to cancer both locally and throughout upper respiratory and oesophageal mucosa. Such dysplastic epithelia may give rise to secondary primary cancer known to kill many successfully treated HNSCC patients [28]. An elevated production of IL-6 from BF-spheroids might be linked to dysplastic-transformed epithelia stimulating MO, as shown by an elevated BF-spheroid co-culture response in patients who died during follow-up.

The importance of IL-6 as to worsening of prognosis of HNSCC patients is in line with published studies both as to properties of serum values of pro-inflammatory cytokines [15] and secretion of pro-inflammatory cytokines by immune cells [16]. The observed significantly higher level of IL-6 in patients with recurrence and/or death might be linked to an IL-6/glycoprotein130 stimulation which activates STAT3 in an EGFR-independent matter [7]. STAT3 activation is associated with cell proliferation and prevention of apoptosis thereby participating in oncogenesis [29] and has shown to be upregulated in HNSCC-tissue and respective in benign mucosa [30]. In this aspect, the present study may be viewed as one of many which show that chronic inflammation may promote cancer growth [31].

Furthermore, numerous previous studies have shown that lymphocytes of the specific immune system express functional changes in cancer patients [1]. It has, for example, been suggested that a high percentage of $CD4^+CD69^+$ T lymphocytes is associated with worse prognosis in HNSCC both as measured among tumour-infiltrating lymphocytes (TIL) [32] and in peripheral blood [33]. The present study adds further strength to the claim that a biologically important interaction takes place between the immune system and malignant cells during tumour development.

Presently, it appears that response above a threshold level of benign co-culture IL-6 secretion is closely associated with recurrence and survival. Above this threshold (RR > 0.5), six of eight patients had recurrence and then subsequently died. No surviving patient had a BF-spheroid IL-6 production above this threshold. Thus, this response may single out patients with high risk of death from cancer disease. These patients could serve as targets for a very cost-efficient follow-up.

A suggestion based on the present work together with our recently published work showing a similar negative prognostic value of increased IL-6 secretion by peripheral blood MO following LPS stimulation [16] together with other published studies may be to employ antibodies against IL-6 as part of HNSCC treatment. Such antibodies have already been implemented in the treatment of juvenile rheumatoid arthritis, and been shown to be safe in that context [5].

Much effort has been put into establishing immunology based treatments of cancer, in particular vaccination [22, 34], and stimulation with biological response modifiers [34] and antibody treatment [22] for the treatment of cancer. The present study adds support to the concept that tumour cells exert influence on the immune system and malignant cells interact with each other. It supports an assumption that also cells of the innate immune system are important elements in the tumour cell-immune system interactions in HNSCC patients. A further disclosure of the interactions between MNP and cancer cells might contribute to development of (adjuvant) immunotherapy in HNSCC.

Acknowledgment

This work has been supported by The Norwegian Cancer Society. We gratefully thank T. Tynning and D.A. Sandnes for excellent technical assistance. We thank statistician Stein Atle Lie, University of Bergen for valuable discussions about statistical methods of analysis for the present work.

References

1 Whiteside TL. Immunobiology of head and neck cancer. *Cancer Metastasis Rev* 2005;24:95–105.
2 Van Ginderachter JA, Movahedi K, Hassanzadeh Ghassabeh G *et al.* Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. *Immunobiology* 2006;211:487–501.
3 Foell J, Hewes B, Mittler RS. T cell costimulatory and inhibitory receptors as therapeutic targets for inducing anti-tumor immunity. *Curr Cancer Drug Targets* 2007;7:55–70.
4 Hume DA. The mononuclear phagocyte system. *Curr Opin Immunol* 2006;18:49–53.
5 Kishimoto T. Interleukin-6: from basic science to medicine–40 years in immunology. *Annu Rev Immunol* 2005;23:1–21.
6 Kamimura D, Ishihara K, Hirano T. IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev Physiol Biochem Pharmacol* 2003;149:1–38.
7 Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gutkind JS. Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system. *Cancer Res* 2003;63:2948–56.
8 Hong SH, Ondrey FG, Avis IM *et al.* Cyclooxygenase regulates human oropharyngeal carcinomas via the proinflammatory cytokine IL-6: a general role for inflammation? *FASEB J* 2000;14:1499–507.
9 Nishino H, Miyata M, Kitamura K. The effect of interleukin-6 on enhancing the invasiveness of head and neck cancer cells in vitro. *Eur Arch Otorhinolaryngol* 1998;255:468–72.
10 Yoshimura A. Signal transduction of inflammatory cytokines and tumor development. *Cancer Sci* 2006;97:439–47.
11 Martinet N, Beck G, Bernard V *et al.* Mechanism for the recruitment of macrophages to cancer site. In vivo concentration gradient of monocyte chemotactic activity. *Cancer* 1992;70:854–60.
12 Heimdal J, Aarstad HJ, Olofsson J. Monocytes secrete interleukin-6 when co-cultured in vitro with benign or malignant autologous fragment spheroids from squamous cell carcinoma patients. *Scand J Immunol* 2000;51:271–8.
13 Gomes RN, Figueiredo RT, Bozza FA *et al.* Increased susceptibility to septic and endotoxic shock in monocyte chemoattractant protein 1/cc chemokine ligand 2-deficient mice correlates with reduced interleukin 10 and enhanced macrophage migration inhibitory factor production. *Shock* 2006;26:457–63.
14 Fidler IJ. Therapy of cancer metastasis by systemic activation of macrophages. *Adv Pharmacol* 1994;30:271–326.
15 De Schutter H, Landuyt W, Verbeken E, Goethals L, Hermans R, Nuyts S. The prognostic value of the hypoxia markers CA IX and GLUT 1 and the cytokines VEGF and IL 6 in head and neck squamous cell carcinoma treated by radiotherapy +/− chemotherapy. *BMC Cancer* 2005;5:42.
16 Heimdal JH, Kross KW, Klementsen B, Olofsson J, Aarstad HJ. Stimulated monocyte IL-6 secretion predicts survival of patients with head and neck squamous cell carcinoma. *BMC Cancer* (in press).
17 Ueno T, Toi M, Saji H *et al.* Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin Cancer Res* 2000;6:3282–9.
18 Marcus B, Arenberg D, Lee J *et al.* Prognostic factors in oral cavity and oropharyngeal squamous cell carcinoma. *Cancer* 2004;101:2779–87.
19 Heimdal JH, Aarstad HJ, Olsnes C, Olofsson J. Human autologous monocytes and monocyte-derived macrophages in co-culture with carcinoma F-spheroids secrete IL 6 by a non-CD14-dependent pathway. *Scand J Immunol* 2001;53:162–70.
20 Kross KW, Heimdal JH, Olsnes C, Olofsson J, Aarstad HJ. Head and neck squamous cell carcinoma spheroid- and monocyte spheroid-stimulated IL-6 and monocyte chemotactic protein-1 secretion are related to TNM stage, inflammatory state and tumor macrophage density. *Acta Otolaryngol* 2005;125:1097–104.
21 Kross KW, Heimdal JH, Olsnes C, Olofson J, Aarstad HJ. Tumour-associated macrophages secrete IL-6 and MCP-1 in head and neck squamous cell carcinoma tissue. *Acta Otolaryngol* 2007;127:532–9.
22 McKechnie A, Robins RA, Eremin O. Immunological aspects of head and neck cancer: biology, pathophysiology and therapeutic mechanisms. *Surgeon* 2004;2:187–207.
23 Kleine-Lowinski K, Gillitzer R, Kuhne-Heid R, Rosl F. Monocyte-chemo-attractant-protein-1 (MCP-1)-gene expression in cervical intra-epithelial neoplasias and cervical carcinomas. *Int J Cancer* 1999;82:6–11.
24 Koide N, Nishio A, Sato T, Sugiyama A, Miyagawa S. Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus. *Am J Gastroenterol* 2004;99:1667–74.
25 Heimdal JH, Olsnes C, Olofsson J, Aarstad HJ. Monocyte and monocyte-derived macrophage secretion of MCP-1 in co-culture with autologous malignant and benign control fragment spheroids. *Cancer Immunol Immunother* 2001;50:300–6.
26 Clinchy B, Fransson A, Druvefors B *et al.* Preoperative interleukin-6 production by mononuclear blood cells predicts survival after radical surgery for colorectal carcinoma. *Cancer* 2007;109:1742–9.
27 Urba S, Wolf G, Eisbruch A *et al.* Single-cycle induction chemotherapy selects patients with advanced laryngeal cancer for combined chemoradiation: a new treatment paradigm. *J Clin Oncol* 2006;24:593–8.
28 Ha PK, Califano JA. The molecular biology of mucosal field cancerization of the head and neck. *Crit Rev Oral Biol Med* 2003;14:363–9.
29 Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. *Oncogene* 2000;19:2474–88.

30 Grandis JR, Drenning SD, Zeng Q *et al.* Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo. *Proc Natl Acad Sci U S A* 2000;97:4227–32.
31 Van Waes C. Nuclear factor-kappaB in development, prevention, and therapy of cancer. *Clin Cancer Res* 2007;13:1076–82.
32 Badoual C, Hans S, Rodriguez J *et al.* Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res* 2006;12:465–72.
33 Aarstad HJ, Heimdal JH, Klementsen B, Olofsson J, Ulvestad E. Presence of activated T lymphocytes in peripheral blood of head and neck squamous cell carcinoma patients predicts impaired prognosis. *Acta Otolaryngol* 2006;126:1326–33.
34 Herold-Mende C, Karcher J, Dyckhoff G, Schirrmacher V. Anti-tumor immunization of head and neck squamous cell carcinoma patients with a virus-modified autologous tumor cell vaccine. *Adv Otorhinolaryngol* 2005;62:173–83.

