MONOCYTES, DENDRITIC CELLS, MACROPHAGES, T CELLS AND HEAD AND NECK CANCER

The effect of a thymic hormone preparation in restoring defective immune functions

MONOCYTEN, DENDRITISCHE CELLEN, MACROFAGEN, T-CELLEN EN HET HOOFD/HALS CARCINOOM

Het effect van een thymushormoonpreparaat op het herstel van gestoorde afweer

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CONTENTS

Chapter 1

GENERAL INTRODUCTION

It is generally accepted that cell mediated immunity (CMI) has more importance in the control of cancer than the antibody-mediated immune response. The cell mediated immune response is the basis of the so-called natural host resistance to cancer, which is also referred to as "immunosurveillance". Although the concept of immunosurveillance has been much debated over the past decades, there is now no doubt that suppression of the immune function increases the incidence of a few types of cancer. Also, spontaneous regression has been observed for some tumors, including melanoma, renal cell carcinoma, and lymphoma, and evidence for a role of immunosurveilance is well supported in these tumors [1-3].

The role of a cell mediated immunosurveilance in patients with a head and neck squamous cell carcinoma (HNSCC) is less clear. Immunosuppressed patients do not develop head and neck cancer more frequently and spontanous regression is at most anecdotal. Despite this, defects of the CMI in HNSCC patients have extensively been documented. One of the first tests to reflect the status of the CMI which was found abnormal in HNSCC patients, was delayed type hypersensitivity (dth) as measured by skin reactions to DNCB (2,4-dinitrochlorobenzene). Ninetyfive percent of the normal adult population react with a dth reaction towards skin-applied DNCB, however such a positive reaction is often absent in HNSCC patients [4]. These observations have not led to a present clinical use of DNCB skin testing, but over the past decades other, more specified defects of the CMI have become clear. These defects are predominantly caused, on one hand by outside factors, like smoking, alcohol and malnutrition, on the other hand are CMI defects caused by factors resulting from, or produced by the tumor itself. In an effort to restore the immune defects, and to neutralize at least some of these immunosuppressive factors, or simply to boost the immune system, various immunotherapies have been attempted in HNSCC patients. These efforts have at present not led to positive clinical results that justify the widespread use of these strategies. Furthermore, it is not at all clear if the incapacity of the immune system to deal with tumor growth is the direct result of these so-called "defects" in the function of its various components and cells, or that the cancers have yet other more important mechanisms to escape a presumed immunosurveillance. Given the more widespread application of CD markers to identify various leucocytes (table I); given the ongoing discovery of new cytokines and growth factors, their role in tumor growth (control) and their potential therapeutic qualities; given the rapid development of new laboratory techniques including tumor genetics, and the lack of progression in treatment outcomes of HNSCC patients over the past 3 decades, head and neck cancer immunology will continue to be an important field of research.

HNSCC tumor antigens, T lymphocyte recruitment and T lymphocyte stimulation

Head and neck carcinomas are infiltrated by lymphocytes referred to as TILs

Table I. **CD markers respectively for T cells, B cells, monocytes/macrophages, dendritic cells and NK cells**

CD	Name	Function
T cells		
CD2	T11 antigen; LFA-2	Receptor for T cell activation; ligand for LFA-3
CD3	T3 antigen	Associated with TCR; signal transduction from TCR to cytoplasm
CD4	T4 antigen	Involved in MHC-class II restricted antigen recognition
CD8	T8 antigen	Involved in MHC-class I restricted antigen recognition
CD11a	LFA-1 antigen	Adhesion molecule binds to ICAM-1 and ICAM-2
CD25	Tac antigen	IL-2 receptor/activation T cells (B cells and macrophages)
CD28	Tp44 antigen	Receptor for B7/BB-1 antigen on activated B-cells/ T cells proliferation
CD45	LCA; T200 antigen	Function unknown, common leukocyte antigen
CD45RO	Restricted LCA	Activated (memory) T cells
CD45RA	restricted LCA	Virgin T cells, monocytes
B cells		
CD5	T1 antigen	Function in T cell proliferation, unknown function in B cells
CD19	Pan-B cell antigen	Function in B cell activation
CD20	B cell antigen	Function in B cell activation
CD22	B cell antigen	Function in B cell adhesion and activation
Smig	Surface membrane immunoglobulin	Binding molecule for antigens
Monocytes/macrophages		
CD11b,c	Adhesion molecule on monocytes/macrophages	MAC-1 antigen, p150-95 antigen; associated with CD18 antigen/adhesion molecule
CD14	Monocytic antigen	LPS receptor
CD68	Macrophage antigen	Function unknown
Dendritic cells		
CD1	T6 antigen	MHC-like protein (antigen-presentation)
CD83	HB15 molecule	Function unknown
S100		intracellular growth factor
NK cells		
CD16	FcγR111	Low affinity Fc receptor for IgG (also present on macrophages)
CD56	NCAM	Function unknown
CD57	Human natural killer cell antigen	Function unknown

CD : Cluster of differentiation
CR : Complement receptor
ICAM : Intercellular adhaesion molecule
IgG : Immunoglobulin G
LCA : Leukocyte common antigen
LFA : Leukocyte function antigen
LPS : Lipopolysaccharide
MHC : Major histocompatibility complex
NCAM : Neural adhaesion molecule
TCR : T cell receptor

Adapted from: "Immunofenotypering in de diagnostiek: indicatiestellingen, uitvoering en interpretatie (Eds. van Dongen JJM, Groeneveld K, Adriaansen HJ, Hooijkaas H), Rotterdam, Erasmus Universiteit, 1994.

(tumor infiltrating lymphocytes). This infiltrating lymphocyte population constists mainly of CD3+CD4+ T cells (with mainly a helper function) and CD8+ T cells (with mainly a cytotoxic function).

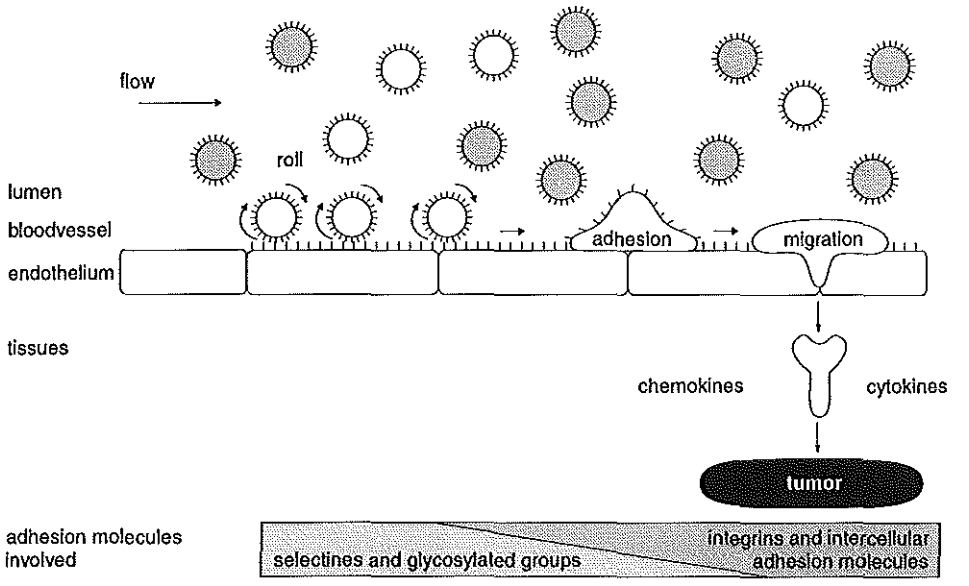

Fig. 1. A schematic representation of the attraction of blood leukocytes to a tumor area. Factors including cytokines and chemokines released by the tumor affect the blood flow and endothelium (expression of adhesion molecules) such that marginating leukocytes start to roll, become activated, adhere and transmigrate. Selectines and glycosylated groups are predominantly involved in the early process of rolling, whereas integrins and intercellular adhesion molecules are predominantly involved in a later firm adhesion and transmigration of the leukocytes. Chemokines released by the tumor direct the migration of the leukocytes towards the cancer cells.

The process of lymphocyte recruitment into a tumor includes different steps (fig. 1): (a) activation of the endothelium in the cancer area and (b) rolling of the lymphocytes along the vessel wall, followed by (c) activation of the lymphocytes resulting in a firm adhesion, and finally (d) extravasation of the cells (crawling along the endothelium, diapedesis, and migration into the carcinomatous tissue), presumably in response to a chemoattractant gradient [5]. In the tumorous area locally released factors may (a) activate the endothelium, (b) attract and/or (c) activate the leukocytes. Factors which can activate the endothelium are cytokines like IL-1α, IFN-γ and TNF-α, leukotrienes, trombin and histamin [6-8]. Activated endothelial cells express high levels of adhesion molecules such as P-selectin (GMP-140, PADGEM), E-selectin, ICAM-1 and VCAM-1 [6,9-11], which make them more adhesive for the passing leukocytes. Furthermore, activation of the endothelium increases vascular permeability and vasodilatation [12]. Chemokines can be trapped on the luminal surface of the endothelial cells and therefore promote attraction and activation of the passing leukocytes [13]. Activation of the passing leucocytes upregulate their integrin expression leading to a much stronger adherence to the endothelium. Hence, a network of activating and chemoattractive factors is emerging that will able marginating lymphocytes to adhere and transmigrate through the endothelial wall into the tissue.

After migration into the tissues the infiltrating T cells must recognize antigens

for further activation. If and how T cells recognize autologous human cancers is not known, but the concept of immunosurveillance implies that T cells are capable of detecting antigens specific for malignant cells, the tumor-associated antigens (TAA). These TAA are in case of head and neck cancers related to various oncofetal antigens, the epidermal growth factor receptor, viral antigens, and a number of surface glycolipids and glycoproteins and other TAA (table II)

Table II. Tumor-associated antigens and monoclonal antibodies raised against these antigens in head and neck malignancies

Antigen	Monoclonal antibody
CEA	anti-CEA-MoAb (clone F023C5)
OFA (phase-specific 44 and 200-kDa antigens)	MoAb 115
22-kD surface antigen	E48 F(ab')2
Poorly differentiated basal cells (50-55 kDa)	K984
Highly differentiated suprabasal cells	K928
A-431 tumor cell line	UCD/AB 6.01
Cell lines 183A, 1483	MoAb R1-EGFR
Nuclear protein	Ki-67
Transferrin receptor	RPN-511
17-1A, epithelial cell surface	K931
Basal membrane structure	UM-A9
Cytokeratin-associated	174H.64
Membrane surface (48 kDa)	SQM1

CEA = carcinoembryonic antigen
OFA = oncofetal antigen
GCDFP = gross cystic disease fluid protein
Adapted from M. Hopsu and K.J.A. Kalremo, Acta Oncol. 32:735, 1993

[14]. Oncofetal antigens include carcino embryonic antigen (CEA) and a 44- and 200 kD oncofetal antigen recognized by a monoclonal antibody (moab) 115. Viral antigens are mainly related to the human papilloma virus (HPV) 16 E gene products, but are only present in a minority of the head and neck cancers [15]. Epstein-Barr Virus (EBV) related antigens do occur only in nasopharyngeal-carcinomas [16].

Aberrant glycosylation is a common feature of malignancy. Alteration in cell surface carbohydrates accompanies malignant transformation which results in the appearance of TAA influencing both tumor growth and spread. Changes in cell surface glycoconjugate expression may result from absence or activation of new tumor-related glycotransferases. It is therefore not surprising that cell-lines from squamous cell carcinomas of the head and neck are characterized by the presence of receptors for various lectins (detecting glycosylated structures) such as for ConA, PNA, DBA and WGA [17].

A considerable number of yet other TAA that are relatively specific for HNSCC has also been discovered via monoclonal antibodies (moabs) that were developed with the aid of HNSCC cell-lines or tissue specimen. These include amongst others the moab E 48, recognizing a 22 kD surface antigen [198; the moab K931, recognizing the so-called 17-IA surface antigen [19]; the moab UM-A9, recognizing a basal membrane structure [20]; the moab 174H.64,

recognizing a cytokeratin-associated antigen [21], and the moab SQM1, recognizing a 48 kD membrane surface antigen [22].

T cells will only recognize TAA when they are presented as short linear peptides bound to major histocompatibility complex (MHC) molecules on the surface of cells (fig. 2) [23]. In this sense, CD4+ T cells are MHC class II "restricted", while CD8+ T cells are MHC class I "restricted". MHC class I molecules are expressed by nearly all cells in the body; MHC class II is mainly expressed on special cells, the so-called professional antigen presenting cells (APCs), including dendritic cells, macrophages, B cells and some subsets of T cells. Dendritic cells (DC) are, however, the most efficient (professional) antigen-presenting cells (APC) due to a variety of factors, amongst which are the earlier mentioned constitutive expression of MHC class II molecules, but also the expression of costimulatory and adhesion molecules, and other characteristics such as motility [24,25].

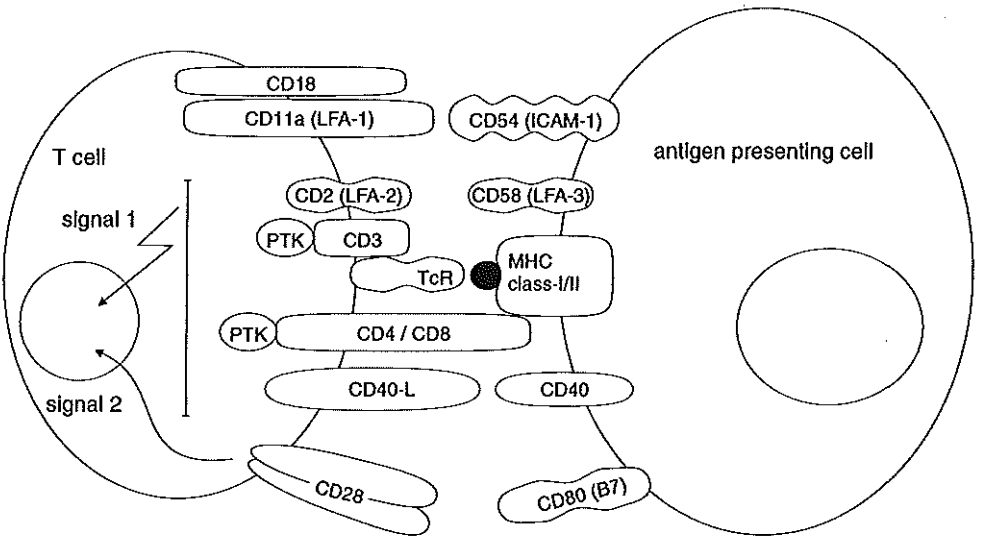

Fig. 2. A schematic representation of the various ligand-receptor interactions between an antigen-presenting cell and a T cell necessary to elicit the effective triggering of the T cell. Firstly there is the antigenic peptide (black dot) in the groove of the MHC-class I or II molecule that specifically triggers the T cell receptor (TcR) via the CD3 complex, also involving CD4 or CD8 signalling (in the case of MHC class II and I respectively). There are further activation signals necessary for giving signal 1 in the T cell in the form of the triggering of the CD2 molecule (by CD58), the CD11/CD18 complex (by CD54) and the CD40-ligand molecule (by CD40).
If only signal 1 is given the T cell becomes anergic. For an effective activation of the T cell signal 2 is necessary. This signal is given via a stimulation of the CD28 molecule (by CD80 or B7-1). PTK = phosphotyrosine kinase 2nd messenger systems.

The genes encoding for both MHC class I and II molecules are members of the immunoglobulin supergene family. These genes in the human species are arranged on chromosome 6 [26]. MHC class I genes encode for the Human Leucocyte Antigens (HLA) A, B, and C. MHC class II genes encode the HLA DP,

DQ, and DR antigens. The encoded HLA molecules are dimers, and comprise an α and β chain. The α-chain of the MHC class I molecules is encoded in the MHC genes, the β-chain is termed beta-2-microglobulin and is encoded on a separate chromosome.
The overall structures of the class I and II MHC molecules are comparable [27]. The molecular confirmation of the chains forms a groove in which the antigenic peptide is presented. Thus the ability of antigenic peptides to be associated with class I or class II MHC molecules is governed by the actual molecular confirmation (tertiary structure) of the antigen-binding groove. It is therefore not surprising that organisms with a particular genetic make-up of MHC class I and II molecules have a special capacity to generate immune responses towards specific microbial and 'self' antigens.
An additional molecule, known as the invariant chain, is intimately involved in the biology of HLA class II molecules. The invariant chain is a membrane glycoprotein, encoded by a non-HLA gene on chromosome 5. The "invariant" designation stems from the observation that, in contrast to the extensive polymorphism of some class II α and all class II β chains, the invariant chain is nonpolymorphic. It forms a trimer with the class II α and β chains in the endoplasmic reticulum during biosynthesis of the MHC-class II molecules and directs the trafficking of the trimer through the posttranslational machinery of the cell to the endosomal compartment. Current evidence indicates that the invariant chain also prevents peptides from binding in the class II groove until the class II molecule is delivered to the endosome. The invariant chain then dissociates from the class II molecule, which can consequently bind antigenic peptides processed from exogenous antigens taken up by the APC and degraded in its lysosomal compartment. The latter fuses with endosomes [28-30]. The complex of the class II molecule with its bound peptide is then transported to the cell membrane; however, the mechanism of transport is still unclear. Due to this intracellular pathway, exogenous antigens are mainly presented in association with MHC class II molecules [31]. Peptides associated with MHC class II molecules can only be presented to CD4+T cells [32], since the CD4 molecule is a special receptor for the MHC class II molecules.
Endogenous antigens, e.g. viral antigens, are degraded by the low molecular mass polypeptide complex present in the cytoplasm [33]. Peptides are then delivered to the MHC class I molecules in the lumen of the endoplasmatic reticulum. After incorporation of the antigenic peptides into the groove and exposure of the MHC class I molecules on the cell surface, only CD8+T cells are able to recognize such peptides, since the CD8 molecule is the special receptor for MHC class I molecules. After recognition, CD8+T cells are able to kill the cell presenting the endogenous antigen [23,34]. Neither class I nor class II MHC molecules can distinguish between 'self' and 'non-self' [35,36].

It must however be noted that the preferential association of exogenous antigens with MHC class II molecules and endogenous antigens with MHC class I, is not an absolute dogma [31,37], and that endogenous TAA can therefore end up in the MHC class II molecules of tumor cells when expressed. They can also end up in the MHC-class II molecules of tumor-infiltrated, professional APCs if tumor antigens are released (for instance from necrotic cells) and

thereafter taken up by these APCs (fig. 3).

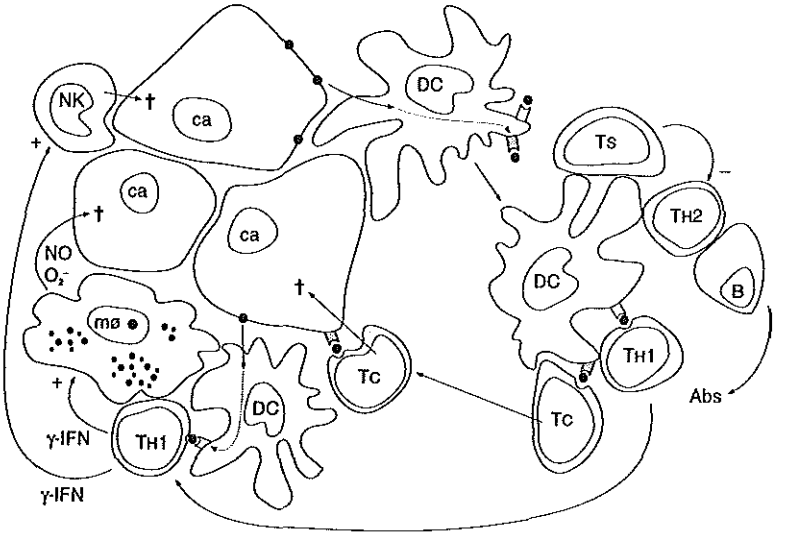

Fig. 3. A schematic representation of the CMI response towards a group of cancer (ca) cells. Antigen presenting cells, in particular dendritic cells (DC) take up tumor-associated antigens (•), degrade these and place antigenic peptides in the groove of either MHC-class II molecules or MHC-class I molecules. These DC stimulate with these antigenic peptides $CD4^+$ T helper 1 (Th1) cells or $CD8^+$ cytotoxic T cells (Tc) respectively. For antibody production (Abs) by B cells (B) the generation of T helper 2 (Th2) cells is necessary. In the immune reaction there exist suppressive forces here symbolized by Ts cells.
When antigen-specific Tc cells recirculate to the tumor they will recognize the antigen-MHC-class I construct on the tumor cell, whereafter they are able to kill the tumor cells.
When antigen-specific Th1 cells recirculate to the tumor they will recognize the antigen-MHC-class II construct on locally present APC, whereafter they start to secrete interferon-γ (γ-IFN) which will activate NK cells and macrophages (mϕ). The latter will produce in these circumstances lethal amounts of oxidative radicals and nitric oxide (NO).

The complex of MHC molecule-antigenic peptide-T cell receptor (TcR) is insufficient for an adequate activation of the T cell (fig. 2). For full activation, the interaction of other accessory molecules on APC with their ligands on T cells is needed, such as the interaction of adhesion molecules [38-40]. The binding produced by these adhesion molecules predominantly strengthens the interaction between the MHC-antigenic peptide-TcR interaction, but also transduces signals that activate the T cell. Important adhesion molecules are Leucocyte Function Antigen 1 (LFA-1) that interacts with Intercellular Adhesion Molecule 1 (ICAM-1), and Leucocyte Function Antigen 3 (LFA-3) that interacts with CD2. Inhibition in this process by monoclonal antibodies to either one of these adhesion molecules inhibits the activation and clonal expansion of T cells [38].
Apart from the adhesion molecule-ligand interaction, the interaction of so-called "costimulatory molecules" on APCs and T cells is essential for further T cell activation and T cell clonal expansion. Costimulating signals are predominantly

provided by the binding of the B7-1 (CD80) molecule on the APC to the CD28 molecule on the T cell (fig. 2) [41,42]. Additional binding of T lymphocytic CTLA-4 (Cytolytic T lymphocyte associated antigen) to B7-2 (CD86) molecules on the APC also takes place but it occurs probably later in the process, since CTLA-4 is primarily seen on the T cells after activation [43]. If these co-stimulatory signals are not provided, the result is T cell anergy (a state of specific non-responsiveness of T cells) [44]. Hence to avoid a state of T cell anergy for a tumor, it is needed that the tumor cell expresses a full picture of the costimulating molecules or that the tumor is sufficiently infiltrated with professional APCs, such as the DC (fig. 3).

Functional impairments of T lymphocytes in HNSCC

The first reports on immunological disorders in HNSCC addressed total lymphocyte counts in peripheral blood. A decreased number of circulating lymphocytes was associated with the presence of a head and neck cancer and correlated with a poor prognosis; later studies made it clear that the total lymphocyte count was a poor predictor of prognosis, but rather reflected the general status of the patient [45]. Furthermore, both decreased and normal B-cell numbers have been described in head and neck cancer patients, and an evaluation of lymphocytic subsets seems to be of little relevance [45].

A number of investigators have considered it a favorable prognostic factor if a solid (head and neck-) tumor is densly infiltrated with T cells [46-48]. However this positive relationship has not been confirmed in later large scale studies, and other studies have contradicted these earlier findings in HNSCC and other tumors [49]. Similarly, a number of studies have tried to correlate the presence of various specific lymphocyte subsets in the tumor to prognosis, again with conflicting results. Snyderman [50] found that tumors with a low CD4/CD8 ratio in the tumor infiltrate had less extensive disease, while Wolf [51] observed a significantly longer survival in patients with a denser CD4+ T cell infiltration. In another study by Wolf [52] on lymphocyte subsets in peripheral blood, an increased T4/T8 ratio was correlated with advanced tumor stage, but others [53] were unable to observe any correlation between intratumoral T cell subsets and prognosis in HNSCC. It is worthy to note that the distribution of T cell subsets in peripheral blood does not correlate with that in tumor infiltrates [50]. T cells infiltrating into a tumor area, may respond to the TAA or to antigens induced by an infection or inflammation associated with tumor growth. With the evolvement of new techniques like the polymerase chain reaction (PCR) and reverse transcribed polymerase chain reaction (RT PCR), it has become possible to analyse the molecular structure of T cell receptor gene arrangements. Recent studies have made it increasingly clear that TILS in head and neck squamous cell carcinomas are at least partly composed of tumor cell-specific CD4+T lymphocytes and CD8+T lymphocytes [54,55], and that unique T cell populations are clonally amplified, which could be the result of an antigen driven selection [56].
However, if such TAA specific T cells do exist in tumors why is it that when a

tumor becomes clinically evident, that the immune system has failed to eliminate the cancer cells? To shed more light on this dilemma, it is of relevance that functional impairments of lymphocytes in HNSCC have been well documented over the past two decades without providing a direct answer to this question. *In vitro,* peripheral blood lymphocytes of healthy individuals proliferate when stimulated with Concanavalin A (Con A) or Phytohemagglutinin A (PHA). This mitogen response has been shown to be decreased in HNSCC patients and the severity of this impairment is correlated to tumor stage [57-63]. More recent studies on functional T cell impairments have focussed on the ability of T cells to lyse cancer cells. TILs isolated from HNSCC and other tumors have poor cytolytic function immediatly after isolation. However after *in vitro* culture of the same cells in the presence of interleukin-2 (IL-2), these cells are potentially capable of eliminating tumor cells [64-69]. Progressive cancer has been associated with a diminished production of IL-2 and other cytokines by TILs as well as by lymphocytes from regional lymphnodes in HNSCC and other tumors [63,70,71]. Other functional defects in the pathway of target cell killing by cytotoxic T cells have been postulated as contributary to the inability of the immune system to erradicate cancer cells in general and HNSCC cells specifically. These functional defects include
a) defects in the function of professional APC (see below),
b) the production of immunosuppressive factors like TGF-β by the tumor [72],
c) a down regulation of cell surface molecules [71] important in the T cell-target cell interaction like intercellular adhesion molecule-1 (ICAM-1), Fas/APO-1 (molecules involved in apoptosis), and the B7-1 and 2 (CD80/CD86) molecules, or
d) a poor expression of MCH class I molecules by cancer cells [1,73].
Little is known about the relevance of these findings, and especially in HNSCC few reports address this matter.

Natural Killer cells in HNSCC

Apart from CD3+T cells there is yet another subset of lymphocytes important in a cell mediated reaction against cancer cells. These are the natural killer (NK) cells (fig. 3). NK cells are lymphocytes with distinct morphological and functional properties. Their cell surface lacks the expression of CD3, CD4, and CD8 surface antigens, but expresses CD6, CD16, and CD56 antigens through which NK-cells can be identified using monoclonal antibodies. NK-cells recognize empty MHC molecules (MHC molecules not loaded with peptide) for target cell interaction, and it is unknown which determinants on the tumor cells cause activation of NK-cells. Direct contact with the target is necessary for target cell lysis through membrane perforating molecules [74]. Most human tumors, including HNSCC, are relatively NK-cell resistant, and NK-cells are rarely present in tumor biopsies [64,66,71,75,76]. In head and neck cancer, high peripheral NK-cell activity, which has been defined as the ability of lymphocytes to lyse tumor cells *in vitro* without the need for activation, has been correlated with a prolonged disease free survival, and it has been reported that patients with low NK-cell activity had a higher risk of developing metastasis [76,77]. NK-cell activity is often considered to play a more important role in control of

metastatic disease than of local disease [44,68,75]. Recent *in vitro* studies [64,69] have shown that in contrast to resting NK-cells, IL-2 activated NK-cells are capable of killing HNSCC cell lines and entering HNSCC spheroids (which are an *in vitro* model for *in vivo* growing tumors) as well as HNSCC implanted in immune deficient mice. The clinical relevance of these findings is however not yet known.

Macrophages and dendritic cells in HNSCC

Macrophages and DC mainly originate from blood monocytes that have lineage restricted precursors in the bone marrow [78]. The majority of monocytes in the peripheral blood express CD14 antigens on their cell membrane, by which they can be identified using anti-CD14 monoclonal antibodies. Once differentiated into macrophages and DC in organs and soft tissues, they lose this marker. Macrophage and DC recruitment is an important phenomenon in CMI, and specific adhesion molecules and chemokines are essential to initiate chemotaxis. A potent chemoattractant for monocytes is complement factor C5a [79,80]. Factors which activate and attract monocytes are leukotriene LTB4, cytokines like TNF-α, TGF-ß, GM-CSF and M-CSF, and various chemokines [5, 80-86]. Major chemokines for monocytes are MCP-1, MCP-2, MCP-3, RANTES, MIP-1α and MIP-1ß [87].

Macrophages can be activated by bacterial products or cytokines, and contribute directly to tumor cell killing (fig. 3). Their main function is scavenging via phagocytosis and pinocytosis. The secretion of cytokines like TNF-α, IL-1, and IL-6 and the production of oxygen and nitrogen metabolites are also important in scavenger function [88]. The exact role of macrophages in tumor control is unknown. Tumor infiltrating macrophages (TAM) are not only known for their cytotoxic/phagocytic capabilities, but also to produce factors that enhance tumor growth [88]. The number of TAM have been linked to enhanced tumor growth in murine sarcomas and carcinomas [89]. On the other hand, other studies have indicated that a large number of macrophages within a tumor was linked to a favorable prognosis [45,90].
Functional impairments of both macrophage and monocyte function have been documented in head and neck cancer patients. Cameron [91] reported a non-toxicity of macrophages towards tumor cells in a majority of HNSCC patients, a phenomenon also documented for other human tumors [92,93]. With respect to monocytes, Garraud [94] reported a decreased expression of HLA-DR (class II) antigens, and a decreased capability to secrete IL-1 when stimulated with LPS. It has also been extensively documented that the monocyte chemotaxis of peripheral blood monocytes of HNSCC patients is impaired [95-99]. The reduced chemotactic capabilities are attributed to a low molecular mass factor with structural homology to p15E, the capsular protein of murine and feline leucemogenic retroviruses, an analogue of which may be produced by HNSCC (see below) [100,101]. Removal of the tumor results in a restoration of monocyte chemotaxis, as measured by the monocyte polarization assay [102].

DC are the most potent, professional antigen presenting cells in the immune system, vital for T cell mediated immunity [24,78]. DC are unique APC in that they are the only APC that are able to effectively stimulate naive ($CD45RA^+$) T cells [103-105]. Recent investigations lead to the idea that the DC population is heterogeneous with respect to ontogenety [106]. It is certainly heterogeneous with respect to morphology, the expression of adhesion molecules and in cytokine production [107-110]. With regard to ontogenety, part of the DC populations is monocyte-derived and closely associated with macrophages, while other DC may have a separate precursor [111]. DC are found in virtually all tissues and organs of the body. The DC of the epidermis and dermis is known as the Langerhans cell. Langerhans cells contain peculiar Birbeck granules that are not seen in DC in other organs, apart from the thymus. Langerhans cells of the skin and DC of the gut-wall are considered as early (immature) stages in the differentiation of the cell, with an excellent capacity to pick up antigens and to degrade these to antigenic peptides and place these peptides in the groove of the MHC-molecules. Skin Langerhans cells and gut DC have been shown to migrate into the afferent lymph as veiled cells to the skin- and gut-draining lymphnodes [24]. These cells can be seen as interdigitating cells in the T cell-areas of these draining lymph nodes, and these stages of the DC are considered as mature stages of the cell with an excellent capacity to stimulate T cells [112]. It seems likely that DC from other peripheral structures, including HNSCC may undergo similar migration and maturation (fig. 3).

Whereas macrophages are a clear source of cytokines like interleukins (IL), such as IL-1, IL-6, and TNF-α, DC have been shown to produce the mRNAs of these cytokines, without a noteworthy production of the actual products [111,113,114]. In general DC are regarded as poor producers of cytokines, and their excellent APC function lies probably in their migratory capacity, their capability to form clusters with T cells via adhesion molecules [115,116] and their high expression of costimulatory molecules such as B7-1 and B7-2 (CD80/CD86) [117].

DC play a role in tumor immunity by presenting TAA to naive T cells. There are no specific monoclonal antibodies that stain for DC's, but a combination of semi-specific monoclonal antibodies, like S100, CD1a, RFD-1 or L25 together with morphological characteristics readily identifies human DC.

A dense infiltration of a tumor with DC has been linked to a favorable prognosis in carcinomas of the stomach [118-120], lung [121], bladder [122], esophagus [123], and uterine cervix [124]. Less work on HNSCC has been done regarding this subject, but similar prognostic correlations were found for nasopharyngeal carcinoma's [52,125-127] and laryngeal carcinoma's [128]. With regard to functionality of these locally accumulated DC again, defects in DC function have been described in animal tumor models, and HNSCC. Gabrilovich [129] showed that DC isolated from tumor-bearing mice showed a reduced ability to induce a cytotoxic T cell response in healthy control animals, while DC from control animals significantly increased specific cytotoxic T cell responses in the tumor-bearing mice. In a study of 44 HNSCC patients, Tas [94] found that in 26 the capability of DC prepared from peripheral blood to form cell clusters with allogeneic lymphocytes was reduced compared to healthy controls. This

impairment could be restored *in vitro* by the addition of the calf thymus extract thymostimulin (TP-1).

Table III. Head and neck cancer-derived (serum)factors and their reported effects on various cells of the CMI.

	T cell function	NK cell function	LAK cell functions	monocyte/ macrophage functions	dendritic cell functions
>75 kd serum factor			↓		
IS	↓				
IAP	↓				
p15E-like factors	↓	↓		↓	↓
phospholipid metobolites (PGE2)	↓	↓	↓	↓	
TGF-β	↓	↓		↓	
IL-10	TH1:↓/TH2:↑				
GM-CSF	↓ (indirect)			↑	
>30kd factor in supernatant of PCI-50	↑	↑			

IS = immunosuppressive substance, IAP = immunosuppressive acidic protein, TGF-β = transforming growth factor-β, IL-10 = interleukin-10, GM-CSF = granulocyte macrophage colony stimulating factor, PCI-50 = head and neck cancer tumor cell line, blank spaces = not tested.

Tumor-derived factors influencing the CMI in HNSCC (table III)

Many factors influence the immunesystem in head and neck cancer. These can be divided into a group of factors related to the presence of the tumor per se and a group of factors related to the life style of HNSCC patients or their treatment. This latter group includes, amongst others, the use of alcohol, smoking habits, malnutrition, age, general health status, anaesthesia, surgery, and radiotherapy [130,131]. These latter factors have recently extensively been reviewed by Wustrow [131], and will not be discussed here.
With regard to the first group of factors it has been recognized for a long time that the sera of HNSCC patients contain molecules that suppress the CMI. In 1978, Berlinger [132] described a deficient mixed leucocyte reaction, which was related to immunosuppressive qualities of macrophages. Wanebo [133], found that approximately half of HNSCC patients have factors in their sera that suppress both lymphocyte proliferation, and NK- and lymphokine activated killer (LAK) cell activity. These factors were not further identified, but may include a soluble factor produced by the tumor with a molecular weight of > 75 kD

[134]. Immunosuppressive Substance (IS; ~ 52 kD), and immunosuppressive acidic protein (IAP) both suppress PHA-induced human lymphocyte blastogenesis, and have been found in serum of head and neck cancer patients [135,136]. However their role in the regulation of the immune respons has not been thorougly investigated, and they have mainly been suggested as a tumor marker. Reports on these factors are lacking in the more recent literature.

Extensive work has been done on the so-called "p15E-like factors". Cianciolo [137,138] was the first to demonstrate the presence of an immunosuppressive low molecular mass factor (LMMF; <25 kD) in both animal and human cancers. This protein shows structural homology with the transmembrane (TM) envelope protein p15E of animal retroviruses, and is referred to as "p15E", "p15E-like LMMF's: or "TM factors". The TM factors can be demonstrated in the sera of HNSCC patients [101,139,140], but are certainly not specific for this disease. In case of other human cancers, as well as a benign diseases like chronic purulent sinusitis and Graves disease these TM factors are also present in patient sera [141-143]. Although it is not exactly known, how and where these factors are produced, it is assumed that chronicly stressed, or malignant transformed epithelial cells of the upper respiratory tract are responsible for their production [144]. Also, these p15E-like factors can be extracted from HNSCC [101], and the tumors stain positively with a number of monoclonal antibodies directed against retroviral p15E [145]. The AA sequence mainly responsible for immunosuppression in retroviral p15E has been identified as a 17 AA long stretch (CKS-17) and this immunosuppressive epitope (CKS-17 epitope) of retroviral p15E has also been found in HNSCC specimen [145,146]. However the CKS-17 sequence is not specific for p15E, and it does also occur in IFN-α [147]. It must be noted that HNSCC also stain for monoclonal antibodies specific for IFN-α [147].
A number of effects on the immunesystem that are mediated by immunosuppressive p15E and CKS-17 are known to date. The molecules suppress the chemotactic capability of peripheral blood monocytes [100,101,148] and the capacity of DC to form cell clusters [143]. Furthermore, the factors suppress monocyte-mediated killing [149], NK-cell activity [150], cytokine dependent T cell proliferation [151-153], and B-cell activation [154], mostly through causing an imbalance of cytokine production, and consequent disturbance of cytokine mediated signal transduction [155-160]. The cytokine dysregulation influences the CMI in many more ways than here described, and we are still far from a full understanding of the importance of p15E-like TM factors in head and neck cancer.

Another important mediator in the immune system that has attracted attention in HNSCC is prostaglandin E2 (PGE2). PGE2 is a product of the cell membrane phospholipid metabolism with a number of known immunosuppressive qualities, including suppression of lymphocyte proliferation and mitogen responses, cytokine production by T helper cells, suppression of activated CD8+ T cell, NK- and LAK cell activity, and suppression of macrophage functions like cytotoxicity and cytokine production [161-168]. PGE2 is probably produced by both tumor cells and tumor infiltrating cells [169-171]. As is the case with

other immunomodulating factors in head and neck cancer, the role of PGE2 in tumor control is unclear. Although it exerts immunosuppressive effects, increased levels of PGE2 have actually been associated with improved survival and were considered to be an indicator of an enhanced immune response [172]. Furthermore, other phospholipid metabolites, like leukotriene B4 (LTB4) and platelet activating factor (PAF) with immune inhibitory properties have been isolated from head and neck tumor environments, and may have been produced by malignant and/or infiltrating cells [173]. They may have synergistic effects to PGE2.

Immuno-suppressive factors other than IS, TM and PGE2/LTB4 have also been demonstrated to be produced by HNSCC and its infiltrating host cells. Transforming growth factor-β (TGF-β) affects B-cells, NK-cells, macrophages and T cells. Although it also has concentration dependent, positive effects on the immune system, it generally is immunosuppressive by - amongst other mechanisms - interference with IL-1 and IL-2 cytokine pathways and inhibiting macrophage activation [71]. HNSCC are also able to release IL-10 which inhibits the production/release of mediators from CD4+ T helper-1 cells (T helper-1 cells are involved in activating and promoting cellular immunity in contrast to T helper-2 cells that are involved in the stimulation of humoral immunity, see also fig. 3). It has been shown that the number of CD8+ T cells in the tumor infiltrate that release IL-10, TGF-β and PGE2 is reduced in head and neck cancers [171]. The same study also reported the release of granulocyte-macrophage colony-stimulating factor (GM-CSF) by tumor- and infiltrating cells, wich is associated with the intratumoral presence of CD34+ GM-progenitor cells. These cells were considered by the authors as natural suppressor cells which were inhibitory to the proliferation of T lymphocytes [174,175].

The above described tumor-derived factors are generally refered to as "immuno-suppressive factors", and although they certainly have these suppressive properties in defined assay systems, immuno-modulation would be a better description for they exert their effects on the immune system through very complicated, not well understood pathways. The same "immunosuppressive" factors may also exert some immunostimulatory functions as well depending on the concentration and environment [71,168].
HNSCC also secrete immuno-modulating factors that are predominantly immuno-stimulatory to the cellular immune response. In a recent study [176] it was found that the supernatant of a HNSCC-cell line (PCI-50) promoted expression of activation markers of NK- and T cells, and sustained proliferation of peripheral blood lymphocytes in culture. NK-cells and T cells cultured in the presence of a combination of IL-2 and the PCI-50 conditioned culture fluid showed an enhanced antitumor cytotoxicity, while cytokine production by NK- and T cells was also induced. These immunostimulative qualities were attributed to (a) soluble factor(s) > 30kD. Little is known about other HNSCC derived immunostimulative factors and studies of these factors are in progress. Suffice to say that the above described findings on tumor-derived factors once more indicate that the immunomodulating role of the tumor itself and its effect on tumor growth and metastatic behaviour is still poorly understood.

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Chapter 2

AIMS OF THE STUDIES

Aims of the studies described in this thesis

The majority of reports on the infiltration of lymphoid cells into head and neck tumors deal with the infiltration of lymphocytes. Reports on the pattern of infiltration of monocytes, macrophages and dendritic cells are scarce. It has been described that patients with a dense infiltration of S100-positive dendritic cells in nasopharyngeal carcinoma are likely to survive longer than those without [1]. No such correlation appears to exist, however, between the density of lysosome-positive macrophage infiltration in or around tumor nests and the prognosis or stage of the tumor [1].

The first objective of this study was to give a detailed analysis of the pattern of tumor infiltration by macrophages and by dendritic cells into head and neck squamous cell carcinoma (HNSCC). The spatial relationship between dendritic cells, macrophages and malignant epithelial cells and the spatial relationship of these cells to infiltrating T lymphocytes and B lymphocytes was studied as well (see chapter 3).

Patients with HNSCC may show certain deficits in their immune functions. The cell-mediated immune system is particularly affected with defects including T cell function [2-4], and impaired function of monocytes, monocyte-derived dendritic cells and macrophages [5-12].

Our group has previously reported in detail impairments of blood monocyte chemotaxis in HNSCC patients. These monocyte chemotactic defects are thougth to be caused by factors of low molecular mass (LMMFs, < 25 kDa) produced by the cancer cells and to appear in the sera of affected patients. Since LMMFs show a structural and functional homology with transmembrane (TM) protein p15E of animal leukemogenic retroviruses, these substances are therefore generally be referred to as "immuno-suppressive p15E-like or TM-like LMMFs" [13,14].

The second objective was therefore to study the relationship of the immunohistomorphological expression of this TM factor in HNSCC cells and the pattern of infiltration of macrophages and dendritic cells (see chapter 3).

In earlier reports our group demonstrated that the abnormal monocyte chemotaxis and the defective cluster capability of dendritic cells in HNSCC could be restored *in vitro* by the addition of a thymic peptide preparation called thymostimulin (TP1) [11]. Clinical trials investigating the effect of TP1 in various malignant disorders have since been carried out. T-lymphocyte levels were found to increase in patients with malignant melanomas and clinical outcomes could be improved with adjuvant TP1 treatment [15]. In cases with large head and neck cancers overall response to chemotherapy improved if TP1 was used as adjuvant therapy [16], while peripheral T cell counts improved under the influence of TP1 in patients with Hodgkin's disease [17].

These in vitro observations were the impetus to conduct a double-blind, placebo-controlled multicenter study to define in vivo effects of TP1 treatment on monocyte chemotactic functions, cluster capability of dendritic cells and levels of serum p15E-like or TM factors in HNSCC patients (third objective).

The studies were in the setting of a dose finding approach. The in vivo effects of TP1 treatment described in this thesis are on:
- *the disturbed monocyte polarisation and dendritic cell clustering in HNSCC patients (chapter 4 and 6);*
- *the infiltration pattern of lymphocytes, macrophages and dendritic cells in HNSCC (chapter 5);*
- *the levels of p15E-like low molecular mass factors in peripheral blood of HNSCC patients (chapter 4 and 6).*

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Chapter 3

Macrophage and dendritic cell infiltration in head and neck squamouscell carcinoma; an immunohistochemical study

Macrophage and dendritic cell infiltration in head and neck squamous-cell carcinoma; an immunohistochemical study

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Abstract. A study was undertaken to help us reach a better understanding of the tumor-infiltrating pattern of lymphoid cells and in particular of monocyte-derived cells, namely the CD68+, acid-phosphatase-expressing scavenger macrophages and the MHC-class-II- and S100-antigen-presenting dendritic cells in head and neck squamous-cell carcinoma. In the stroma of the tumors distinctive small fields of lymphocytes were found, the T cell areas of these fields being intermingled with dendritic cells. Intra-epithelial dendritic cell infiltration was low. The infiltrative pattern of macrophages was similar to patterns described in earlier studies with substantial stromal invasion and inconsistent intra-epithelial invasion, but small granuloma-like structures of CD68+ macrophage-like cells, found in the stroma of tumors, have not been reported before. The histochemical localization of the tumor-infiltrated dendritic cells and macrophages supports the view that the former cells are involved in the sensitization to tumor antigens, whereas the latter cells are involved in tumor cytotoxicity/scavenging of tumor cell debris. Although it has been shown in the past that transmembranal (TM) factors (p15E-like factors) present in the serum and tumor of patients with cancer of the head and neck have suppressive effects on monocyte/macrophage/dendritic cell function, a relationship between the intensity of epithelial staining for TM factors and the infiltrative pattern of monocytes/macrophages/dendritic cells could not be demonstrated.

Key words: Macrophages – Dendritic cells – Head and neck cancer – Squamous-cell carcinoma

Introduction

There is experimental evidence that the cell-mediated immune system plays an important role in the defence against head and neck squamous carcinoma cells [4, 8, 9, 15, 22]. Cell-mediated immune functions are executed by T lymphocytes, monocytes, macrophages and dendritic cells. It is therefore of relevance that defects in the function of these cells have been described in patients with head and neck cancer.

Dendritic cells play a role in tumor defence by presenting tumor-associated antigens to the immune system. Dendritic cells are monocyte-derived cells, with a distinctive morphology and a characteristic marker pattern in immunohistology. The cell shows long cytoplasmic veils or dendrites, has a kidney-shaped nucleus and usually reacts strongly with MHC-class-II-directed antibodies amongst which are L25 and RFD1. The cell is also often positive for the protein S100. Dendritic cells have a weak expression (sometimes only in the form of a paranuclear spot) of CD68, CD14 and of the enzyme acid phosphatase. To execute its antigen-presenting function, the dendritic cell forms clusters with lymphocytes and it is therefore of importance that, in patients with head and neck cancer, the capability of blood dendritic cells to form clusters with allogenic lymphocytes has been described as impaired [19].

Macrophages are also monocyte-derived cells and mainly have a killer and scavenger function. Macrophages are strongly positive for CD68, and the enzyme acid phosphatase; occasionally, they are positive for MHC class II markers. Morphologically, they are large, rounded cells lacking long cytoplasmic protrusions. Defects in the tumoricidal capacity of macrophages have also been reported in patients with head and neck cancer [4].

The majority of reports on the infiltration of lymphoid cells into head and neck tumors deal with the infiltration of lymphocytes. Reports on the pattern of infiltration of monocytes, macrophages and dendritic cells are scarce. It has been described that patients with a dense infiltration of S100-positive dendritic cells in nasopharyngeal carcinoma are likely to survive longer than those without [16]. No such correlation appears to exist, however, between the density of lysosome-positive macrophage infiltration in or around tumor nests and the prognosis or stage of the tumor [16].

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Table 1. Monoclonal antibodies

Epitope	Antibody	Dilution	Specificity	Source
RFD1		1:1000	Active dendritic cells and subset of B cells (class-II-MHC-associated antigen)	Kindly provided by L. Poulter, London
L25		1:1000	Dendritic cells and B cells	Kindly provided by Ishii, Sapporo
S100		1:500	Dendritic cells (mainly intra-epithelial)	Dakopatts, Glostrup
CD68	Ki-M7	1:200	Macrophages (strong cytoplasmic expression)	Behring, Marburg, Germany
CD14	LeuM3	1:500	Monocytes, macrophages	Becton Dickinson, San Jose, Calif.
CD3	Leu4	1:60	Immature and mature functional T cells	Becton Dickinson, San Jose, Calif.
CD19	CLB-B4	1:25	B cells	Central Laboratory of the Blood Transfusion Service, Amsterdam
4F5		1:200	p15E	Kindly provided by G. J. Cianciolo

The first objective of this study is to give a detailed analysis of the pattern of tumor infiltration by macrophages, defined as large rounded cells strongly positive for CD68 and CD14 and by dendritic cells defined as stellate-shaped cells positive for L25, RFD1 and S100. The spatial relationship between dendritic cells, macrophages and malignant epithelial cells and the spatial relationship of these cells to infiltrating T lymphocytes and B lymphocytes was studied as well.

Carcinoma cells, including head and neck squamous carcinoma cells are capable of producing factors that exert a variety of suppressive effects on different types of immune cells. Some of these tumor factors also demonstrate a suppressive effect on the chemotactic capability of monocytes/macrophages and the capability of dendritic cells to form clusters with allogeneic lymphocytes. The factors produced show a structural and functional homology with a retroviral transmembranal (TM) protein called p15E [5], hence the name p15E-like factors. It is not yet clear if any relationship exists between the degree of TM factor expression in head and neck cancer cells in vivo and the infiltration pattern of macrophages and dendritic cells in and around the malignant cells. We therefore also studied the relationship of the immunohistomorphological expression of this TM factor in head and neck squamous carcinoma cells and the pattern of infiltration of macrophages and dendritic cells.

Materials and methods

Patients and tissues. Samples of fresh tumor tissue were obtained from surgically removed tumors of 18 patients with squamous-cell carcinoma of the head and neck; 7 carcinomas of the oral cavity, 6 oropharyngeal carcinomas, 3 carcinomas of the hypopharynx and 2 of the larynx were included. Small representative tissue parts of the removed tumor were frozen in liquid nitrogen and stored at –80°C. Tissue was taken from the central solid part of the tumor to avoid the effect of superficial bacterial infection on lymphoid cell infiltration; signs of infection were not present in any of the tumor biopsies.

Immunohistology. Serial 6-µm cryostat sections were cut and mounted on slides, air-dried and fixed in acetone. Normal rabbit serum (Dakopatts, Copenhagen, Denmark) was added and gently removed by tapping the slides after 15 min. The slides were thereafter incubated for 1 h at room temperature with the monoclonal antibodies listed in Table 1, to identify the various infiltrating lymphoid cells. All antibodies were diluted in phosphate-buffered saline (PBS), pH 7.4 enriched with 1% bovine serum albumin (Sigma, St. Louis, Mo.) before incubation. After incubation the sections were gently rinsed with PBS to wash away excess monoclonal antibodies for at least 15 min and thereafter incubated with horseradish-peroxidase-conjugated rabbit anti-(mouse Ig) serum (Dakopatts, Copenhagen, Denmark) with 1% human pooled serum for 30 min. Subsequently, the sections were rinsed with PBS for another 15 min and stained for peroxidase activity with 3.3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, Mo.) in PBS containing 0.01% H_2O_2. The sections were rinsed in tap water, counterstained with hematoxylin for 30 s, dehydrated and mounted in malinol. All sections were additionally tested for acid phosphatase activity with naphthol AS-BI phosphatase as substrate and hexazotized pararosaniline as diazonium salt.

The slides were studied by two of the authors (J. K. and H. D.) using a Zeiss light microscope at various magnifications. Tumors were studied for morphology and lymphoid cell infiltration.

Cell infiltration was semiquantitatively scored as follows: –, no cells present; ±, few scattered cells present; +, diffuse compartmental infiltration by solitary cells; ++, marked compartmental cell infiltration with no apparent organization; +++, heavy cell accumulation with a clear infiltration architecture; ++++, massive clotting of cells.

Results

Studying the immunohistological appearance of specimens of head and neck carcinoma, we could clearly make a distinction between areas composed of malignant squamous epithelial cells (parenchymal tumor nests) and areas of surrounding stromal connective tissue.

In the stromal connective tissue three types of lymphoid cell infiltration could be identified: (a) an infiltration by scattered and mostly single lymphoid cells. (b) small areas of densely packed lymphoid cells with a certain degree of organization and (c) small granuloma-like structures. The first type of stromal infiltration was the most predominant.

In the malignant epithelial areas (the tumor nests) there was also a scattered infiltration of single lymphoid cells.

The infiltration pattern of scattered, mostly single lymphoid cells in the stromal compartment of the tumor (Table 2)

Many CD68+ large cells were present, which morphologically varied from monocyte-like cells to very large, round, strongly staining macrophages. A similar pattern of distribution was seen for CD14+ cells.

Table 2. Lymphoid cell infiltration in the stromal compartment and into malignant epithelial areas

Cells	Areas of scattered lymphoid cell infiltration	Areas of dense packed lymphoid cell accumulations	Granulomatous structures	Cancer nest infiltration
Dendritic cells				
S100	+	++	–	+
CD1a	+	±	–	–
RFD1/L25	++	++	+	± to +
Macrophages				
CD68	+++	+++ (particularly in	++++	++
CD14	++	++ B cell areas)	++	+
T cells				
CD3	+ to ++	+++ to ++++	–	±
B cells				
CD19	–	– to +++	–	–

RFD1+ stellate-shaped cells were also clearly present, however, generally in fewer numbers than the CD68+ macrophages. The RFD1+ dendritic cells were also positive for other MHC class II molecules (as identified with the mAb OKIa); the cells were, however, less positive for L25 and markedly less positive for CD1a and the S100 protein (though they were positive).

The number of scattered single CD3+ lymphocytes varied from tumor to tumor, there being no apparent relationship between CD3+ lymphocyte infiltration and the differentiation grade of the tumor. A large proportion of the small round CD3+ T lymphocytes were also positive for MHC class II and RFD1. CD19+ B cells were not present in this type of infiltrate.

Areas of dense, packed lymphoid cells in the stromal compartment (Table 2)

Small areas of dense packed lymphoid cell infiltration were present in 8/18 of the studied tumor specimens (Fig. 1). These dense lymphoid cell areas were randomly localized and not particularly located in the vicinity of, or at the borders of the epithelial tumor nests. Inter-tumoral variation was extensive, but intra-tumoral variation was limited with respect to the number of such areas.

The areas of dense lymphoid cells had a certain degree of architecture with identifiable areas of CD3+ T cell accumulation and, in an occasional tumor, a central area of CD19+ B cells. These latter areas are reminiscent of early B cell follicles. RFD1+ and L25+ stellate-shaped cells were strongly represented within the CD3+ T cell areas. In the S100 staining, the presence of dendritic cells within the T cell zones was also obvious; CD1a positivity was, however, almost absent in these areas. With respect to the central B cell areas it was clear that in particular CD68+ and CD14+ monocytes/macrophages could be identified within these areas.

Granulomatous structures within the stromal compartment (Table 2)

Granulomatous structures were mostly small and always located near the borders of the malignant epithelial areas. They were present in 7/18 of the specimens studied (Fig. 2). The presence or absence of these lymphoid cell structures was again not dependent on tumor differentiation, nor was there a relationship with the presence of the dense, packed lymphoid cell accumulations. The cells forming the granulomas were strongly positive for the macrophage marker CD68, while being only weakly positive for CD14, RFD1 and L25. T lymphocytes (CD3+) and B lymphocytes (CD19+) were not present within these structures.

Scattered infiltration of lymphoid cells in the parenchymal areas of malignant epithelial cells (Table 2)

CD68+ macrophage infiltration was markedly present in most tumors, within the parenchymal areas of malignant epithelial cells, though, less than in the stromal areas

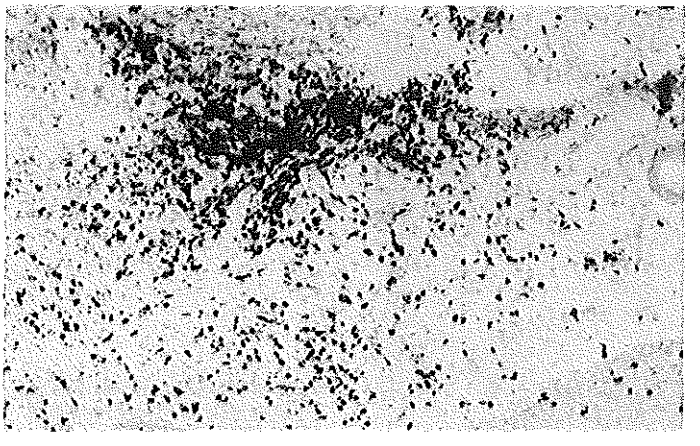


Fig. 1. Area of dense CD3+ lymphoid cell infiltration in stromal compartment. Magnification: 36×

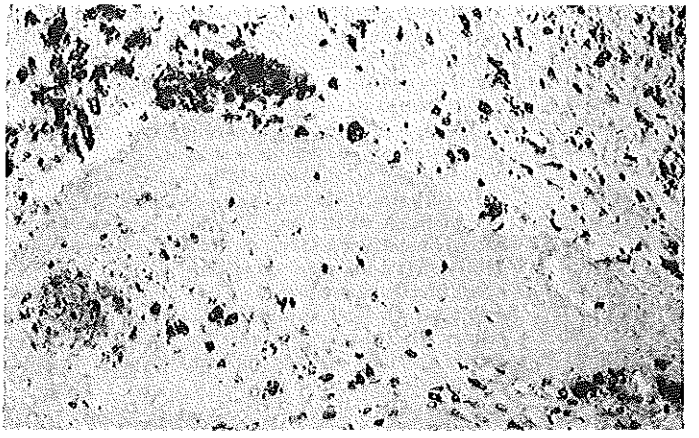
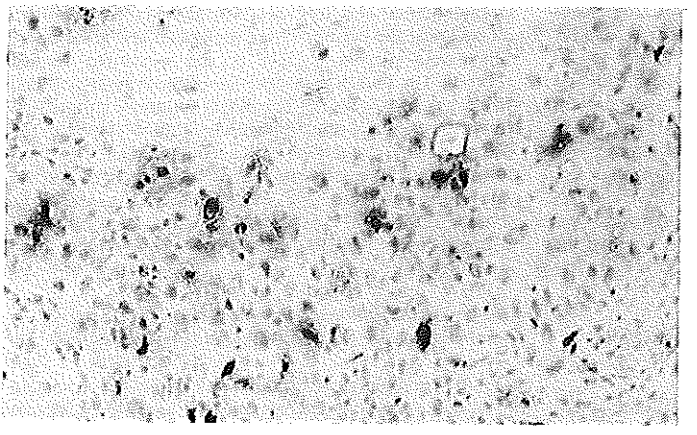


Fig. 2. Granulomatous CD68+ structure within the stromal compartment. Note also intratumoral CD68+ macrophage infiltration. Magnification: 36×



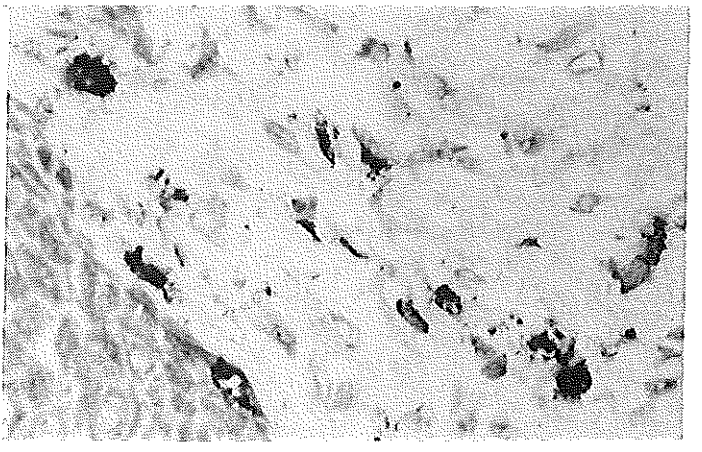


Fig. 3. S100+ dendritic cell infiltration into malignant epithelial area and surrounding stroma. Magnification: A 80×; B 200×

(Fig. 2). The pattern of infiltration between the different tumors, but also within an individual tumor was often variable. There was a stronger and more diffuse infiltration in poorly differentiated tumors, the CD68+ macrophages often reaching the central areas of the tumor nests. In well-differentiated tumor tissue the infiltration was less extensive, the CD68+ macrophages staying closer to the outer zones of the tumor. A similar pattern of CD14+ monocyte/macrophage infiltration was found although numbers of such monocytes/macrophages were considerably lower. Very few CD1a+, RFD1+ and L25+ cells with a classic dendritic cell morphology were present in well-differentiated malignant epithelial areas; however, in poorly differentiated tumors more RFD1+ cells were identifiable. The few infiltrating dendritic cells in the well-differentiated tumors stayed at the borders of the tumor nests. Intra-epithelial infiltration of S100+ dendritic cells was considerable and comparable to stromal S100+ dendritic cell infiltration (Fig. 3 A, B).

CD3+ lymphocytes also basically infiltrated the outer zones of the tumor nests (similar to the CD1a+, RFD1+ and L25+ dendritic cells in the well-differentiated tumors), again in considerably lower numbers compared to their infiltration into the stromal compartment. Infiltration of CD3+ lymphocytes into poorly differentiated tumors was more extensive than into well-differentiated tumors. CD19+ B cells were virtually absent from these malignant epithelial areas.

In all tumors, the malignant epithelial structures stained clearly positive for the anti-p15E antibody 4F5 to an extent that mostly depended on the differentiation grade of the tumor. Expression varied from weak to strong between tumors and sometimes even within tumors. There was, however, no relationship between the intensity of p15E expression and the severity of lymphoid cell infiltration of any kind.

Discussion

The dendritic cells in the antigen-presenting accessory cell *par excellence* [2, 10, 11]. The cell exposes on its long cytoplasmic extensions a high density of MHC class I and II molecules (the antigen-presenting molecules), and actively seeks contact with surrounding cells including lymphocytes to present the antigens. During this antigen presentation the cell forms cell clusters with the lymphocytes. Apart from this cluster formation, in which a micro-environment suitable for antigen presentation is created, the dendritic cell is capable of producing a series of cytokines that activate T and B lymphocytes. Dendritic cells do not only occur in the T cell areas of spleen and lymph nodes as so-called interdigitating dendritic cells, but also occur as the Langerhans cells in the epidermis and dermis of the skin and mucosal surfaces. It is now generally accepted that the dendritic cells of the skin and mucosal surfaces travel via the lymph to the draining lymph nodes, transporting antigens from the periphery to the lymphoid organs to present the antigens to lymphocytes to initiate immune reactivity in the lymphoid spleen [12].

Although no specific mAb for the staining of dendritic cells exists, RFD1 and L25 can be used adequately (together with classical morphological characteristics such as shape) to identify the lymphoid dendritic cells present in the T cell areas of spleen and lymph nodes. S100 and CD1 are reasonably characteristic for the Langerhans cells of the skin (these marker proteins are also present in some dendritic cells of the lymphoid tissues).

In this study we found that RFD1+ and L25+ dendritic cells were markedly present within the T cell areas of lymphoid cell accumulations present in the stromal compartment between tumor nests. These dendritic cells were virtually negative for the markers S100 and CD1. The position of these lymphoid dendritic cells indicates their antigen-presenting function and suggests an active stimulation of the immune system in the dense lymphoid areas in at least some tumors. RFD1+ and L25+ dendritic cells were also diffusely present in the stromal compartment, but only scarcely within the well-differentiated malignant epithelial areas. The intra-epithelial infiltration of S100+ dendritic cells was considerable and comparable to stromal S100+ dendritic cell infiltration. This observation is in agreement with the classical notion that intra-epithelial dendritic cells, often referred to as Langerhans cells, are mainly S100-positive, and our data thus suggest a more Langerhans-cell-like characteristic of the dendritic cells that have infiltrated the malignant epithelial cell areas.

Our observations are hence by and large in agreement with the study of Nomori et al. [16] on nasopharyngeal carcinoma. These authors found S100+ dendritic cells primarily located within the tumor nests, and found few of these cells in the surrounding connective tissue. Similar findings have been reported by Nakano et al. [14] in squamous-cell carcinoma of the uterine cervix; they described that the S100+ dendritic cells were mainly located in between the malignant epithelial tumor cells. In gastric adenocarcinoma Tsujitani et al. [20, 21] described clusters of S100+ dendritic cells in the tumor stroma as well as an infiltration in between the cancer cells. In all these studies S100 only was used to identify dendritic cells, and it is therefore difficult to draw any conclusions concerning the dendritic cells that are negative for this protein (the majority of the dendritic cells present in T cell areas of the focal lymphocyte infiltrates found in our study). It is of importance to note that all three of the above-mentioned authors correlated a marked dendritic cell infiltration with a better prognosis. These findings indicate that dendritic cells probably play a more important role in the immune defense against cancer than do scavenger macrophages. The number of patients and length of follow-up period in our study are so far insufficient for correlation studies, but later reports will address this issue.

Macrophages with a scavenger and cytotoxic function are mainly involved in the efferent arm of the immune response. Such cells are markedly positive for CD68 and lysosomal enzymes such as acid phosphatase. In our study CD68+ macrophages were strongly represented both within the connective tissue compartment as well as in the malignant epithelial areas; however, the infiltration into the latter area varied widely between the various tumors, some showing hardly any, others showing massive intra-epitheli-

al invasion. The latter pattern may indicate a stronger cytotoxic response to these tumors; however, histologically clear tumor cell destruction was not seen.

Ki-M7 (a monoclonal antibody that reacts with CD68, used in our study) stains mature CD68+ macrophages [7]. Despite the fact that macrophages have in vivo and in vitro a cytotoxic/cytolytic effect when activated by lymphokines or bacterial products, their precise role in tumor immunology in vivo remains uncertain. Cameron and Stromberg [4] found a defect in tumoricidal capacity in head and neck cancer; half of the patients in their study possessed macrophages that were non-cytotoxic toward tumor cells in vitro. Allen [1] found no histological evidence for a cytotoxic role of macrophages in colorectal tumors. In murine sarcomas and carcinomas, tumor-associated macrophages were even thought to stimulate tumor growth [13]. It has been suggested [17] that tumor cells may become insensitive to cytolysis in vivo by activated macrophages by building up a resistance to tumor necrosis factors, which are mediators in the lymphokine activation of macrophages.

Neuchrist et al. [15], also using immunohistology, showed that most tumor-infiltrating macrophages are of a functionally mature phenotype. The distribution these authors described is similar to our findings, with an extensive stromal macrophage invasion and some infiltration into the areas of the malignant epithelial cells (with a wide inter-tumoral variation). These authors, however, did find (in contrast to us) a correlation between the extent of CD3+ lymphocyte infiltration and the infiltration by macrophages. Others have also stressed the important role of T-cell-activating macrophages in direct tumor defence [23]. It must be noted, however, that Neuchrist et al. used markers such as a-HLA-DR, a-Fcy receptors I, II, III, and Rm3/1 to identify the macrophages. We consider HLA-DR+ cells not as classical macrophages but, when stellate-shaped, as dendritic cells; this might explain the discrepancy between our results and those of others. We did find a relationship between the infiltration of HLA-DR+ dendritic cells and T cells.

In nasopharyngeal carcinoma Nomori [16] found that most lysozyme-antibody-stained macrophages surrounded the tumor nests although he did not describe the granulomatous structures of our study. In the granulomatous structures present in the stromal compartment close to the tumor nests all cells were strongly positive for CD68, in fact the histology suggests to us that these areas were almost predominantly composed of large, fused macrophages supporting the classically held view that granuloma's are mainly involved in the degradation of persistent antigens or non-degradable materials. A marked intra-epithelial infiltration of macrophages in adenocarcinoma of the stomach and in breast cancer was found by Tsujitani et al. [20, 21] and Zuk et al. [24]. We also found a marked infiltration with such cells mainly in poorly differentiated head and neck squamous-cell carcinomas.

Cianciolo [6] demonstrated in 1980 the presence of a low-molecular-mass (low-M_r) factor in both animal as well as human cancers. This factor had an inhibitory effect on monocyte chemotaxis and appeared later to be absorbable by any of three different mAb to a retroviral capsular protein, namely p15E. Tan [18] showed that the p15E-like low-M_r factors were also present in the tumors of patients with head and neck cancer as well as in the serum of these patients. The p15E-like factors, presently called TM factors, have a suppressive effect not only on monocyte chemotaxis but also on the function of various cells of the immune system. The factors inhibit IL-2 dependent T cell proliferation, they inhibit the blastogenic responses to mitogens and antigens, they suppress the natural killer cell activity, they hamper the clustering capacity of dendritic cells and they suppress the respiratory burst of human monocytes. All tumors in our study showed positivity for the anti-TM-factor mAb 4F5. However, no relationship between p15E expression and any particular infiltrative pattern could be demonstrated. In our earlier report we were able to correlate tumor infiltration with chemotactic ability [3]. Important to note is that positive staining of cancer cells for anti-TM-factor mAb does not necessarily mean that the tumor produces or secretes these factors. Furthermore, recent work in our group [19] has suggested that the anti-TM-factor mAb may not be as specific for p15E as was believed earlier: it has been shown that these mAb cross-react with interferon α and possibly other cytokines. Future work will address this matter.

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Chapter 4

In vivo effects of thymostimulin treatment on monocyte polarization, dendritic cell clustering and serum p15E- like trans-membrane factors in operable head and neck squamous cell carcinoma patients

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In vivo effects of thymostimulin treatment on monocyte polarization, dendritic cell clustering and serum p15E-like trans-membrane factors in operable head and neck squamous cell carcinoma patients

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Abstract Head and neck squamous cell carcinoma patients have been characterized by impairments in their cell-mediated immune system, particularly by decreased chemotactic function of monocytes and impairments in the function of the monocyte-derived dendritic cells (viz, a decreased capability to form cell "clusters"). These impairments are thought to be due to immunosuppressive factors of low molecular mass released by tumor, the so-called p15E-like factors. These suppressive effects of p15-like factors can be neutralized in vitro by thymic peptides, such as thymostimulin (TP1). In a randomized double-blind, placebo-controlled multicenter trial in the Netherlands, 41 patients with operable head and neck squamous cell carcinomas (HNSCC) were treated for 10 days prior to surgery with intramuscular TP1 in one of three dosages (0.5 mg/kg; 1.0 mg/kg or 2.0 mg/kg body weight) or treated with placebo. Assessment of monocyte chemotaxis, the capability of dendritic cells to form clusters and the presence of p15E-like low-molecular-mass factors (LMMFs) in serum was performed before TP1 treatment and on the day of surgery. Findings demonstrated that TP1 in a dose of 1.0 mg/kg and 2.0 mg/kg resulted in normalization of impaired monocyte chemotactic capability. Although the cluster capability of dendritic cells after TP1 treatment improved, values only reached statistical significance for the 0.5 mg/kg group. Serum p15E-like LMMF levels were not affected by TP1 treatment in any of the patient groups. Contrary to expectations we found no correlation between elevated immunosuppressive LMMFs and defective monocyte chemotaxis or cluster capability of dendritic cells. We conclude that treatment with TP1 can improve monocyte chemotaxis in HNSCC patients but an effect on the production of p15E-like factors by carcinoma cells could not be demonstrated.

Key words Head and neck carcinoma · Immunotherapy · Monocytes · Dendritic cells · Thymostimulin

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Introduction

Patients with head and neck squamous cell carcinoma (HNSCC) may show certain deficits in their immune functions. The cell-mediated immune system is particularly affected with defects including T-cell function [20, 24, 44], and impaired function of monocytes, monocyte-derived dendritic cells and macrophages [3, 5, 8, 14, 16, 37, 43, 45].

We and others have previously reported in detail impairments of blood monocyte chemotaxis in HNSCC patients. These monocyte chemotactic defects are thought to be caused by factors of low molecular mass (LMMFs, < 25 kDa) produced by the cancer cells and appearing in the sera of affected patients. These chemotactic defects can be mimicked in vivo in animal models as well as in vitro using healthy donor monocytes exposed to such tumor-isolated LMMFs [4, 36, 38]. Since LMMFs show a structural and functional homology with transmembrane (TM) protein p15E of animal leukemogenic retroviruses, these substances are therefore generally be referred to as "immunosuppressive p15E-like- or TM-like LMMFs" [9, 38].

The function of monocyte-derived, antigen-presenting dendritic cells is frequently disturbed in the presence of HNSCC. This defect may also be the result of p15E-like LMMFs [43], since such factors in vitro exert a negative effect on the ability of dendritic cells to form cellular clusters. In general, monocyte-derived dendritic cells are class II MHC-positive mononuclear cells with long cytoplas-

mic extensions that act as excellent accessory cells in immune responses [2, 13, 19, 26, 35].

In an earlier report we demonstrated that abnormal monocyte chemotaxis and a defective cluster capability of dendritic cells could be restored in vitro by the addition of a thymic peptide preparation called thymostimulin (TP1) [43]. Clinical trials investigating the effect of TP1 in various malignant disorders have since been carried out. T-lymphocyte levels were found to increase in patients with malignant melanomas and clinical outcomes could be improved with adjuvant TP1 treatment [7]. In cases with large head and neck cancers overall response to chemotherapy improved if TP1 was used as adjuvant therapy [34], while peripheral T-cell counts improved under the influence of TP1 in patients with Hodgkin's disease [25]. These various observations were the impetus for us to conduct a double-blind, placebo-controlled multicenter study to define in vivo effects of TP1 treatment on monocyte chemotactic functions, cluster capability of dendritic cells and levels of serum p15E-like factors in HNSCC patients.

Patients and methods

Forty-one patients with operable squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx or larynx agreed to participate in the trial. All patients met the inclusion criteria listed in Table 1 and gave informed consent according to university requirements for human research in The Netherlands. All patients were entered randomly in a double-blind fashion in one of the four following treatment groups: group I – patients receiving placebo treatment; group II – patients receiving 0.5 mg/kg TP1; group III – patients receiving 1.0 mg/kg TP1; group IV – patients receiving 2.0 mg/kg TP1. To avoid investigator bias, TP1 in different dosages or placebo was (double-blind) injected intramuscularly in each patient once daily for 10 consecutive days prior to definitive operation. All injections were well tolerated and no adverse side effects were seen. Table 2 summarizes the patients' ages and sexes, tumor locations and differentiations, TNM classification and subsequent treatment given. No statistical differences were found for age, TNM classification or tumor differentiations.

Bovine thymic extract (TP1)

Thymostimulin was prepared as a bovine thymic extract using the following procedure described by Bergesi and Falchetti [6] and Falchetti et al. [12]. Calf thymus glands were minced and extracted with ammonium acetate. The extract was then heated to 70°C, filtered, and precipitated with ammonium sulfate. The precipitate was dissolved in water and subjected to ultrafiltration on an Ami-

Table 1 Inclusion criteria for patients with operable head and neck squamous cell carcinomas (HNSCC) for determining effects of TP1 treatment on monocyte polarization, dendritic cell clustering and serum p15E-like transmembrane factors

- Untreated squamous cell carcinoma of oral cavity, oropharynx, hypopharynx or larynx, with no evidence of metastatic disease
- Curative treatment possible by surgery or surgery combined with radiotherapy
- No other malignancies
- White blood count > 4.10^9/l; platelets > 100.0^9/l and hematocrit > 30%
- No serious concurrent non-malignant systemic or infectious diseases
- No treatment with corticosteroids

Table 2 Clinical profiles of patients with HNSCC prior to TP1 treatment (*W* well differentiated, *M* moderately differentiated, *P* poorly differentiated, *TL* total laryngectomy, *CR* composite resection, *P* pharyngectomy, *PP* partial pharyngectomy)

Patients	Sex	Age	TNM classification	Localization	Differentiation grade	Planned treatment
Placebo (n = 9)						
1	♀	46	T1N1M0	Larynx	M	TL
2	♀	51	T3N0M0	oropharynx	P	CR
3	♂	67	T3N1M0	oral cavity	W	CR
4	♂	59	T4N2M0	hypopharnyx	P-M	TL + P
5	♂	45	T2N1M0	oropharynx	M	CR
6	♂	65	T4N2bM0	oral cavity	M	CR
7	♂	67	T4N0M0	oral cavity	P	CR
8	♂	45	T3N2M0	oropharynx	W	CR
9	♂	60	T3N2M0	oral cavity	P	CR
0.5 mg/kg TP1 (n = 10)						
10	♀	62	T4N0M0	Oral cavity	M	CR
11	♀	61	T2N0M0	oropharynx	M	CR
12	♂	46	T2N0M0	oral cavity	P-M	CR
13	♂	56	T2N0M0	oral cavity	P	CR
14	♂	68	T1N2cM0	larynx	M	TL
15	♂	72	T2N0M0	oral cavity	W	CR
16	♂	52	T3N0M0	oropharynx	W	CR
17	♂	44	T2N0M0	oral cavity	P	CR
18	♂	60	T2N2aM0	oropharynx	P	CR
19	♀	74	T2N1M0	oral cavity	W	CR
1.0 mg/kg TP1 (n = 13)						
20	♂	61	T3N1M0	Oropharynx	M	CR
21	♂	45	T2N0M0	oral cavity	M	CR
22	♂	70	T3N2M0	oropharynx	M	CR
23	♂	47	T2N2aM0	hypopharynx	M	TL + PP
24	♀	56	T4N2M0	larynx	M	TL
25	♂	78	T1N0M0	oral cavity	W	CR
26	♀	65	T3N2bM0	oral cavity	M	CR
27	♂	44	T2N1M0	oral cavity	M	CR
28	♀	52	T3N0M0	oropharynx	W	CR
29	♂	66	T4N1M0	oropharynx	W	CR
30	♂	56	T3N1M0	larynx	M	TL
31	♂	64	T4N1M0	larynx	P	TL
32	♂	50	T4N2M0	hypopharynx	P	TL + PP
2.0 mg/kg TP 1 (n = 9)						
33	♂	67	T4N0M0	Oral cavity	M	CR
34	♀	58	T2N2bM0	oropharynx	M	CR
35	♂	59	T3N0M0	oropharynx	W	CR
36	♂	72	T3N2bM0	oropharynx	M-W	CR
37	♀	61	T2N0M0	oral cavity	M	CR
38	♂	51	T3N2cM0	larynx	M-W	TL
39	♂	51	T4N2M0	oral cavity	M-P	CR
40	♀	49	T3N2bM0	oropharynx	P	CR
41	♀	37	T2N0M0	oral cavity	W	CR

con PM-10 membrane. The filtrate was desalted on Sephadex G-25 and gel-filtered on Sephadex G-50. Fractions showing characteristic bands at RF 0.22 and 0.24 on polyacrylamide gel electrophoresis were combined and termed "thymostimulin" (TP1). This extract was next lyophilized and its activity expressed as units of T-cell-rosette formation per milligram of protein. No endotoxin was contained in this extract, as tested in doses up to 100 mg/kg when administered to mice for 21 days or rats for 31 days, or when given to cats or dogs for 180 days in doses up to 50 mg/kg [12]. Additionally, the extract failed to alter neuromuscular transmission in vitro or in vivo [6].

Isolation of peripheral blood monocytes

Peripheral blood mononuclear cells from patients and healthy controls were isolated by Ficoll-Isopaque density gradient centrifugation. Cells were washed twice in phosphate buffered saline (PBS) at pH 7.4, containing 0.5% bovine serum albumin (BSA). Cells were then counted in suspension employing positive staining with non-specific esterase (NSE) as described by Mullink et al. [29]. Monocytes in the Ficoll-Isopaque isolated fraction were enriched by Percoll gradient centrifugation [31]. After washing, the Ficoll-isolated cell pellet containing both monocytes and lymphocytes was resuspended in RPMI 1640 supplemented with 10% fetal calf serum (GIBCO, Breda, The Netherlands), 2 m*M* glutamine and antibiotics (penicillin and streptomycin), and carefully underlaid with an equal volume of Percoll 1.063 (Pharmacia, Uppsala, Sweden). After centrifugation at 400 *g* for 40 min, all cells were collected from the interface, washed twice in medium for 10 min and counted. This latter suspension now contained 70–95% NSE-positive cells. This suspension was used for direct monocyte polarization and maturation to obtain dendritic cells (see below).

Formation of dendritic cells

Dendritic cells were prepared from blood monocytes according to the method described by Kabel et al. [21]. Metrizamide (Serva, Heidelberg, Germany) was dissolved in RPMI supplemented with 10% fetal calf serum. Cells from the isolated monocyte fractions were exposed to 14.5% metrizamide in suspension culture for 30 min (using 5% CO_2 at 37°C and 100% humidity). Thereafter, cells were washed by slowly adding culture fluid to prevent osmotic lysis. Cells were then further cultured under non-adhering conditions for 16 h in polypropylene tubes (with 5% CO_2 and 100% humidity at 37°C). This procedure yielded 40–80% cells with a dendritic morphology, showing class II MHC positivity, decreased expression of the monocytic CD14 determinant, decreased phagocytic capability, but enhanced stimulator capability in the mixed leukocyte reaction (MLR). The full technical details of this method are described by Kabel et al. [21] and in Mooy et al. [28].

Clustering of dendritic cells

The cluster assay as described by Austyn et al. [2] was performed with modifications used by Kabel et al. [21]. Approximately 5×10^4 dendritic cells prepared from peripheral blood monocytes were exposed to metrizamide and allowed to cluster for 4 h in 5% CO_2 at 37°C in 250 µl flat-bottomed wells. Formed clusters were counted using an inverted microscope and values were expressed as the number of clusters per 6 microscopic fields (× 200). A cluster was defined as an accumulation of 4–25 cells in three-dimensional configuration. An insufficient amount of blood was received to obtain enough dendritic cells for the cluster assay in 8 cases.

Monocyte polarization assay

The Cianciolo and Snyderman [10] assay for monocyte polarization was performed with slight modifications [38] to rapidly test monocyte chemotaxis. Outcomes of the assay previously proved to correlate well with outcomes of the conventional Boyden chamber assay to measure chemotaxis to casein [38]. Repeat 0.2 ml aliquots of the Percoll or elutriator purified cell suspension containing 0.2×10^6 monocytes were added to 12–75 mm polypropylene tubes (Falcon Labware Division, Becton Dickinson, Oxford, Calif., USA) containing 0.05 ml of either medium or formylmethionylleucylphenylalanine (fMLP) in medium, to reach a final concentration of 10 n*M*. All experiments were carried out in duplicate. Tubes were incubated in a waterbath at 37°C for 15 min. Incubation was stopped by addition of 0.25-ml ice-cold 10% formaldehyde in 0.05 *M* PBS (pH 7.2). Cell suspensions were kept at 4°C until counted in a hemocytometer under a light microscope (magnification, × 250). Each test was read "blindly" by two persons and 200 cells were counted from each tube. A cell was considered to be "polarized" if one of the following occurred: elongated or triangular shapes, broadened lamellipodia or membrane ruffling.

Chemotactic responsiveness of a monocyte population was expressed as the percentage of polarized monocytes in the presence of fMLP minus the percentage of polarized monocytes in the absence of fMLP. The percentage of polarized monocytes was calculated as follows:

$$\frac{\%\ \text{total cells polarized}}{\%\ \text{NSE-positive cells}} \times 100\%$$

Lymphocytes were excluded by their lack of any polarization activity in this assay [9].

Determination of p15E-like immunosuppressive factors in patients' sera

Sera for immunological evaluation was collected by venipuncture prior to the first injection of TP1 and 1–2 h before surgery. In a number of events insufficient sera were received due to logistical problems. Sera of healthy hospital staff members served as control sera during all tests. Sera were diluted 1 : 1 in saline and subjected to ultrafiltration through Amicon CD25 Centriflo cones (Amicon Corp., Danvers, Maine, USA) for 15 min at 800 *g* (molecular mass "cut-off point", 25 kDa). Residues were resuspended and stored at –20°C until further use.

The capability of serum fractions to inhibit fMLP-induced polarization of healthy donor (elutriator-purified) monocytes was determined by incubating (1×10^6/ml) healthy donor monocytes for 15 min at 37°C either with fMLP alone or with fMLP in combination with a serum fraction (final dilution, 1 : 60). Details of the technique have been described by Tas et al. [43]. The percentage of inhibition was calculated as follows:

$i = (1 - (Ff\text{-}fo/Mf\text{-}fo)) \times 100\%$, where Ff = % monocytes polarized after incubation with fMLP and LMMF; Mf = % monocytes polarized after incubation with fMLP alone; and fo = % spontaneously polarized monocytes.

Spontaneous polarization was not affected by addition of serum fractions to non-fMLP stimulated donor monocytes.

To validate the p15E-like character of LMMF adsorption, experiments were carried out by neutralizing serum fractions at 4°C for 16 h before testing effects on monocyte polarization by using a combination of two p15E-specific mAbs (4F5 and 19F8 as anti-p15E isotypes IgG2a and IgG2b). The final mAb dilution was 1 : 200 and final IgG concentration 50 µg/ml. Following neutralization, Amicon ultrafiltration was performed to remove complexes. The adsorption/neutralizing procedure was carried out twice. As control antibodies irrelevant anti-human IgG2a and IgG2b were used.

Statistical analysis

Statistical analysis was performed using the Wilcoxon signed rank test; $P < 0.05$ was taken as the level of significance.

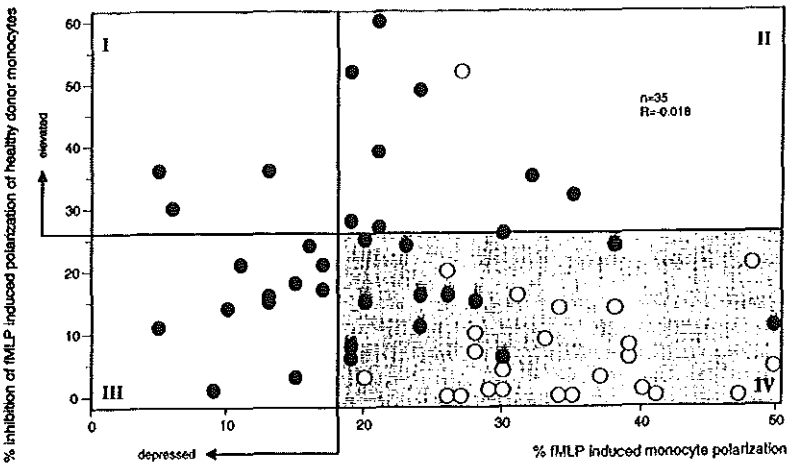

Fig. 1 Serum p15E-like low-molecular-mass factor (LMMF) levels and monocyte polarization before treatment. *I* elevated serum LMMFs and depressed monocyte polarization; *II* elevated serum LMMFs and normal monocyte polarization; *III* non-elevated serum LMMFs and depressed monocyte polarization; *IV* non-elevated serum LMMFs and normal monocyte polarization (● patients, ○ healthy controls)

Results

Defects of monocyte polarization and dendritic cell clustering capability in relation to serum p15E-like LMMF levels

Figure 1 shows pretreatment levels of p15E-like LMMFs in the sera of individual HNSCC patients in comparison to their individual monocyte chemotactic capabilities. Levels of p15E-like LMMFs in patient sera were determined indirectly by measuring their suppressive effects on fMLP-induced monocyte polarization of healthy blood donors.

In the control group of healthy volunteers the mean monocyte polarization was 34.2% ± 7.9 (n = 23), while the mean bioactive inhibitory level of p15E-like LMMFs was 7.3% ± 9.3 (n = 23). Monocyte polarization < 18.4% (mean –2 SD) was therefore considered to be defective, and LMMF levels > 25.9% inhibition (mean + 2 SD) were considered elevated. According to these criteria, 16 patients (40%) had defective monocyte polarization, viz chemotaxis of lower than 18.4% towards the chemoattractant fMLP prior to treatment. Twelve patients (31%) had elevated p15E-like LMMF levels. The patients with defective monocyte chemotaxis were evenly distributed over the group that had normal serum LMMF levels and the group that had elevated serum LMMF levels, and no correlation was possible between elevated LMMF levels and disturbed monocyte chemotactic capability (n = 35, r = 0.018, $P > 0.1$).

The mean dendritic cell cluster assay in the healthy controls was 133.5 ± 42 clusters/6 microscopic fields (n = 17). A value of less than 92 clusters/6 microscopic fields (mean – 1 SD) was considered to be abnormal. According to this criterion 16 patients had defective dendritic cell clustering capability before treatment. A correlation between dendritic cell clustering capability and p15E-like LMMF levels could not be detected (n = 34, r = –0.10, $P > 0.5$). Furthermore no correlation existed between dendritic cell clustering capability and monocyte polarization (n = 34, r = 0.08, $P > 0.5$).

Effects of TP1 treatment on monocyte polarization, dendritic cell clustering capability and serum p15E-like LMMF levels

Figure 2a shows fMLP-induced polarization of monocytes isolated from the blood of HNSSC patients before surgery (day 0) and 10 days after surgery (day 10) and administration of either placebo or a TP1 dosage. In all four groups the mean initial value (day 0) was markedly lower than the 34.2% mean of the polarized monocytes found in the healthy control group.

Treatment with 1.0 mg/kg TP1 resulted in a restoration of disturbed monocyte polarization from 17.6% ± 6.3 (n = 13) to a near-normal value of 30.9% ± 13.8 (n = 13) at day 10 ($P < 0.01$). Similar effects were seen with 2.0 mg/kg TP1 ($P < 0.05$). Monocyte polarization improved to 32.0% ± 15.8 (n = 9) from an initial 17.8% ± 18.2 (n = 9). Treatment with 0.5 mg/kg TP1 showed no significant improvement (20.9% ± 7.0 to 23.0% ± 8.1, n = 10). Placebo treatment also showed no significant effect on the impaired monocyte polarization (24.0% ± 14.2 to 25.7% ± 8.3, n = 9).

Figure 2b shows the number of clusters formed by monocyte-derived dendritic cells from the sera of HNSSC patients. At day 0, patients of all four groups showed lower values than normal, although this was least marked for the placebo group. All three TP1-treated groups showed an increase in mean dendritic cell clustering capability after treatment (0.5 mg/kg group: 86.0 ± 37.0 to 121.1 ± 38.2, n = 9; 1.0 mg/kg group: 99.7 ± 34.2 to 116.6 ± 23.0, n = 11; 2.0 mg/kg group: 98.4 ± 30.5 to 113.3 ± 23.0, n = 9). However, only the change in the 0.5 mg/kg

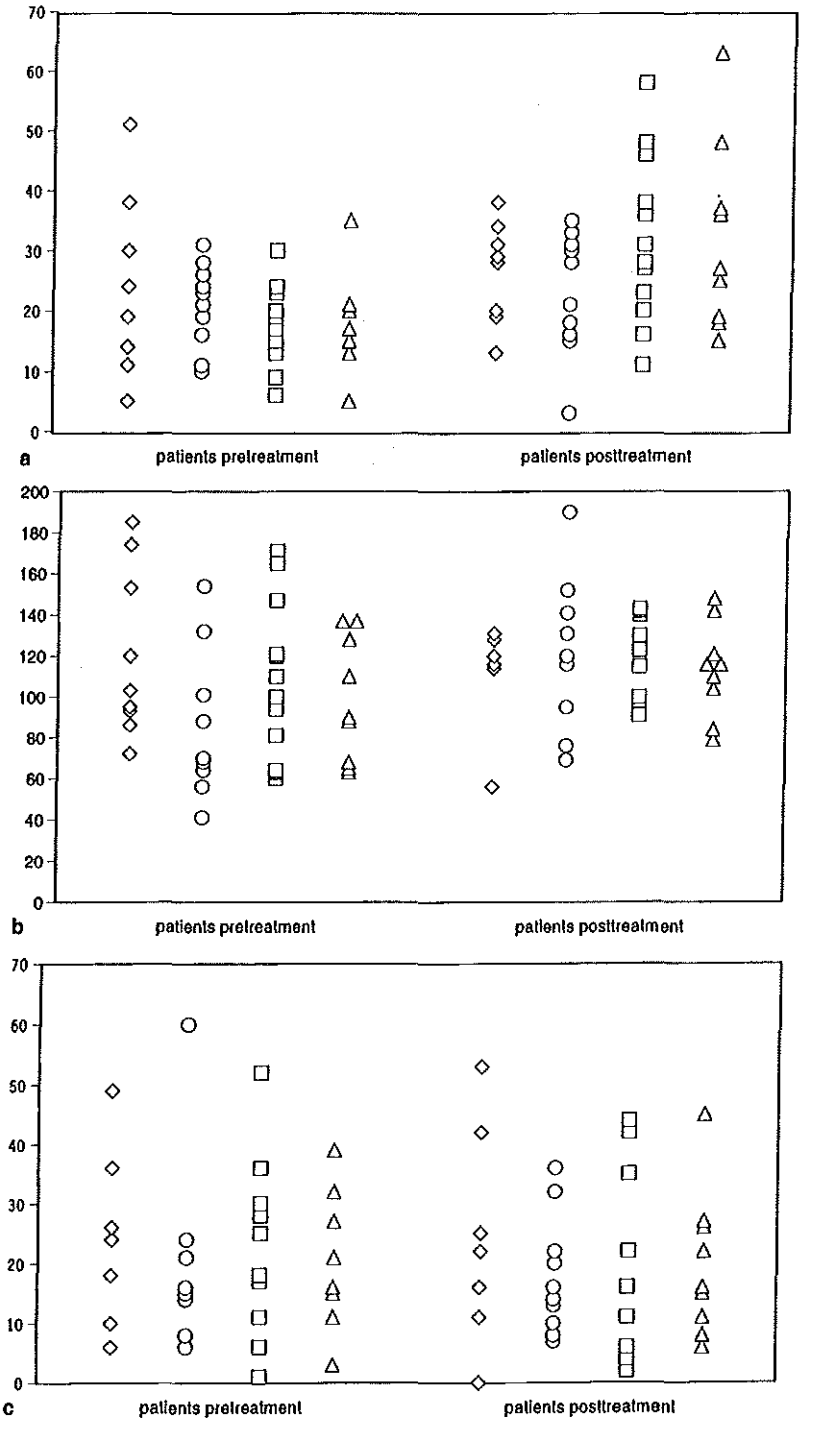

Fig. 2 a Percentage of formylmethionylleucylphenylalanine (fMLP)-induced monocyte polarization before and after 10 days of TP1 treatment (◇ placebo, ○ 0.5 mg/kg TP1, □ 1.0 mg/kg TP1, △ 2.0 mg/kg TP1). **b** Number of dendritic cell-lymphocyte clusters per 6 microscopic fields (× 200) obtained with dendritic cells from patient sera before and after 10 days of TP1 treatment. (◇ placebo, ○ 0.5 mg/kg TP1, □ 1.0 mg/kg TP1, △ 2.0 mg/kg TP1). **c** Percentage inhibition of fMLP-induced polarization of healthy donor monocytes by LMMFs prepared from patients before and after 10 days of TP1 treatment (◇ placebo, ○ 0.5 mg/kg TP1, □ 1.0 mg/kg TP1, △ 2.0 mg/kg TP1)

group reached statistical significance ($P < 0.05$). The placebo group showed some decrease in the clustering capability of dendritic cells, but this decrease was not statistically significant.

Pretreatment levels of p15E-like LMMFs and values found after 10 days of TP1 treatment are shown in Fig. 2c. No significant changes were found in the levels of serum p15E-like factors in group I (0.5 mg/kg, $n = 7$), group II (1.0 mg/kg, $n = 15$), group III (2.0 mg/kg, $n = 9$) or placebo ($n = 9$).

Discussion

Suppression of cell-mediated immunity in HNSCC is thought to be at least partially due to immunosuppressive LMMFs produced and released by tumors [9]. These factors show a structural homology to retroviral protein p15E. The p15E-like LMMFs and a 17-amino-acid peptide synthesized from MuLV p15E (anti-CKS-17) exert a suppressive effect on various immune cells. Suppression has been demonstrated in vitro on monocyte chemotactic responses [9, 36]; monocyte cytotoxicity by inactivating interleukin-1 (IL-1) [15, 22]; the respiratory burst of human monocytes [17]; activation of feline neutrophils [23]; IL-2- or IL-1-dependent proliferations of T-cells and their blastogenic responses to mitogens and allo-antigens [11, 30, 33], activity of human natural killer (NK) cells [18]; polyclonal activation of B-cells [27] and the capability of dendritic cells to form clusters per se and with T-cells [32, 41].

The presence of immunosuppressive p15E-like factors can be detected in the serum of HNSCC patients via a bioassay using healthy donor monocytes. In previous studies we showed that removal of a tumor often resulted in a postoperative decline of the level of these serum p15E-like LMMFs [42], and restoration of monocyte chemotactic responsiveness [36]. We assumed that there was a direct effect on monocyte chemotaxis by immunosuppressive p15E-like LMMFs produced by tumor and circulating in serum, since direct contact between monocytes and the serum p15E-like factors seemed inevitable. Our present data, however, have failed to demonstrate any positive correlations between defective monocyte chemotaxis or dendritic cell clustering and serum levels of p15E-like LMMFs.

An explanation for our present findings may be due to heterogeneity in sensitivity of monocytes of individual HNSCC patients to immunosuppressive effects of p15E-like factors. It is also possible that other immunomodulating factors produced in our patients and stimulating or suppressing monocyte chemotaxis may play an additional role. A third explanation might be that defective monocyte polarization and dendritic cell clustering might not be a direct result of elevated p15E-like LMMFs in serum, but that these immunosuppressive factors could possibly exert such effects as hampered maturation of fully active monocytes and dendritic cells at the level of the bone marrow. In contrast, healthy individuals may have relatively high serum levels of p15E-like LMMFs without showing clinical or experimental signs of immune suppression [39].

Our results do show restoration of defective monocyte chemotactic responsiveness in HNSCC patients due to treatment with TP1. Treatment with 1.0 mg/kg or 2.0 mg/kg body weight administered intramuscularly for 10 consecutive days prior to operation has given near-normal values for monocyte responsiveness on the day of operation. Statistically, 1.0 mg/kg was found to be the most optimal dose for restoration of the monocyte polarization, which has also been reported to be the dose most frequently used to treat patients with various other disorders [1, 7, 40]. An improvement of the dendritic cell clustering capability after TP1 treatment was seen for all TP1-treated groups; however only in the 0.5 mg/kg group did this reach statistical significance. A plausible explanation for this may be that the mean pretreatment values of the dendritic cell cluster assay of the 1.0 mg/kg and 2.0 mg/kg groups were less disturbed than those of the 0.5 mg/kg group. Therefore the possible improvement that could be achieved was less pronounced. Tas et al. [40] treated patients with chronic purulent rhinosinusitis and found a similar restorative effect of TP1 treatment on monocyte chemotactic functions accompanied by a concomitant decrease in serum p15E-like LMMF levels as well as clear clinical improvement.

In our current study, p15E-like LMMF levels in sera from HNSCC patients were unaffected by TP1 treatment. These findings suggest that TP1 counteracts the immunosuppressive effects of p15E-like factors at a cellular level and TP1 does not affect production of p15E-like factors by tumor. The decrease in detectable serum LMMF levels reported in patients with rhinosinusitis after TP1 treatment could also be explained by a lower production due to improved clinical status, rather than a direct effect of TP1 on the production or immunosuppressive properties of these p15E-like LMMFs. Conclusions concerning clinical effects of TP1 in the treatment of HNSCC cannot be drawn from our study at this time and will be addressed in a later report on the long-term results of TP1 treatment.

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Chapter 5

Thymostimulin enhancement of T cell infiltration into head and neck squamous cell carcinoma

THYMOSTIMULIN ENHANCEMENT OF T-CELL INFILTRATION INTO HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Background. Head and neck squamous cell carcinoma (HNSCC) produces immunosuppressive low-molecular-mass factors (LMMFs) responsible for defects in the cell-mediated immune system. These defects include impaired monocyte chemotaxis and an impaired capability of dendritic cells (DC) to form cellular clusters. It has been shown previously that the immunomodulating drug thymostimulin (TP1) restores these defects in vitro.

Methods. An immunohistochemical study was performed on tumors of 18 patients with HNSCC who had preoperatively been treated with TP1 in one of three dosages (0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg body weight). Additionally, tumors of 4 patients who had been treated with a placebo and 12 patients who had not received any preoperative treatment were studied. A relative surface area of infiltration, meaning the percentage of stromal or epithelial tissue covered by infiltrating cells in histologic sections, was calculated using an image analysis system (VIDAS RT) for CD3+ T-cells, CD14+/CD68+ monocytes/macrophages and L25+/CD1a+ dendritic cells for each tumor.

Results. A highly significant, denser T-cell infiltration into the stromal tissue area of tumors of patients who had been treated with TP1 when compared with tumors of non–TP1-treated patients was observed for all three dosages. None of the other tumor-infiltrating cell types was affected by TP1. In addition, a correlation was found between the tumor T-cell infiltration and capability of DCs in the peripheral blood to form clusters with T-cells. No correlation existed between CD3+ T-cell numbers in peripheral blood and T-cell infiltration into the tumor; nor were monocyte chemotactic functions in peripheral blood correlated with tumor infiltration by monocytes or monocyte-derived macrophages and DCs.

Conclusions. Preoperative treatment of HNSCC patients with TP1 appears to strongly enhance tumor–T-cell infiltration. The number of tumor-infiltrating DCs was not affected by TP1, but a positive correlation between tumor–T-cell infiltration and DC clustering capability suggests that the functional status of DCs is important in improved cell-mediated immunity.

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The cell-mediated immune system is assumed to play a significant role in defense against squamous cell carcinoma of the head and neck (HNSCC). It is therefore of interest that abnormalities in functional behavior of lymphoid cells have been described in these patients, including impairments of the function of T-lymphocytes, monocytes, and the monocyte-derived dendritic cells (DCs) and macrophages.[1–8] In previous studies we reported on defects in monocyte chemotaxis and the defective capability of antigen-presenting monocyte-derived DCs to form clusters with themselves and T-cells.[4,8–10] Although tumors may pro-

duce various immunostimulatory and immunosuppressive factors, and heretofore-described defects are at least partly due to an immunosuppressive low-molecular-mass factor (LMMF) produced by the squamous carcinoma cells.[11] Cell-mediated immune defects could be induced in vitro after isolation of the LMMF from the tumor or the serum of HNSCC patients, and neutralization experiments showed the factor to belong to the IFN-α/p15E family of immunosuppressive peptides.[12] Thymostimulin (TP1), a thymic hormone preparation, was shown to counteract the immunosuppressive action of these LMMFs in vitro, and to restore the migratory capability of blood monocytes as well as the clustering capability of DCs.[9]

In vitro and in vivo TP1 treatment improved the defective monocyte chemotaxis and DC clustering capability in HNSCC patients. Recently, we reported a restoration of monocyte chemotactic functions and an improvement of DC clustering capability on the day of operation of patients who had been treated with daily TP1 injections for a period of 10 days prior to surgery.[10] This report addresses the effect of TP1 treatment on the infiltration of T-lymphocytes, monocytes/macrophages, and DCs into the tumor itself, studying the same group of patients as referred to above, of whom the data on the number and performance of peripheral monocytes, DCs, and T-cells were available.

PATIENTS, MATERIALS, AND METHODS

Patients. Thirty-four patients with operable squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx consented to participate in the multicenter study. The study had been approved by the medical ethical committee of all participating centers. All patients met the following inclusion criteria: Squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx, with no evidence of distant metastatic disease. No other malignancies were present. Intent of curative treatment by surgery or surgery and radiotherapy. A white blood count $>4.10^9$/L, platelets $>100.0^9$/L, and hematocrit >30%. Patients had received no previous radiotherapy or surgery in the head and neck area or previous chemotherapy, nor had they been treated with corticosteroids. They were without serious nonmalignant systemic disease or serious infectious diseases.

Twenty-two patients comprised a subpopulation of a larger study group who had been entered in a multicenter, double-blind, placebo-controlled trial studying the effects of TP1 treatment of HNSCC patients. These 22 patients came from 3 of the 5 participating centers of the larger study on the restoration of monocyte chemotaxis and DC clustering (dose-finding study). Tumor tissue was taken from these 22 patients for immunohistochemistry during surgery. They were, through a random and double-blind selection, entered into one of the following four treatment groups: Group I, patients receiving placebo treatment (n = 4); group II, patients receiving 0.5 mg TP1/kg body weight (n = 4); group III, patients receiving 1.0 mg TP1/kg body weight (n = 6); and group IV, patients receiving 2.0 mg TP1/kg body weight (n = 8).

TP1 or placebo was injected intramuscularly (arm or leg) once daily for 10 consecutive days prior to surgical removal of the tumor. The intramuscular injections were well tolerated, and no adverse side effects were seen. At the time of surgery, blood was drawn for the tests described in detail previously[9,10] and in short below. An additional 12 patients (group 0) who met the inclusion criteria but did not agree to participate in the treatment protocol were not treated preoperatively; however, surgical specimens as well as peripheral blood cells on the day of surgery were studied (these patients had agreed to this type of investigation).

Patients in the various treatment groups were comparable for age, TNM classification, and primary site of the tumor (Table 1). The number of patients in each group depended on the availability of tumor tissue (not all participating centers in the larger trial provided tumor tissue), explaining the unbalanced number of patients between the groups. Because the number of patients in the respective treatment groups was small, the three groups of TP1-treated patients and the two groups of nonimmunologically treated patients were combined, when appropriate, for statistical evaluation.

Materials and Methods. Thymostimulin is a bovine thymic extract prepared by Serono, according to Bergesi and Falchetti[13] and Falchetti et al,[14] using the following procedures: calf thymus glands are minced and extracted with ammonium acetate. The extract is heated to 70°C, filtered, and precipitated with ammonium sulfate. The precipitate is dissolved in water and subjected to ultrafiltration with an Amicon PM-10 membrane. The filtrate is desalted on Sephadex G-25 and gel-filtered on Sephadex G-50. The fractions which on polyacryl-

Table 1. Clinical profiles of patients.

Patients	TNM classification	Site
Placebo (n = 4)		
1	T2N1M0	Oropharynx
2	T4N2M0	Hypopharynx
3	T4N0M0	Larynx
4	T3N0M0	Oropharynx
0.5 mg TP1/kg (n = 4)		
5	T4N0M0	Oral cavity
6	T2N0M0	Oral cavity
7	T2N2M0	Oropharynx
8	T2N1M0	Oral cavity
1.0 mg TP1/kg (n = 6)		
9	T3N1M0	Oropharynx
10	T2N2M0	Hypopharynx
11	T2N1M0	Oral cavity
12	T3N0M0	Oropharynx
13	T4N1M0	Oropharynx
14	T4N1M0	Larynx
2.0 mg TP1/kg (n = 8)		
15	T2N2M0	Oropharynx
16	T3N0M0	Oropharynx
17	T3N2M0	Oropharynx
18	T2N0M0	Oral cavity
19	T3N2M0	Larynx
20	T4N2M0	Oral cavity
21	T2N0M0	Oral cavity
22	T3N2M0	Oropharynx
Untreated (n = 12)		
23	T4N1M0	Oral cavity
24	T3N2M0	Larynx
25	T3N1M0	Oropharynx
26	T3N1M0	Hypopharynx
27	T4N0M0	Larynx
28	T4N0M0	Oral cavity
29	T1N2M0	Hypopharynx
30	T2N0M0	Oral cavity
31	T4N2M0	Hypopharynx
32	T4N2M0	Larynx
33	T4N0M0	Larynx
34	T3N1M0	Oropharynx

amide gel electrophoresis show two characteristic bands with Rf 0.22 and 0.24 are combined and termed thymostimulin (TP1). The extract is lyophilized, and its activity is expressed as units of T-cell–rosette formation per milligram of protein. It does not contain endotoxin, as tested with the pyrogen test in rabbits. In toxicologic studies, the extract does not cause any toxic or other side effects in doses up to 100 mg/kg when administered to mice for 21 days or to rats for 31 days, or when administered to cats or dogs for 180 days in doses up to 50 mg/kg.[14] The extract does not alter neuromuscular transmission either in vitro or in vitro.[13] In this study one batch of TP1 was used throughout. Placebo consisted of 5 mg mannitol in 2 mL saline.

Samples of fresh tumor tissue were obtained from surgically removed primary tumors. Small parts of tissue without macroscopic signs of necrosis, and representative of the removed tumor, were frozen in liquid nitrogen and stored at −80°C. Serial 6 μm-cryostat sections were cut and mounted on slides, air dried, and fixed in acetone. Normal rabbit serum (Dakopatts, Copenhagen, Denmark) was added and gently removed by tapping the slides after 10 minutes. The slides were then incubated for 1 hour at room temperature with the monoclonal antibodies listed in Table 2, to identify the various infiltrating lymphoid cells. All antibodies were diluted in phosphate-buffered saline (PBS) pH 7.4 enriched with 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO) and 0.01% azide before incubation. After incubation, the sections were gently rinsed with PBS to wash away excess monoclonal antibodies for at least 15 minutes and thereafter incubated with horseradish peroxidase (HRP)–conjugated rabbit anti-mouse serum (Dakopatts, Copenhagen, Denmark) with 1% human pooled serum for 30 minutes. Subsequently, the sections were rinsed with PBS for another 15 minutes and stained for peroxidase activity with 3.3′-diaminobenzidine-tetra-HCL (Sigma) in PBS containing 0.01% H_2O_2. The sections were rinsed in tap water and counterstained with hematoxylin for 30 seconds, dehydrated, and mounted in depex mounting medium.

Analysis of tissues was done using a Vidas RT image analysis system (Kontron, Munich, Germany). At magnification × 200, 5–10 randomly chosen fields of tumor stroma and 3–7 randomly

Table 2. Monoclonal antibodies.

Epitope	Antibody	Dilution	Specificity/source
CD1a	OKT6	1:100	Dendritic cells (mainly intraepithelial) (kindly provided by T. Godthelp, Rotterdam)
L25		1:1000	Dendritic cells and B cells (kindly provided by Ishii, Sapporo)
CD68	Ki-M7	1:200	Macrophages (strong cytoplasmic expression) (Behring, Marburg, Germany)
CD14	Leu-M3	1:500	Monocytes, macrophages (Becton Dickinson, San Jose, CA)
CD3	Leu-4	1:60	Immature and mature functional T-cells (Becton Dickinson)

chosen fields of malignant epithelial fields were analyzed in one or two (depending on the amount of tumor tissue available) representative sections of the tumor (no necrosis, clearly recognizable malignant squamous cell tissue). For each such field, the area covered by lymphoid cells positive for the specific marker was measured via quantitative morphometric analysis by Vidas RT system, which is based on color differences in the section (dark areas being marker-positive). The area covered by marker-positive cells was calculated as a percentage of the total stromal or epithelial area analyzed. Subsequently, a mean was calculated for each tumor and designated the "relative surface area of infiltration." If a relative surface area of infiltration was less than 0.5%, it was stated as such and no exact figure was given. If statistical analysis had to be performed in such a case, 0.4% was taken as the figure.

The isolation of blood monocytes and the polarization assay have previously been described.[9,10] The latter assay is a rapid method to reflect monocyte chemotaxis.[12] In brief, heparinized blood (from patients and healthy controls) was used for the enrichment of fresh monocytes. After Ficoll-Isopaque, and subsequently Percoll density gradient centrifugation, we obtained 60–80% monocytes as determined by enzymatic nonspecific esterase positive (NSE+) staining. These monocytes were tested in the monocyte polarization assay by exposing them to a chemoattractant (10 n*M* fMLP) for 15 minutes at 37°C, then arresting this incubation with 10% formaldehyde. A monocyte was microscopically scored "polarized" if any of the following features occured: (a) elongated or triangular shape, (b) broadened lamellipodia, or (c) membrane ruffling. The outcomes were calculated as follows: % total cells polarized/% NSE positive cells × 100%.

Both the cluster assay and preparation of DCs from blood monocytes were performed according the methods described by Kabel et al.[15] In brief, monocytes were incubated with 14.5% (w/v) metrizamide for 30 minutes at 37°C/5% CO_2, followed by thorough washing. After a 16-hour culture period under nonadherent conditions, this method yielded 40–80% DCs.[16] These blood monocyte–derived DCs were allowed to cluster with allogeneic T-lymphocytes (from healthy controls) for 4 hours at 37°C/5% CO_2 in 250 μL flat-bottomed wells. The number of clusters (aggregates of 4–25 cells) were counted and expressed per six microscopic fields (inverted microscope, magnification × 200).

Heparinized peripheral blood samples were obtained from subjects and analyzed the same day. Analyses were performed using a FAC scan flow cytometer (Becton Dickinson, San Jose, CA). Whole blood was incubated with optimally titrated fluorescein isothiocyanate (FITC)–conjugated anti-CD3 antibody (Leu-4 FITC, Becton Dickinson) for immunofluorescence staining. As a rule, irrelevant IgG2 FITC-conjugated McAbs were used as negative controls. Before flow cytometric analysis, red blood cells were lysed using FACS lysing solution (Becton Dickinson). The whole blood forward and side scatter patterns, and the percentages of granulocytes, monocytes and lymphocytes (Leucogate) were determined. Data analysis was performed on the lymphocyte population thus defined. This lymphocyte population represented at least 95% of the total number of lymphocytes and contained less than 3% monocytes. White blood cell counts were performed with a Coulter counter model ZM (Coulter Electronics, Hialeah, FL). Percentages as well as absolute numbers of the CD3+ lymphocytes were determined.

Sera for immunologic evaluation were collected a few hours before surgery, and Sera of healthy hospital staff members served as controls during the tests. Sera were collected from the patients and healthy controls by venipuncture and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CD25 Centriflo cones (Amicon) for 15 minutes at 800*g* (molecular mass "cut-off point" 25 kD). The residues were resuspended and stored at −20°C until further use.

Statistical analysis was performed using the nonparametric, unpaired Mann-Witney test (Instat computer program); $p < .05$ was taken as the level of significance.

RESULTS

Tumors of 18 patients who had received TP1 treatment (4 patients received 0.5 mg/kg, 6 patients received 1.0 mg/kg, 8 patients received 2.0 mg/kg body weight) and 16 patients who had not received TP1 treatment (4 patients received placebo, 12 patients were left untreated preoperatively) were analyzed for stromal and epithelial macrophage/monocyte, DC, and T-cell infiltration.

With regard to CD68+ macrophage infiltration, CD14+ monocyte infiltration, and CD1a+ and L25+ DC infiltration, there were no statistically significant differences between any of the groups studied: Table 3 shows mean relative sur-

Table 3. Relative surface area of macrophage, monocyte and dendritic cell infiltration in TP1-treated and non-TP1-treated HNSCC patients.

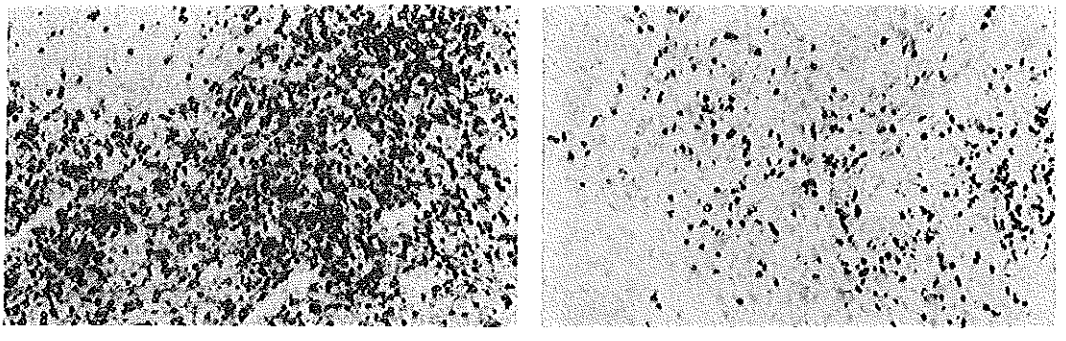
	TP1-treated patients		Non-TP1-treated patients	
	Stromal infiltration (%)	Epithelial infiltration (%)	Stromal infiltration (%)	Epithelial infiltration (%)
CD68+ macrophages	8.8 (n = 17 SD = 2.4)	2.9 (n = 17, SD = 2.6)	8.6 (n = 13, SD = 2.4)	2.5 (n = 13, SD = 1.9)
CD14+ monocytes	8.5 (n = 17, SD = 2.4)	1.7 (n = 17, SD = 1.1)	7.2 (n = 13, SD = 2.3)	2.0 (n = 13, SD = 1.1)
CD1a+ dendritic cells	<0.5 (n = 17)	1.0 (n = 17, SD = 0.9)	<0.5 (n = 15)	1.8 (n = 14, SD = 1.6)
L25+ dendritic cells	2.8 (n = 15, SD = 1.0)	0.7 (n = 15, SD = 0.5)	3.0 (n = 12, SD = 1.4)	0.5 (n = 12, SD = 0.3)

n = Number of tumors studied.

face areas of infiltration for each of these cells in tumor stroma and malignant epithelium. In this table patients are divided into two groups for reasons of simplicity only (a TP1-treated and a non–TP1-treated group).

However, T-cell infiltration into the stroma of tumors was much denser in patients treated with TP1 than in patients who had not received TP1 (Figure 1a and 1b, Table 4): the mean relative surface area of TP1-treated patients was 10.7% (n = 18, SD = 4.0) and 4.8% (n = 16, SD = 1.54) for the non–TP1-treated patients. This difference is highly statistically significant ($p < .0001$). With regard to the various subgroups: Mean stromal CD3+ cell infiltration for the 0.5 mg/kg, 1.0 mg/kg, and 2.0 mg/kg subgroups were 10.3% (n = 4, SD = 2.25), 13.0% (n = 6, SD = 2.03), and 9.11% (n = 8, SD = 5.10), respectively, each separately being significantly higher than the non–TP1-treated group ($p < .001$, $p < .0005$, $p < .05$). Intraepithelial infiltration of CD3+ T-cells was much less than stromal infiltration, but also appeared to be denser in the tumors of TP1-treated patients as compared with the tumors of non–TP1-treated patients (1.2% versus < 0.5%). However, this difference did not reach statistical significance.

Correlations were studied between the presence in the tumor of the various infiltrating cells and the outcome of the polarization assay on peripheral blood monocytes, the cluster capability of DCs isolated from the peripheral blood, and the numbers of blood CD3+ T-cells. Positive significant correlations could not be established between the presence in the tumor of CD68+ macrophages, CD14+ monocytes, or CD1a+ and L25+ DCs and either the monocyte polarizing or DC clustering capability. However, there was a significant positive correlation between the number of T-cells infiltrating into the stromal compartment of the tu-



A

FIGURE 1. (A) CD3+ T-cell infiltration in an oropharyngeal carcinoma (T2N2M0) of a TP1-treated patient. Note the dense infiltration of T-cells into the stromal tissue (relative surface area of infiltration, 18.3%). **(B)** CD3+ T-cell infiltration in an oropharyngeal carcinoma (T3N0M0) of a non–TP1-treated patient. The infiltration of T-cells is significantly lower than in tumors of TP1-treated patients (relative surface area of infiltration, 4.0%).

Table 4. Mean relative surface area of CD3+ T-cell infiltration in tumors of TP1-treated and non-TP1-treated patients.

	Stromal T-cell infiltration (%)	Epithelial T-cell infiltration (%)
Untreated (group 0)	5.0 (n = 12, SD = 1.62)	<0.5 (n = 12)
Placebo (group I)	4.1 (n = 4, SD = 0.8)	<0.5 (n = 4)
Non-TP1-treated (group 0 + I)	4.8 (n = 16, SD = 1.54)	<0.5 (n = 16)
0.5 mg/kg TP1 (group II)	10.3 (n = 4, SD = 2.25, $p < .001$)	2.0 (n = 4, SD = 2.25)
1.0 mg/kg TP1 (group III)	13.0 (n = 6, SD = 2.03, $p < .0005$)	0.9 (n = 6, SD = 0.43)
2.0 mg/kg (group IV)	9.11 (n = 8, SD = 5.10, $p < .05$)	1.7 (n = 8, SD = 1.80)
TP1 treated total (groups II, III, IV)	10.7 (n = 18, SD = 4.0, $p < .0001$)	1.5 (n = 18, SD = 1.3)

n = Number of tumors studied; p-value is given in regard to the non-TP1-treated group.

mor (these T-cells are found clustering around infiltrated DCs, as illustrated in Figure 2 and earlier reported in detail[17]) and the number of clusters found in the cluster assay using peripheral blood DCs ($r = .4, n = 25, p = .05$). No correlation existed between CD3+ T-cell infiltration into the tumor and CD3+ T-cell numbers in peripheral blood, nor between tumor T-cell infiltration and monocyte polarization.

DISCUSSION

This study shows that HNSCC samples from patients treated with TP1 had a denser infiltration of T-cells into the stromal compartment of the tumors than from patients who were untreated or those who received a placebo. Our methodology allows comparison only between treatment groups (and not individual patients), because it is based on the analysis of only 1 or 2 sections of each tumor (there may be significant differences within tumors); nevertheless our data give a strong indication that TP1 enhances T-cell infiltration into HNSCC. Effect of TP1 treatment was not seen on the pattern and distribution of infiltrating monocytes, macrophages, or DCs, the infiltration patterns and distributions within the tumor being similar to earlier reports.[17,18] The enhancing effect on T-cell infiltration of TP1 occurred in all three dosages used. In our earlier report we showed that 1.0 mg TP1/kg and 2.0 mg TP1/kg body weight were the most effective dosages for the restoration of the defective chemotáxis of blood monocytes in HNSCC patients,[9,10] although 0.5 mg TP1/kg body weight was most effective in restoring the defective DC clustering capability. In the present study 1.0 mg/kg TP1 had the greatest effect, but 0.5 mg and 2.0 mg TP1/kg were also effective.

A dense infiltration of DCs has been related to a better prognosis in laryngeal,[19] nasopharyngeal,[20] and esophageal carcinoma,[21] as well as in uterine cervical and gastric carcinoma.[22–24] Dendritic cells have an important immune function by presenting (tumor-associated) antigens to T-cells. In this process they form clusters with the T-cells. Regarding this function it seems of no surprise that a correlation between numbers of tumor-infiltrating DCs cells and T-cells has been described in the past.[25] In our study an enhancement of T-cell infiltration into the tumor after TP1 treatment was not accompanied by a denser DC infiltration. We did, however, establish a positive correlation between T-cell tumor infiltration and the capability of blood DCs to form cellular clusters with T-cells, thereby suggesting that not only numerical infiltration of DCs, as found in earlier studies, but also the functional status of the cells is of importance in cell-mediated tumor-related immunologic events. This is very much in line with the earlier-described

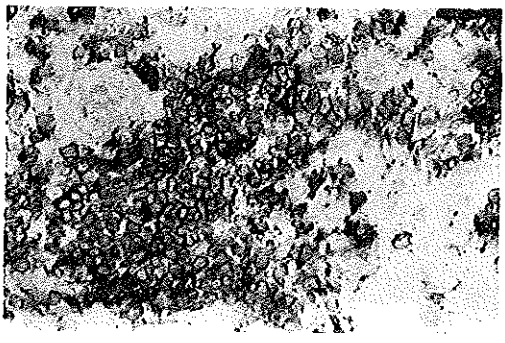


FIGURE 2. Double staining of HNSCC for CD3+ T-cells (3,3'-deaminobenzidine-tetra-HCL, brown-colored cells) and L25+ dendritic cells (fast blue, blue-colored cells) forming a cell cluster.

improvement of DC clustering capability after TP1 treatment,[10] and the currently reported enhancement of T-cell infiltration into the tumors of the same TP1-treated patients. The mechanisms by which DC functions are influenced are not understood. First, DCs form a heterogenous cell population with probably more than one precursor cell. For monocyte-derived DCs it has been shown that TP1 stimulates the maturation of DCs from monocytes.[9] Safer et al[26] demonstrated that the thymic factor Thymosin Beta 4 is important in the polymerization of actin in highly motile cells such as blood platelets and neutrophils. DCs are also highly motile cells, containing abundant actin filaments as contractile proteins.[27] Similar to the platelets and neutrophils, the actin filaments of DCs may be influenced by thymic hormones such as TP1. Because the clustering process of DCs is an active process that is highly dependent on the motility of the cells, it is possible that TP1 stimulates DC clustering by stimulation of polymerization/depolimerization of the actin filaments.

Reports concerning the clinical relevance of a dense T-cell infiltration into tumor areas are inconclusive so far. A dense stromal T-cell infiltration has been correlated to a better prognosis in HNSCC patients[28,29] as well as in stage I malignant melanoma.[30] However, Gallo[31] found no correlation between T-cell infiltration and prognosis in nasopharyngeal carcinoma, whereas in papillary carcinoma of the thyroid and renal cell carcinoma an enhanced T-cell infiltration has been associated with an unfavorable prognosis.[32] Therefore, the clinical relevance of the findings of our present study is as yet unclear.

Controversy also exists about tumoricidal capacity of macrophages,[17] but in nasopharyngeal carcinoma intratumoral infiltration of monocytes/macrophages has been related to a favorable prognosis.[25,31] Patients with HNSCC are often characterized by a defective chemotactic capability of blood monocytes,[4,7] which has been correlated to a decreased infiltration of acid phosphatase-positive macrophages into the tumor area.[4] We previously reported a restoration of monocyte chemotactic function in patients treated with TP1.[10] This study shows that TP1 treatment did not result in a significantly denser infiltration of monocytes/macrophages into the tumor, nor did a correlation exist between the presence of CD68+ macrophages and CD14+ monocytes in the tumor and the monocyte chemotactic ability as measured with the monocyte polarization assay. It seems inevitable to conclude from this study that no direct relationship appears to exist between monocyte chemotactic functions in the peripheral blood on the day of surgery and the number of monocytes and monocyte-derived cells present in the tumor.

In conclusion, this study confirms our earlier observations[9,10] that TP1 treatment with a thymic hormone preparation results in a discrete stimulation of the cell-mediated immune system in HNSCC patients. In addition to the earlier-reported restoration of impaired monocyte motility and DC clustering capability, TP1 treatment also resulted in a highly significant denser T-cell infiltration into the tumor. It cannot be concluded from the present study (in principle, a dose-finding study) if the described stimulation of the immune system by TP1 results in a better clinical outcome. A preliminary follow-up of our patient population suggests effects of TP1-treatment neither on recurrence of the disease nor on morbidity. Larger prospective studies designed to address this question are necessary.

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Chapter 6

The effects of thymostimulin on immunological function in patients with head and neck cancer

The effects of thymostimulin on immunological function in patients with head and neck cancer

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Patients with head and neck carcinoma show deficits of cellular immunity, probably due to low molecular mass factors (LMMFs) released by the tumour. Thymostimulin (TP1) restores the defective monocyte chemotaxis and dendritic cell clustering capability in the presence of the tumour when administered pre-operatively. In the present study we investigated these immune parameters in 39 patients treated with TP1 for 10 days pre- and 6 months post-operatively and in 22 patients who were not treated with TP1 for 6 months after operation. Removal of the tumour in non-TP1-treated patients also resulted in a restoration of monocyte and dendritic cell functions, while TP1 treatment gave no additional effect. LMMF levels in the blood of both TP1-treated and non-TP1-treated patients remained elevated even after removal of the tumour, and it is therefore concluded that it is unlikely that depression of cellular immunity is a direct effect of these LMMFS.

Keywords *head and neck carcinoma immunotherapy thymostimulin monocytes dendritic cells p15E*

Introduction

Patients with head and neck squamous cell carcinoma are immunologically compromised and show deficits in their cell-mediated immune system, including defective skin tests on specific and non-specific stimuli, defective chemotaxis of monocytes and defective interaction of dendritic cells with lymphocytes.[1–8] Dendritic cells are important immune accessory cells that act by presenting antigens to T cells. The defects in cellular immunity are thought to contribute to tumour growth, since the immune system is important in host defence against malignancies. With regard to the origin of the defective function of monocytes, dendritic cells and T cells low molecular mass factors (LMMF) produced by the tumours may be important. Previously we demonstrated that these factors, which show a structural homology with the retroviral protein p15E and with IFN-alpha 2b,[9,10] depress, not only T cell and NK cell function, but the ability of monocytes to change shape (polarization), the latter function being related to monocyte migratory and chemotactic capabilities.[5] The defective capability of dendritic cells to form clusters with lymphocytes has also been ascribed to the suppressive effect of the tumour-derived LMMFs.[8,11,12] A direct correlation between serum levels of LMMFs in patients with head and neck cancer and defective monocyte polarization or dendritic cell-lymphocyte

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clustering could not, however, be demonstrated in an earlier report.[13]

Thymostimulin (TP1) is an immunostimulating drug prepared from calf thymus extracts. When TP1 was added to the monocytes or dendritic cells of patients with head and neck cancer *in vitro*, it resulted in the restoration of defective monocyte polarization and dendritic cell clustering.[8] *In vivo*, also a restoration of cell-mediated immunity was seen at the time of surgical removal of the tumour when the drug had been given pre-operatively for 10 days. It was also remarkable that the tumours of these patients treated preoperatively with TP1 showed a denser infiltration of $CD3^+$ T-lymphocytes (intermingled with dendritic cells) when compared with tumours of non-TP1-treated patients.[14]

This study investigates the effects of thymostimulin on monocyte function, dendritic cell function and numbers of T-lymphocytes, T-lymphocyte subsets and NK-cells in a period of up to 180 days after surgical removal of the tumour. In a randomized trial patients were, in addition to conventional therapy (surgery or surgery and radiotherapy), treated with one of three regimes of TP1 treatment (0.5 mg/kg; 1.0 mg/kg; 2.0 mg/kg) or a placebo, from 10 days prior to surgical removal of the tumour to 6 months post-operatively. A group of patients not immunologically treated was also included. Monocyte polarization, the capability of the monocyte-derived dendritic cells to cluster with allogeneic lymphocytes, serum p15E-like LMMF levels and numbers of blood lymphocytes and lymphocyte subsets were monitored for the entire period of treatment. Although the study was not designed to evaluate the prognostic relevance of the treatment, outcome in terms of recurrence will nevertheless be considered, since the majority of patients have now been followed-up for a period ranging from 30 months to 5 yr.

Material and methods

PATIENTS

Forty-one patients with a squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx and larynx agreed to participate in a multicentre trial. Inclusion criteria have been listed before in detail;[13] most important, patients with co-existent disease were excluded. The patients were randomly entered into one of the four following groups: patients receiving placebo treatment (group A); patients receiving 0.5 mg/kg TP1 (group B); patients receiving 1.0 mg/kg TP1 (group C); and patients receiving 2.0 mg/kg TP1 (group D). This dosage scheme was chosen since 1.0 mg/kg TP1 has been reported to be effective in several immunodeficiency disorders.[15-17] In the placebo group one patient developed an unexplained thrombosis and one patient developed pulmonary complications post-operatively. Both patients were excluded from further study, leaving 39 patients for evaluation. Treatment consisted of 10 daily pre-operative intramuscular injections, and 21 daily post-operative injections, after which treatment was continued for 21 weeks twice weekly.

Additionally, 22 patients operated upon in the same period and who did not receive TP1 or placebo were studied as an additional control group (group E). Patients were entered in group E if they met the inclusion criteria, but declined to participate in the trial (they did consent to serve as controls). In this group, four patients consented to pre-operative laboratory tests only, one patient was lost to follow-up and in two patients a macroscopical incomplete resection was performed; the post-operative data on these three patients were therefore excluded from evaluation (leaving 19 patients for evaluation in group E).

TNM classification ranged from T1N1 to T4N2. Localization and classification were evenly distributed over all groups of patients entered into the trial, as well as patients in group E. Primary treatment consisted of surgery alone (15 patients) or surgery followed by radiotherapy (43 patients).

Blood was taken before starting the TP1 injections (day −10), on the day of operation (day 0) and on day 21, day 90 and day 180 post-operatively for the laboratory tests described below. One patient in the trial population developed a second primary head and neck carcinoma within the follow-up period, post-operative laboratory data were therefore excluded from evaluation concerning the prognostic value for recurrence.

Clinical follow-up was until recurrence or for a minimum of 30 months up to 5 yr.

BOVINE THYMIC EXTRACT (THYMOSTIMULIN, TP1)

Thymostimulin was prepared as a bovine thymic extract using the following procedure, described by Bergesi and Falchetti[18] and Falchetti *et al.*[19] Calf thymus glands were minced and extracted with ammonium acetate. The extract was then heated to 70°C, filtered, and precipitated with ammonium sulphate. The precipitate was dissolved in water and subjected to ultrafiltration on an Amicon PM-10 membrane. The filtrate was desalted on Sephadex G-25 and gel-filtered on Sephadex G-50. Fractions showing characteristic bands at RF 0.22 and 0.24 on polyacrylamide gel electrophoresis were combined and termed 'thymostimulin' (TP1). This extract was next lyophilized and its activity expressed as units of T-cell-rosette formation per milligram of protein. No endotoxin was contained in this extract, as tested in doses up to 100 mg/kg when administered to mice for 21 days or rats for 31 days, or when given to cats or dogs for 180 days in doses up to 50 mg/kg.[19] Additionally, the extract failed to alter neuromuscular transmission *in vitro* or *in vivo*.[18].

ISOLATION OF BLOOD MONOCYTES AND MONOCYTE POLARIZATION ASSAY

The isolation of blood monocytes and the polarization assay have previously been described in detail.[8,13] The latter assay

correlates to monocyte migration and chemotaxis.[5] In brief, heparinized blood (from patients and healthy controls) was used for the enrichment of fresh monocytes. After Ficoll-Isopaque, and subsequently Percoll density gradient centrifugation, we obtained 60–80% monocytes as determined by enzymatic non-specific esterase positive (NSE+) staining. These monocytes were tested in the monocyte polarization assay by exposing them to a chemo-attractant (10 nM fMLP) for 15 min at 37°C, and arresting this incubation with 10% formaldehyde. A monocyte was microscopically scored 'polarized' if any of the following features occurred: (a) elongated or triangular shape; (b) broadened lamellipodia; and (c) membrane ruffling. The outcome was calculated as follows: % total cells polarized/% NSE+cells × 100%.

MONOCYTE-DERIVED DENDRITIC CELLS AND THEIR CLUSTERING CAPABILITY

Dendritic cells were maturated from blood monocytes and their cluster behaviour with lymphocytes was assayed according to the methods described by Kabel *et al.* in detail.[20] In brief, monocytes were incubated with 14.5% (w/v) metrizamide for 30 min at 37°C/5% CO_2, and thoroughly washed. After a 16-h culture period, under non-adherent conditions, this method yielded 40–80% dendritic cells.[21]

These monocyte derived dendritic cells were allowed to cluster with allogeneic T-lymphocytes (from healthy controls) for 4 h at 37°C/5% CO_2 in 250 µl flat-bottomed wells. The number of clusters (aggregates of 4–25 cells) were counted and expressed per six microscopic fields (inverted microscope, 200× magnification).

NUMBER OF BLOOD LYMPHOCYTES AND LYMPHOCYTE SUBSETS

Details on the technique can be found elsewhere.[22] Heparinized peripheral blood samples from patients and controls were obtained and analysed. Whole blood was incubated for the detection of surface markers by the use of optimally titrated monoclonal antibodies. Isotype identical irrelevant monoclonal antibodies, conjugated with either FITC or PE, were used as negative controls. We used the markers CD3 (Leu-4 FITC, Becton Dickinson), CD4 (Leu-3 PE or Leu-3 FITC, Becton Dickinson) and CD8 (LEU-2 PE, Becton Dickinson) to label T cells. For natural killer (NK) cells, the marker CD167 (Leu-11c PE, Becton Dickinson) was used. CD14 (MY4 PE, Coulter Clone) was used to determine the percentage of granulocytes, monocytes, and lymphocytes. Data analyses were performed on the lymphocyte population thus defined. This population represents at least 95% of the total number of lymphocytes and contained less than 3% contamination with monocytes. White blood cell counts were performed with a Coulter counter. The surface membrane labellings were measured and analysed using a FACScan flow cytometer and FACScan research software.

DETERMINATION OF P15E-LIKE LMMFS IN SERA

Sera were collected prior to the first TP1 injection and 1–2 h before surgery. Sera of healthy hospital staff members served as controls. Sera were collected from the patients and healthy controls by venipuncture and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CD25 Centriflo cones for 15 min at 800 g (molecular weight 'cut off point' 25 kD). The residues were resuspended and stored at −20°C until further use.

The capability of the serum fractions to inhibit fMLP-induced polarization of healthy donor monocytes (elutriator purified) was determined by incubating the monocytes (1×10^6/ml) for 15 min at 37°C, either with fMLP alone or with fMLP in combination with a serum fraction (final dilution 1:60). The percentage of inhibition was calculated as follows:

$$i = (1-(Ff\text{-}fo/Mf\text{-}fo)) \times 100\%$$

Ff = % monocytes polarized after incubation with fMLP and LMMF, Mf = % monocytes polarized after incubation with fMLP alone, fo = % spontaneously polarized monocytes. Addition of serum fractions to non-fMLP stimulated donor monocytes did not affect the spontaneous polarization.

To validate the p15E-like character of LMMF adsorption, experiments were carried out by neutralizing serum fractions at 4°C for 16 h before testing the effects on monocyte polarization by using a combination of two p15E specific monodonal antibodies (4F5 and 19F8 as anti-p15E isotypes IgG2a and IgG2b). The final mAb dilution was 1:200 and final IgG concentration 50 µg/ml. Following neutralization, Amicon ultrafiltration was performed to remove complexes. The adsorption/neutralizing procedure was carried out twice. Irrelevant anti-human IgG2a and IgG2b were used as controls.

Statistical analysis

Statistical analysis was performed using the Wilcoxon matched pairs signed ranks test and the Kruskal Wallis test.

Results

MONOCYTE POLARIZATION

Table I shows the percentages of monocytes capable of polarizing on exposure to the chemo-attractant fMLP for the four groups of patients entered into the trial, as well as for the group of conventionally treated patients (group E). There was a significant improvement in monocyte polarization from 17.6% to 30.9% after 10 days of pre-operative treatment with

Table 1. Monocyte polarization (in percentages)

Day	Group A	Group B	Group C	Group D	Group E
−10	24	20.9	17.6*	17.8†	
	(n = 9; SD 14)	(n = 10; SD 7.0)	(n = 13; SD 8.2)	(n = 9; SD 8.2)	
0	25.7	23	30.9*	32†	19.5‡
(operation)	(n = 9; SD 8.3)	(n = 10; SD 10.1)	(n = 13; SD 13.8)	(n = 9; SD 15.7)	(n = 22; SD 7.9)
21	32.1	24.9	32.2	23.7	25.4
	(n = 7; SD 14.7)	(n = 8; SD 7.4)	(n = 11; SD 11.9)	(n = 9; SD 10.6)	(n = 16; SD 9.7)
90	35.1	27.9	30.5	28.8	30.7‡
	(n = 7; SD 12.0)	(n = 10; SD 12.2)	(n = 13; SD 12.0)	(n = 8; SD 10.7)	(n = 11; SD 7.3)
180	24	30.6	27.9	29.3	18.3
	(n = 5; SD 12.2)	(n = 10; SD 9.5)	(n = 12; SD 7.9)	(n = 8; SD 12.7)	(n = 10; SD 5.3)

Group A = placebo; group B = 0.5 mg/kg TP1; group C = 1.0 mg/kg TP1; group D = 2.0 mg/kg TP1; group E = conventionally treated.
*$P < 0.05$
†$P < 0.05$
‡$P < 0.05$

1.0 mg/kg TP1 and from 17.8% to 32.0% for 2.0 mg/kg TP1 (as has been reported before).[13] Such improvements were not seen in the placebo and 0.5 mg/kg TP1 groups.

With regard to the long-term effects of TP1, it is essential to note that the group of conventionally treated patients (group E) showed a significant ($P < 0.05$, Wilcoxon) improvement in monocyte polarization after operation: a value of 19.5% polarized monocytes on day 0 (day of operation) rose to 30.7% on day 90 post-operatively. This restoration of monocyte polarization after surgery is a phenomenon described before[5] and the removal of the tumour with its suppressive factors is held responsible for this restoration. The mean pre-treatment monocyte polarization of the placebo group (24%) was only relatively mildly disturbed (Table 1); therefore the improvement to 35.1% after surgery was not statistically significant. TP1 treatment (groups B, C, D) had no further stimulating effects on the already restored monocyte polarization after surgical removal of the tumour; at day 90 there was therefore no difference between the groups.

At the end of the treatment period (day 180) the Kruskal Wallis test showed a difference ($P < 0.05$) between groups, suggesting a renewed disturbance of monocyte polarization in the non-TP1 treated patients (Table 1). The number of patients at day 180 in the various treatment groups was too small to evaluate.

DENDRITIC CELL CLUSTERING

Table 2 shows the mean values of the dendritic cell cluster assay for the five treatment groups. The pattern is similar to the outcome of the monocyte polarization assay. A significant preoperative improvement ($P < 0.05$) from 86 to 121 was found for the 0.5 mg/kg group.[13] For both the 1.0 mg/kg and 2.0 mg/kg group the pre-operative improvement was not statistically significant. At day 90 post-operatively, a significant improvement ($P < 0.05$) of the dendritic cell clustering capability compared to the pre-operative value (day 0) was again seen for the untreated group (Table 2) and the phenomenon was now also evident in the placebo-treated group. Such improvements were also recorded for the 1.0 mg/kg and the 2.0 mg/kg group; however, in the latter this did not reach statistical significance. In the 0.5 mg/kg group, pre-operative improved values did not rise further from day 0 to day 90 post-operatively.

At day 180 after operation no statistical significant differences for the dendritic cell cluster assay were found between any of the five groups.

p15E-LIKE LMMFS

Table 3 shows the mean levels of p15E-like LMMFs in the serum of the five treatment groups. In all groups pre-operative values of serum LMMFs (measured by percentage inhibition of polarization of healthy donor monocytes) were considerably higher than the mean percentage inhibition of donor monocytes by p15E-like LMMFs in the group of healthy controls. TP1 treatment had no effects on these levels, and on the day of operation similarly elevated mean serum levels of LMMFs were found in groups A, B and C, as well as in groups D and E. The mean LMMF serum-levels did not drop post-operatively in any of the groups and remained elevated for 6 months post-operatively. If patients were divided into a recurrence and a non-recurrence group (for details see further) as is shown in Figure 1, pre-operative p15E-like LMMF levels were similar in both groups and we did not see the post-operative decline in LMMF levels in the non-recurrence group as was reported in an earlier study.[23]

LYMPHOCYTES AND LYMPHOCYTE SUBSETS

The values of the number of blood $CD3^+$ T cells, $CD4^+$ and $CD8^+$ lymphocyte subsets, $CD16^+$ NK cells and $CD14^+$

Table 2. Dendritic cell clustering

Day	Group A	Group B	Group C	Group D	Group E
−10	120	86†	100	98	
	(n = 9; SD 40.8)	(n = 9; SD 37.0)	(n = 12; SD 34.2)	(n = 9; SD 30.5)	
0	111*	121†	117‡	113	102§
(operation)	(n = 6; SD 27.7)	(n = 9; SD 38.2)	(n = 11; SD 23.0)	(n = 9; SD 23.0)	(n = 20; SD 23.5)
21	122	125	126	103	119
	(n = 5; SD 15.5)	(n = 8; SD 36.3)	(n = 10; SD 18.5)	(n = 9; SD 30.1)	(n = 13; SD 29.0)
90	139*	128	144‡	134	142§
	(n = 6; SD 21.2)	(n = 8; SD 34.1)	(n = 13; SD 32.5)	(n = 7; SD 50.3)	(n = 9; SD 21.7)
180	117	115	123	140	115
	(n = 4; SD 40.6)	(n = 9; SD 26.2)	(n = 11; SD 18.6)	(n = 8; SD 34.5)	(n = 8; SD 20.3)

Group A = placebo; group B = 0.5 mg/kg TP1; group C = 1.0 mg/kg TP1; group D = 2.0 mg/kg TP1; group E = conventionally treated.
*$P < 0.05$
†$P < 0.05$
‡$P < 0.05$
§$P < 0.05$

Table 3. p15E-like LMMF levels (in percentage, fMLP-induced donor monocyte polarization inhibition)

Day	Group A	Group B	Group C	Group D	Group E
−10	24	20	22	22	
	(n = 7; SD 14)	(n = 10; SD 15)	(n = 10; SD 15)	(n = 8; SD 9)	
0	24	18	20	19	16
(operation)	(n = 7; SD 18)	(n = 10; SD 10)	(n = 10; SD 16)	(n = 8; SD 13)	(n = 8; SD 19)
21	26	22	24	22	17
	(n = 5; SD 22)	(n = 8; SD 13)	(n = 8; SD 18)	(n = 8; SD 19)	(n = 7; SD 14)
90	26	29	15	28	23
	(n = 5; SD 9)	(n = 10; SD 11)	(n = 10; SD 9)	(n = 6; SD 15)	(n = 5; SD 19)
180	23	26	17	23	18
	(n = 5; SD 14)	(n = 8; SD 15)	(n = 8; SD 18)	(n = 7; SD 17)	(n = 5; SD 15)

Group A = placebo; group B = 0.5 mg/kg TP1; group C = 1.0 mg/kg TP1; group D = 2.0 mg/kg TP1; group E = conventionally treated.

monocytes are not separately shown. No statistically significant difference in the numbers of any of these cells was found between patients and healthy controls, nor between different treatment groups at any time. Thus, neither for TP1 treatment (in the presence or absence of the tumour), nor for operation, could an effect on the number of these cells be demonstrated.

RECURRENCES

The number of patients in this study is too small to do a valid statistical analysis on the effect of TP1 treatment and immune performance on recurrence rates (the study was not designed for this purpose). However, we felt it important to evaluate trends for the design of possible further studies.

In the placebo group 5/7 (71%) of the patients had a recurrence of disease within 30 months, whereas in the conventionally treated group only 4/19 (21%) had a recurrence, giving in total a recurrence rate of 9/26 (34.6%) in the non-immunologically treated patients (however, the range between the two groups is not acceptable). With regard to the TP1 treated groups, 2 of 10 (20%) patients in group B (0.5 mg/kg), 7/13 (54%) in group C (1.0 mg/kg), and 3/9 (33%) in group D (2.0 mg/kg) developed recurrences. Hence in total 12/32 (37.5%) TP1 treated patients developed a recurrence, and this is not different from the non-immunologically treated patients.

For the purpose of further analysis, patients were divided in two groups: patients who developed a recurrence and those who did not. Localization of tumour and T stage were similar in both groups; the recurrence group had slightly more neck node involvement. Patients who received TP1 were equally distributed over both groups: 55% versus 51% (of the patients with a recurrence, 37.5% received TP1 in any of the three regimes and 34.6% did not receive TP1). We could not demonstrate a correlation between the presence of defective monocyte polarization (defined as a value of $< 18.4\%$)[11] pre-operatively or at any time post-operatively and the development of a recurrence, neither was there such a correlation between defective dendritic cell-lymphocyte clustering (defined as a value of < 92 clusters)[13] and recurrence of disease.

It must be noted, however, that of the only three patients

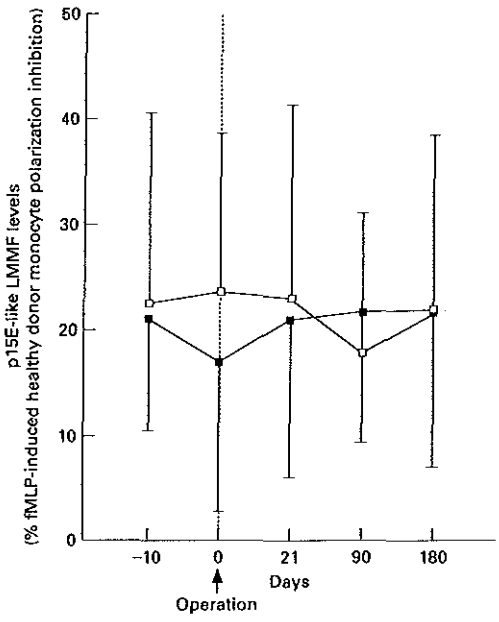

Figure 1. Serum p15E-like LMMF levels in the group of patients who developed a recurrence and the group of patients who did not develop a recurrence. □ = recurrence; ■ = non-recurrence.

who had both defective monocyte polarization and defective dendritic cell-lymphocyte cluster capability at the end of the treatment period, two developed a recurrence thereafter, while the third patient developed a squamous cell carcinoma of the lung. On the other hand, of the patients ($n = 3$) who had fully restored monocyte polarization (defined as a value at the upper limit of normality; > 30%) and a fully restored dendritic cell-lymphocyte clustering (defined as a value at the upper limit of normality; > 130 clusters) on day 180, none developed a recurrence.

Discussion

The present study and an earlier one[13] have shown that thymostimulin (TP1) treatment for 10 days prior to surgery, did restore to normal defective monocyte polarization and defective cluster capability of dendritic cells. We also reported on an earlier occasion that tumour infiltration with $CD3^+$ T cells was much denser in the patients treated with TP1 preoperatively.[14] It must be noted, however, that statistically significant differences were reached with different TP1 doses; 1.0 mg/kg and 2.0 mg/kg bodyweight were the best doses to restore monocyte polarization, while 0.5 mg/kg was best to restore the dendritic cell cluster capability. The enhanced T cell infiltration of the tumour itself was seen with all three doses of TP1. Whether these findings on dose-effects are real or only due to the small numbers of patients studied in each group needs further investigation.

The present data on the long-term effects of TP1 treatment (90 and 180 days post-operatively) take our earlier observations further and confirm that ablative surgery in itself results in a restoration of monocyte polarization and dendritic cell clustering capability.[5] The study also shows that TP1 treatment has no further immune stimulating effect in the 90 days following operation. Hence, TP1 does not seem to stimulate monocytes and dendritic cells directly, but seems to restore them to normal in the pre-operative period when the tumour is still present. A rationale for TP1 treatment after surgical removal of the tumour is hence lacking. It must also be noted that neither the presence of the tumour nor treatment with TP1 had any short or long-term effects on the number of T lymphocytes, T lymphocyte subsets, B cells or NK cells in the peripheral blood.

Despite the fact that conclusions concerning clinical effects may not be drawn from the present trial, we did not find any sign of evidence for an improvement in disease outcome in TP1 treated patients. If would, however, be incorrect to conclude at this stage that TP1 is an ineffective adjuvant in the treatment of head and neck cancer. TP1 may turn out to be a beneficial adjuvant, especially for selected patients with a clearly disturbed cellular immune system before operation. Further studies could focus on the use of thymic hormone treatment under these circumstances.

We and others[1,3,4,5,6,13,24,25] have extensively reported the defective monocyte polarization and the related defective monocyte chemotactic functions in head and neck cancer, as well as on the defective dendritic cell clustering capability. These various defective functions of the cell mediated immune system were assumed to be caused by an immunosuppressive protein produced by the tumour and released into the serum. Cianciolo[26] was the first to demonstrate that human tumours release such low molecular mass factors with *in vitro* immunosuppressive capabilities.[5,6] These LMMFs were later shown to have a strong functional and structural homology with the retroviral protein p15E and were therefore referred to as p15E-like LMMFs. Tumours and sera of patients contain these LMMFs, and they were shown to inhibit monocyte polarization *in vitro*.[7] The cellular mechanism by which p15E-like LMMFs influence monocyte motility is, however, not clear. Moreover, p15E-like LMMFs can be produced by cells other than squamous carcinoma.[11,12,15] Recently, we could not demonstrate a direct correlation between serum LMMF levels and defective monocyte polarization *in vivo*.[13] This argues against a direct effect of tumour-released LMMFs present in the circulation of a patient on the monocytes present in the same circulation. This notion is further supported by the present finding that the p15E-like LMMF levels were not affected by the TP1 injections, while there was a clear effect on the monocyte and dendritic cell functions.

In the present study we could not confirm earlier data on a predictive value of p15E levels with regard to recurrence. This may be explained by the fact that in the earlier study only small numbers of patients were studied.

In conclusion, the migrating function of monocytes and monocyte-derived dendritic cells is suppressed in patients with head and neck cancer. Although it is likely that factors released by the tumour are responsible for this suppression, this study did not show a simple correlation between the presence of p15E-like LMMFs in serum (most likely tumour-derived) and a disturbed function of the patient's monocytes. Hence more factors are involved. TP1 treatment did restore the suppressed function of monocytes and monocyte-derived dendritic cells before surgical removal of the tumour; however, TP1 treatment after surgical removal did not contribute to a further improvement other than that produced by removal of the tumour.

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Chapter 7

GENERAL DISCUSSION

Objectives

Despite advances in surgical- and radiation techniques, as well as the introduction of various chemotherapeutic agents, survival of HNSCC patients has not improved over the past 3 decades. Intervention with the immune system using biologic response modifiers (BRM's), commonly called immunotherapy, has more recently become available, and has yielded reasonable succes rates in a number of cancers, especially in malignant melanoma and renal cell carcinoma [1,2]. Cytokine treatments (IL-2, IFN-α) have been reported to achieve partial remissions in about 10-20% of patients with these tumors. Complete remissions are however rare and the partial remissions are not longlasting [3].

Significant clinical results from the use of BRM's have so far not been achieved in HNSCC patients. The *in vivo* studies, focussing on the immunomodulating drug thymostimulin (TP-1), described in this thesis show that:

1. a recognizable pattern of lymphoid cell infiltration exists in head and neck squamous cell carcinoma's. Distinctive small stromal fields of T lymphocytes intermingled with dendritic cells can be seen, while tumors are generally densly infiltrated with macrophages. The T cell component of this infiltrate can significantly be enhanced by preoperative TP-1 treatment.
2. preoperative TP-1 treatment of head and neck squamous cell carcinoma patients results in
   - a normalization of the disturbed monocyte polarisation
   - a restoration of the disturbed dendritic cell clustering capability.
3. removal of the tumor similarly results in a restoration of the disturbed dendritic cell clustering capability, and confirm earlier studies that after removal of the tumor a normalization of monocyte polarization occurs.
4. neither TP-1 treatment nor removal of the tumor affects p15E-like low molecular mass factor levels in blood of head and neck squamous cell carcinoma patients, and that there is no spatial relationship of p15E-like factor positive cells in HNSCC and the infiltration with macrophages or dendritic cells.
5. show that TP-1 treatment does not further enhance restoration of immune functions accomplished by surgery on a short or long-term basis.

TP-1 has thus been shown in our studies to be a potent BRM. However TP-1 is certainly not the only immunomodulating drug able to enhance immune functions in head and neck squamous cell carcinoma patients. A discussion of other potent BRM's follows below.

Other BRMs used in HNSCC

The most widely studied agent in head and neck cancer is IL-2. IL-2 is a 15 kD glycoprotein, produced by activated CD4+ T lymphocytes, which is critical in the activation of cytotoxic cells that can mediate host anti-tumor activity. In HNSCC, IL-2 has been administered systemically as well as locally. The disadvantage of systemic administration is its dose-limiting severe toxicity [4]. Therefore, most studies have concentrated on local administration, which can be either intratumoral, peri-lymphatic, or intra-arterial through the external carotid artery and the main tumor arterial supply [5,6]. Although Matijssen [7] found no clinical responses and no immunohistochemical changes in the tumor-infiltrate from perilymphatic IL-2 injections, most studies report differently. Denser infiltration of T lymphocytes [8,9], of CD1a+ dendritic cells [8], of NK-cells [9], of LAK-cells [10], and of eosinophils [5], as well as an enhanced CD25 (IL-2 receptor) expression [8-10] and TIL cytolytic activity [9] has been reported. However the few clinical responses are generally not long lasting [5]. Recent laboratory studies have shown some promising prospectives regarding IL-2 treatment. Firstly, a combination of IL-2 with IL-12, a cytokine that particularly stimulates the T helper-1 (TH1) response and also induces lymphocyte cytolytic activity, was *in vitro* found to activate cytolysis more than either cytokine alone [11]. Secondly, the use of mutant IL-2 molecules with various amino acid subtitutions resulted in less toxicity, and this might make systemic therapy more attractive [4]. Thirdly, in a study by Whiteside et al. [12], tumors arising from a HNSCC cell line transfected with the IL-2 gene, that were induced in immunosuppressed nude mice, regressed spontaneously. Translating this model to a clinical situation, it might mean that after removing cancer cells from a patient; the transduction of tumor cells ex vivo with cytokine genes, and a reintroduction of genetically engineered cells into the patient might lead to an immune response to the tumor. Since this approach is technically demanding, large scale clinical applications of this method may not be expected in the near future.

Another group of cytokines that has been studied in the treatment of HNSCC, either alone or in combination with IL-2 are the interferons IFN-α and IFN-γ. By and large, IFN-α augments MHC class I expression, while IFN-γ upregulates class II antigens. Systemic IFN-α treatment in combination with IL-2 [13,14], as well as IFN-α alone [5,15] have been reported to have anti-tumor activity and to induce partial or complete responses in a low percentage of patients with head and neck cancer; however, improved survival rates due to these treatments have not been shown. IFN-γ by itself has so far been found ineffective in the treatment of (head and neck) cancer [16,17].

Kimura [18] reported a significantly better 5 year cumulative survival rate in a group of head and neck cancer patients treated with Sizofilan (SPG) as adjuvent therapy, compared to a matched control group. SPG is a glucan produced by *Schizophyllum commune Feries* (a Basidiomycetes species), and is considered to stimulate the induction of a number of effector cells against tumors.

Unfortunately, the study mentioned above is on a small group of patients (15), and so far no larger studies have been reported confirming these findings. OK-432 is a preparation acquired from Streptococcus pyogenes A3. It stimulates the cytotoxic activity of macrophages, NK-cells, LAK-cells, and T cells; it also induces the production of a number of cytokines, including IL-1, IL-2, IL-6, IFN, TNF, NKAF, and TGIF, a tumor growth inhibitory factor [19-21]. Patients preoperatively treated with local injections of ex vivo OK-432-cultured blood lymphocytes in combination with radiotherapy or chemotherapy showed a dense infiltration of T lymphocytes in the tumor on immunohistochemical examination, and an overall response rate was observed in 88% of the patients [19]. A control group was however not mentioned in this study. In another study by Fukazawa [22], 78 patients received peri-operative OK-432 treatment (intramuscular injections) in combination with BCG, and Ge-132 (β-carboxyethyl germanium sesquioxid, an interferon inducer). A significant better 5 year survival rate was observed compared to a historical control group (57.3% vs 28.6%). Clearly, randomized, controled studies are needed to fully evaluate the use of OK-432 in the treatment of head and neck cancer.

Although research concerning the use of BRM's in HNSCC treatment regimes has been going on for more than a decade, it has so far not yielded a widespread clinical application. It has become clear that it is certainly possible to boost the immune response, resulting in for instance an enhanced tumor infiltration by mononuclear cells and an enhanced cytotoxicity (see TP-1 and OK-432 treatments). This enhanced immune reactivity has however not yet been translated into a better survival of patients. Ongoing work on the use of ex vivo cultured and stimulated (tumor specific) cytotoxic cells, and on the use of gene-therapy may result in future more powerful immunomodulating treatments. In the meantime however we should also concentrate on disclosure of the tumor mechanisms of "immune resistance", viz the production of immunosuppressive agents by the cancer cells. This awareness of immunesuppressing capabilities of the HNSCC particularly applies to yet another new approach in immunotherapy, i.e. the vaccination with tumor-antigen loaded DC [23]. Recent research indicates that such vaccination indeed has a favourable outcome in both animal models of tumor growth as well as in cancer patients [24,25]. If - in addition to such vaccination - immunosuppressive TM-factors influencing DC function could be neutralized with thymic factors or α-TM antibodies even better outcomes might be expected.

Conclusion

Head and neck squamous cell carcinomas that become clinically evident are apparently resistant to the specific and non-specific arm of the cell mediated immune response. If this is a direct result of the well documented functional defects of CMI in HNSCC patients, which are, in part caused by factors produced by the tumors themselves, is not clear. Other deficits in the defence

mechanisms, like for instance the lack of expression of MHC-class II costimulatory molecules on tumor cells; the release of tumor (super-) antigens that anergize cytotoxic cells or an inadequate expression of MHC-class I molecules by the tumor cells may also play a role, while there may be many other unknown factors.
Immunomodulating therapies have yielded some encouraging results (enhanced T cell infiltration, restoration of defective CMI), but have so far not resulted in an improved disease free survival in the majority of studies. Currently, ex vivo culture of tumor specific cytotoxic cells, the transduction of genes into tumor cells and the vaccination with tumor-peptide loaded DC seem to be the most promising strategies for the near-future. Research should further focus on the isolation of tumor infiltrated and TAA-specific lymphocytes, culturing these cells outside the body, and define optimal ways to activate and stimulate these cells with various cytokines like IL-2 and IL-12 before re-introducing them into the patient. Transduction of cytokine producing genes in such T cells which seems to work in animal models is already finding its application in humans, but the procedure using retroviral or adeno-associated vectors is complex. Furthermore, it must be kept in mind that small tumors may well be more susceptible to immuno-modulating therapy than large tumors, and that most trials have focussed on patients with advanced stage disease, while small tumors are often well managed by traditional techniques. In this respect (a combination of) agents may be more benificial in a perioperative adjuvant setting in case of smaller tumors. However, in view of the many different escape mechanisms cancers possess, it will be very difficult to find a generally acceptable, effective immuno-modulating therapy based on natural cytotoxicity.

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SUMMARY

In the general introduction (chapter 1), the basics of host defense are explained with special emphasis on tumor immunology, and on head and neck cancer in particular. The role of the various cells of the cell mediated immune system is discussed. Impairments of immune functions in head and neck squamous cell carcinoma patients are well documented, and a detailed discussion on such impairments is part of this chapter. Furthermore, immunosuppressive factors related to head and neck squamous cell carcinoma's are being eluded on.

In chapter 2 the objectives of the studies described in this thesis are listed:

1. The first objective was to give a detailed analysis of the pattern of head and neck cancer infiltration by macrophages, dendritic cells and T cells.
2. The second objective was a study on the pattern of infiltration by macrophages and dendritic cells in relation to the immunohistomorphological expression by the tumor cells of low molecular mass factors (so-called p15E-like or TM factors) that are capable of suppressing monocyte, macrophage and dendritic cell functions.
3. The third objective was a study on the in vivo effects of a thymic hormone preparation (TP-1) on the
   - disturbed monocyte polarization and dendritic cell clustering of HNSCC patients
   - infiltration pattern of lymphocytes, macrophages and dendritic cells in HNSCC
   - levels of the p15E-like factors in the peripheral blood of HNSCC patients.

In chapter 3, a study is described which was designed to determine if a consistent infiltration pattern of lymphoid cells in previously untreated head and neck squamous cell carcinoma's could be detected. In the majority of tumors a recognizable pattern was seen. The stroma of the tumors contained distinctive small fields of T lymphocytes, intermingled with $S100^+$/$RFD1^+$/$L25^+$dendritic cells. Intra-epithelial T cell and dendritic cell infiltration was low, the intra-epithelial dendritic cell infiltration mainly being $S100^+$ dendritic cells. $CD68^+$ macrophage infiltration was markedly present in both stroma and tumor nests, although the latter being less dense than the stromal infiltration. In nearly half of the specimens studied granulomatous structures of densly packed macrophages, intrastromally located at the borders of tumor nests were seen. The histochemical localization of the tumor infiltrating dendritic cells and macrophages supports the view that the former cells are involved in the sensitization to tumor antigens, whereas the latter cells are involved in tumor cytotoxicity/scavenging of tumor cell debris. In all tumors, the malignant epithelial cells stained positive to at least some degree for the anti-p15E antibody 4F5. The expression varied from weak to strong between tumors and was associated with the histological grade of the tumors. There was no relationship between the intensity of p15E expression and the severity of lymphoid cell infiltration of any kind.

Chapter 4 describes the *in vivo* effects of 10 days of preoperative thymostimulin (TP-1) treatment of head and neck squamous cell carcinoma patients on a) the polarization capability of peripheral blood monocytes as a measure of chemotaxis, b) the capability of peripheral blood dendritic cells to form cell clusters, and c) the level of p15E-like trans-membrane factors in blood. This study firstly confirmed earlier findings that a significant number of head and neck squamous cell carcinoma patients have a depressed monocyte polarisation assay, a defective capability of dendritic cells to form cell clusters with T-cells, and elevated levels of immunosuppressive p15E-like low molecular mass factors (LMMF's). Patients were treated in a double blind fashion with intramuscular injections of either TP-1 in one of three dosages (0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg body weight) or a placebo. Assessment of monocyte polarization, the capability of dendritic cells to form clusters, and the presence of p15E-like LMMF's in serum was performed before TP-1 treatment and on the day of surgery. Findings demonstrated that TP-1 in a dose of 1.0 mg/kg and 2.0 mg/kg bodyweight resulted in a normalization of the impaired monocyte polarization. Although the cluster capability of dendritic cells improved after TP-1 treatment, values only reached statistical significance for the 0.5 mg/kg treatment group. Serum p15E-like LMMF levels were not affected by TP-1 treatment in any of the patient groups. No correlation was found between elevated immunosuppressive LMMF's and defective monocyte polarization or cluster capability of dendritic cells.

Chapter 5 describes the infiltration pattern of lymphoid cells into head and neck squamous cell carcinoma's of patients preoperatively treated with the immunomodulating drug thymostimulin (TP-1) as compared to non TP-1-treated patients. For all dosages of TP-1 used (0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg), a highly significant denser T cell infiltration into the stromal tissue of the tumors was observed compared to tumors of patients not treated with TP-1. Intraepithelial infiltration of T cells appeared to be denser in TP-1 treated patients, this was however not statistically significant. The quantitive infiltration of macrophages and dendritic cells was not affected by TP-1. Correlations between the presence of the various tumor infiltrating cells, and the outcome of the monocyte polarization assay as an indicator of monocyte chemotaxis, and dendritic cell cluster capabilily of peripheral blood monocytes/dendritic cells, as well as numbers of peripheral blood T cells, were also studied. A positive correlation was found for tumor T cell infiltration and the capability of peripheral blood dendritic cells to form cell clusters with T cells. No correlation existed between T cell numbers in peripheral blood and T cell infiltration into the tumor; nor was the monocyte polarization of peripheral blood monocytes correlated with tumor infiltration of monocytes or monocyte-derived macrophages or dendritic cells.

In chapter 6 the long-term effects of thymostimulin (TP-1) treatment of head and neck squamous cell carcinoma patients on monocyte polarization and

dendritic cell clustering of peripheral blood monocytes/dendritic cells are described. These immune parameters were studied in 39 patients treated with TP-1 in one of three dosages (0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg bodyweight) for 10 days pre- and 6 months postoperatively, and in 22 patients who were not treated with TP-1.
In the non-TP-1 treated group we confirmed that surgical removal of the tumor resulted in a restoration of monocyte and dendritic cell functions (as was preoperatively seen for patients treated with TP-1, see chapter 3); TP-1 treatment gave no additional effect to this restoration. At the end of the treatment period (day 180), a difference of monocyte polarization between TP-1 treated patients and non-TP-1 treated patients could be demonstrated, suggesting a renewed disturbance of monocyte polarization in the non-TP-1 treated patients; however, for the dendritic cell cluster assay no differences between the 2 groups were found at that time. P15E-like LMMF levels in the blood of both TP-1 treated and non-TP-1 treated patients remained elevated, even after removal of the tumor. It is therefore unlikely that depression of cellular immunity is a direct effect of these LMMF's. A comment on the clinical effects (survival) of TP-1 is made in this chapter: beneficial effects were not recorded in this dose-finding study.

The general discussion (chapter 7) firstly lists again the outcomes of our studies and further focusses on biologic response modifiers (BRM's) in use in respect to head and neck cancer. These BRM's concern Thymostimulin, IL-2, interferons IFN-α and IFN-γ, Sizofilan, and OK-432. Although encouraging laboratory results were achieved for most of these BRM's, clinical effects have generally been disappointing. Future strategies are discussed in this chapter. We conclude that it will be very difficult to find a generally acceptable, effective immunomodulating therapy based on natural cytotoxicity, due to the many escape mechanisms the cancers possess.

SAMENVATTING

In de introductie (hoofdstuk 1), wordt de basis van het afweermechanisme uitgelegd met speciale nadruk op tumorimmunologie en die van hoofd/hals-kanker in het bijzonder. De rol van de verschillende cellen van de cellulaire immuniteit wordt besproken. Verstoringen van de immuunfunctie van patiënten met een hoofd/halscarcinoom zijn goed gedocumenteerd en een gedetailleerde discussie is deel van dit hoofdstuk. Daarbij worden met name immunosuppressieve factoren behandeld in relatie tot dit carcinoom.

In hoofdstuk 2 worden de doelstellingen van dit proefschrift beschreven:

1. De eerste doelstelling was een gedetailleerde beschrijving te geven van het infiltratiepatroon van hoofd/halscarcinomen door macrofagen, dendritische cellen en T-cellen.
2. De tweede doelstelling betrof het infiltratiepatroon van deze cellen in relatie tot de immunohistochemische expressie van laag moleculaire gewichts-factoren (zgn. p15E-achtige of transmembranale (TM) factoren) door tumorcellen. Deze factoren zijn in staat om monocyt-, macrofaag- en dendritische celfuncties te verstoren.
3. De derde doelstelling was het bestuderen van de in vivo effecten van een thymushormoonpreparaat (TP-1) op
   - de verstoorde monocytenpolarisatie en dendritische celclustering bij hoofd/halscarcinoompatiënten.
   - het infiltratiepatroon van lymfocyten, macrofagen en dendritische cellen van hoofd/halscarcinomen.
   - de concentratie van de p15E-achtige factoren in het perifere bloed van hoofd/halscarcinoompatiënten.

In hoofdstuk 3 wordt een basale studie beschreven die ontworpen is om te bepalen of een constant patroon van celinfiltratie van lymfoïde cellen in voorheen onbehandelde hoofd/halscarcinomen vastgesteld kon worden. In het merendeel van de tumoren werd een duidelijk herkenbaar patroon gezien. Het stroma van deze tumoren bevatte omschreven kleine veldjes van T-lymfocyten, waartussen zich S100+/RFD1+/L25+ dendritische cellen bevonden. Intra-epitheliale T-cel en dendritische celinfiltratie was gering; de intra-epitheliale dendritische celinfiltratie bestond voornamelijk uit S100+ dendritische cellen. CD68+ macrofageninfiltratie was uitgesproken aanwezig in het stroma en de tumornesten. In het epitheel was de infiltratie duidelijk minder dicht dan in het stroma. In bijna de helft van de tumoren werden granulomateuze structuren van zeer dicht opeengepakte macrofagen gezien die zich in het stroma dicht tegen de tumornesten aan bevonden. Deze immunohistochemische localisatie van tumor infiltrerende dendritische cellen en macrofagen steunen de opvatting dat de eerstgenoemde cellen betrokken zijn bij de sensibilisatie tegen tumorantigenen, terwijl de laatstgenoemde cellen betrokken zijn bij tumorcytotoxiteit en opruimen van tumorceldébris. In alle tumoren kleurde het maligne plaveiselcelepitheel aan voor het anti-p15E antilichaam 4F5. P15E is een eiwit dat geassocieerd wordt met immunosuppressieve eigenschappen van

hoofd/halstumoren. De expressie hiervan varieerde van zwak tot sterk en was geassocieerd met de differentiatiegraad van de tumoren. Er was geen relatie tussen de intensiteit van p15E expressie en de graad van lymfoïde celinfiltratie.

Hoofdstuk 4 beschrijft de in vivo effecten van 10 dagen pre-operatieve behandeling met thymostimuline (TP-1) van patiënten met een plaveiselcel-carcinoom in het hoofd/halsgebied op a) de monocytenpolarisatie van perifere bloedmonocyten als een maat voor chemotaxis, b) de clustervormende eigenschappen van dendritische cellen in het perifere bloed en c) de hoeveelheid van p15E-achtige TM factoren in het bloed. Deze studie bevestigde eerdere bevindingen dat een significant aantal van hoofd/halscarcinoompatiënten een gestoorde monocytenpolarisatie heeft, een verminderde clustervorming van dendritische cellen met T-cellen en een verhoogde hoeveelheid van immunosuppressieve p15E-achtige laag moleculaire gewicht factoren (LMMF's) in het bloed. Patiënten werden behandeld met intramusculaire injecties van ofwel TP-1, in een van drie doseringen (0,5 mg/kg, 1.0 mg/kg of 2.0 mg/kg lichaamsgewicht) of een placebo. De monocytenpolarisatie, de dendritische celclustervorming en de aanwezigheid van p15E-achtige LMMF's in het serum werden bepaald vóór TP-1 behandeling en op de dag van chirurgie (meestal dag 10). TP-1 in een dosering van 1.0 mg/kg en 2.0 mg/kg lichaamsgewicht resulteerde in een normalisatie van de verstoorde monocytenpolarisatie. De dendritische celclustervorming verbeterde ook na TP-1 behandeling; dit bleek echter alleen significant voor de 0.5 mg/kg groep. Serum p15E-achtige LMMF's werd niet beïnvloed door TP-1 behandeling (in geen van de doseringen). Er werd geen correlatie gezien tussen een verhoogde hoeveelheid immunosuppressieve LMMF's in het bloed en een verstoorde monocytenpolarisatie of dendritische celclustering.

Hoofdstuk 5 beschrijft het patroon van infiltratie van lymfoïde cellen in hoofd/halscarcinomen van patiënten die pre-operatief behandeld zijn met thymostimuline (TP-1), vergeleken met tumoren van niet met TP-1 behandelde patiënten. Voor alle doseringen TP-1 die gebruikt zijn (0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg lichaamsgewicht), werd een hoog significant dichtere T- celinfiltratie van het tumorstroma gezien vergeleken met tumoren van patiënten die niet met TP-1 behandeld waren. Ook intra-epitheliale infiltratie van T-cellen leek dichter te zijn in tumoren van met TP-1 behandelde patiënten; dit bleek echter een niet statistisch significant verschil te zijn. De kwantitatieve infiltratie van macrofagen en dendritische cellen werd niet door TP-1 beïnvloed. Voorts werden correlaties bestudeerd tussen de aanwezigheid van verschillende tumorinfiltrerende cellen en 1) het resultaat van de monocytenpolarisatie (als een indicator van de monocytenchemotaxis), 2) de dendritische celclustering en 3) aantallen van perifere T-cellen. Een positieve correlatie werd gevonden voor de T-celinfiltratie van tumoren en de mogelijkheid van dendritische cellen uit het perifere bloed om celclusters met T-cellen te vormen. Er was geen correlatie tussen T-cel aantallen in het perifere bloed en T-celinfiltratie in de tumor, noch was de

monocytenpolarisatie van perifere monocyten gecorreleerd met infiltratie in de tumor door macrofagen of dendritische cellen.

Hoofdstuk 6 gaat over de effecten op lange termijn van thymostimuline (TP-1)-behandeling van patiënten met een hoofd/halscarcinoom op de monocytenpolarisatie en dendritische celclustering. Deze immunoparameters werden bestudeerd bij 39 patiënten die TP-1 kregen in 1 van de 3 doseringen (0,5 mg/kg, 1,0 mg/kg, 2,0 mg/kg lichaamsgewicht) gedurende 10 dagen pre-operatief en 6 maanden postoperatief. Deze parameters werden tevens bestudeerd bij 22 patiënten die niet met TP-1 behandeld werden. In de groep patiënten die niet met TP-1 behandeld werden resulteerde het chirurgisch verwijderen van tumor in een herstel van de monocytenpolarisatie en de dendritische celclustering (zoals pre-operatief reeds werd gezien bij patiënten die met TP-1 behandeld werden, zie hoofdstuk 3); TP-1 behandeling gaf geen additioneel effect. Aan het eind van de behandelingsperiode (dag 180), werd een verschil tussen de monocytenpolarisatie van TP-1 behandelde patiënten en niet met TP-1 behandelde patiënten gezien, hetgeen suggereert dat de monocytenpolarisatie bij niet met TP-1 behandelde patiënten opnieuw verstoord zou zijn. Voor de dendritische celclustering konden op dat moment geen verschillen aangetoond worden. P15E-achtige LMMF concentraties in het bloed van met TP-1 behandelde en niet met TP-1 behandelde patiënten, bleven verhoogd, zelfs na verwijdering van de tumor. Het is daarom onwaarschijnlijk dat de depressie van de cellulaire immuniteit een direct effect is van deze LMMF's. De mogelijke klinische effecten van TP-1 worden van commentaar voorzien in dit hoofdstuk; positieve klinische effecten werden niet vastgesteld in deze dose-finding studie.

De discussie (hoofdstuk 7) vat onze studieresultaten samen en spitst zich verder toe op "biologic response modifiers" (BRM's) die (in experimentele opstelling) gebruikt worden bij de behandeling van hoofd/halscarcinomen. Deze BRM's betreffen Thymostimuline, IL-2, de interferonen IFN-alpha en IFN-gamma, Sizofilan en OK-432. Alhoewel de cellulaire immuniteit in gunstige zin wordt beïnvloed door de meeste van deze BRM's, blijven de klinische effecten daarbij teleurstellend achter. Mogelijke toekomstige onderzoeks- en behandelingsstrategieën op gebied van tumorimmunologie van het hoofd/halsgebied worden in dit hoofdstuk besproken. Geconcludeerd wordt echter dat het zeer moeilijk zal zijn een acceptabele, op natuurlijke cytotoxiteit gebaseerde, effectieve immunomodulerende therapie te vinden vanwege de vele ontsnappingsmechanismen die deze carcinomen bezitten.

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