Interactions between Malignant Keratinocytes and Fibroblasts

Studies in Head and Neck Squamous Cell Carcinoma

MALIN HAKELIUS
Abstract

Carcinoma growth requires a supportive tumor stroma. The concept of reciprocal interactions between tumor and stromal cells has become widely acknowledged and the connective tissue activation seen in the malignant process has been likened to that of a healing wound. Little is, however, known about the specific characteristics of these interactions, distinguishing them from the interplay occurring between epithelial and stromal cells in wound healing. In order to study differences in the humoral effects of malignant and benign epithelial cells on fibroblasts, we used an in vitro coculture model with human oral squamous cell carcinoma cells (SCC) or normal oral keratinocytes (NOK) on one side of a semi-permeable membrane and fibroblasts seeded in gels on the other. Pro-collagens α1(I) and α1(III) were more downregulated in NOK cocultures compared to SCC cocultures. IL-1α was identified as a major keratinocyte-derived soluble factor behind the effects observed. We concluded that SCC are less antifibrotic compared to NOK. There was also a differential expression among enzymes involved in ECM turnover. The urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) were both upregulated by NOK, but not by SCC. Here, rIL-1ra caused further upregulation of PAI-1. Global gene expression in fibroblasts was assessed using Affymetrix™ arrays. In total, 82 transcripts were considered differentially expressed; 52 were up- and 30 were downregulated in SCC compared to NOK cocultures. Among the differentially expressed genes there was an enrichment of genes related to collagens and to a nonspecific, innate-type response. The innate response marker pentraxin (PTX3) was upregulated by keratinocyte-derived IL-1α in both NOK and SCC cocultures. We observed a considerably higher IL-1α / IL-1ra quotient in SCC cocultures, however, while PTX3 mRNA upregulation was higher in SCC cocultures, there was no difference in the level of PTX3 secreted protein. Taken together, we concluded that NOK and SCC regulate genes important for ECM composition and for the innate immune-response differentially. IL-1α was identified as one important mediator of the observed effects. In general, SCC appeared to be more profibrotic in their effects on fibroblasts.

Keywords: coculture, extracellular matrix, interleukin-1 alpha, tumor stroma, differential gene regulation, innate response

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List of Papers

The thesis is based on the following publications. They are referred to in the text by their Roman numerals.


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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BCS</td>
<td>Bovine calf serum</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
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<td>HPV</td>
<td>Human papilloma virus</td>
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<td>IL-1α</td>
<td>Interleukin 1-α</td>
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<td>IL-1β</td>
<td>Interleukin 1-β</td>
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<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
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<tr>
<td>IL-1Ra</td>
<td>Interleukin 1 receptor antagonist</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>NOK</td>
<td>Normal oral keratinocyte</td>
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<tr>
<td>OSM</td>
<td>Oncostatin M</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activator-1</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>rIL-1Ra</td>
<td>Recombinant IL-1Ra</td>
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<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
<td>DAMP</td>
<td>Danger-associated molecular pattern</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<td>SAP</td>
<td>Serum amyloid A</td>
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<td>PTX3</td>
<td>Pentraxin 3</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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Introduction

Carcinoma constitutes a major cause of disability and death. Of all possible locations for primary carcinomas, the head and neck region is responsible for only a minor fraction, far less than carcinomas of the lung, intraabdominal organs and prostate. Nevertheless, it has been estimated that more than 500 000 new head and neck cancers are diagnosed annually throughout the world, whereof approximately 75% occur in males and 25% in females. The expected number of deaths is more than 300 000, and the most common head and neck cancer site is the oral cavity (1).

The present thesis focuses on basic mechanisms related to the communication between cancer cells, here in the form of head and neck carcinoma squamous epithelium cell lines, and the underlying connective tissue stroma, where a comparison is made with the corresponding interplay between normal oral squamous epithelial cells, i.e. keratinocytes, and the stroma. An accepted truth is that carcinomas are a consequence of an accumulation of initiating somatic mutations in epithelial cells. A prerequisite for the growth of carcinomas is a delicate cooperation between malignant cells, irrespective of origin, and their microenvironment, particularly a supportive stroma, which grows with the tumor and which has characteristics that enable generations of tumor cells to proliferate and spread. The old concept of this interaction was that of a one-direction communication between tumor cells and the surrounding cells of the supportive stroma. More recently this has evolved into a concept of a reciprocal tight relation whereby tumor cells influence stromal cells, and stromal cells reciprocally affect the behavior of the tumor cells (2-5).

One approach to gaining further knowledge about the characteristics of this cellular interplay is to investigate the interactions between non-malignant epithelial cells and the underlying stromal cells, and to analyze in what way this interplay is affected when the epithelial phenotype is altered.

The present study is an extension of previous studies on the humoral communication between normal cutaneous keratinocytes and dermal fibroblasts in vitro, with the above-mentioned approach in mind.
Background

Squamous cell carcinoma

Solid tumors have two interdependent compartments, the tumor cell parenchyma and the stroma. In epithelial tumors, and in many other tumors, a basal lamina separates the tumor cells from the stroma. This barrier becomes incomplete during tumor invasion (6).

General features

Squamous cell carcinoma (SCC) is a malignant tumor that may appear at any anatomical location with a squamous epithelium. Common sites are skin, lips, mouth, esophagus, urinary bladder, prostate, lungs, vagina, and cervix. It results from a progressive multi-step process during which a squamous epithelial cell, and subsequently a group of cells, accumulate multiple and consecutive genetic alterations, leading to morphologic deviations from normal architecture, and during which the interplay with the surrounding stroma gradually alters (7). The first morphologic deviation is an expansion of immature epithelial cells with a corresponding decrease in the number of mature cells, “dysplasia”. This is gradually followed by a state in which the epithelial organization and normal maturation process is lost, “carcinoma in situ”. Finally there are signs of infiltration of such cell groups through the basal lamina, “true carcinoma”, representing a state where the fully evolved carcinoma has obtained the ability to invade surrounding tissues. In this state the carcinoma breaks through at least one basement membrane zone, grows into the mesenchyme at the primary site and may metastasize to distant sites (8).

Head and neck squamous cell carcinoma

The entity “head and neck carcinomas” refers to a number of biologically similar cancers originating in the lip, oral cavity, nasal cavity, paranasal sinuses, pharynx and larynx. Ninety percent of the head and neck tumors are squamous cell carcinomas and at least 75% of them are caused by consumption of a combination of tobacco and alcohol (9). Head and neck carcinomas are characterized by a high morbidity and mortality. Available treatment modalities are surgery, cytotoxic chemotherapy and radiation (10).
Established risk factors for head and neck squamous cell carcinomas (HNSCC) are use of alcohol and tobacco (11), and several studies report synergistic effects of alcohol and tobacco consumption (12-14). In recent times human papilloma virus (HPV) has been identified as a cause of squamous cell carcinoma in the oral cavity and oropharynx (9, 15, 16). HPV type 16 is considered to be the only genotype that is cancer-causing in the head and neck region (17-19).

Epithelial-mesenchymal interactions

Tissues with a lining surface are characterized by an ecto- or entodermal derived epithelium, a mesodermal stroma, and a basal lamina that separates these two compartments. Development, growth and subsequent turnover of the tissue components are under the control of complex and delicate interactive processes between cell types and stromal structures, where basic cell programs and mechanical factors interact. For reviews see (20-24). The present investigation relates to interactions in a particular epithelial-stromal complex, located in the skin and in the upper airways, which are covered by squamous epithelium. Therefore the focus of the following presentation is on those sites.

Epithelium

The epithelium lines surfaces. It is characterized by densely packed cells, leaving very little intercellular space, and rests on a basal membrane (24). Epithelial cells are connected to each other by specialized structures important for paracellular communication between the epithelial cells and for creating an epithelial-specific microenvironment (25). Recently, this microenvironment has been suggested to be involved in the regulation of epithelial cell proliferation (26).

While a simple epithelium with only one cell layer covers areas with considerable transport of water and solutes, a stratified, several-cell-layer-thick epithelium covers areas with mechanical demands, e.g. the skin (27) and upper gastrointestinal tract.

The epithelium has no vascularity and receives its nutrition through diffusion across the basal lamina.

The basement membrane (BM) constitutes the interphase between the epithelium and underlying connective tissues and has an important function as a barrier between these tissue compartments. It serves as a support-structure for the overlying epithelium and its functional and structural integrity is critical for the mechanical resistance of the epithelial surface. With electron microscopy the BM is visualized as having two laminas, the electron dense lamina densa and the electron lucent lamina lucida. In the skin, both dermal
fibroblasts and epidermal keratinocytes contribute to the build-up of the BM (28), mainly composed of laminins, collagen type IV, nidogen, and the proteoglycan perlecan (29). In healthy tissue, the BM impedes cell-cell contact between the epithelium and the connective tissue stroma, and paracrine mechanisms exerted by secreted soluble factors constitute the main mode of communication between epithelium and underlying cells (30). These interactions regulate the turnover and adaptation of the ECM during normal tissue maintenance and growth, and also during inflammation and tissue repair (20).

**Connective tissue stroma**

The basic characteristic of connective tissues is the abundance of a significant extracellular component composed of ground substance, an amorphous gel-like structure, and the fibrillar extracellular matrix (ECM). Cells are distributed in this environment and anchored to the structural components of the ECM. The connective tissue is a supportive structure for the overlying epithelium, and contains vascular networks and neuronal structures. The connective tissue density and structure vary in relation to physiological needs. It is dense, rich in collagen and low in water in the dermis, and more loose and rich in water in environments characterized by extensive water and solute transport. Indeed, the connective tissue stroma is not only an important structural compartment in organs, it is also active in maintaining fluid homeostasis and filtration. Further, innate immunity, inflammatory processes, cancer invasion and wound healing occur in and are highly dependent on connective tissue.

Several cell types populate the connective tissue. Fibroblasts are the dominating cell type. They are anchored to the ECM and become activated at sites of damage. Activated fibroblasts are responsible for the synthesis, deposition and remodeling of the ECM as well as for the production of soluble paracrine growth factors that regulate cell proliferation, morphology, survival and death (4). The connective tissue also contains inflammatory and immune regulating cells and peripheral nerve cells.

**Connective tissue turnover**

Connective tissue has the capacity to adapt its structure to changing mechanical demands by turnover of ECM components and by structural reorganization. Moreover, inflammation-driven activation of the connective tissue compartment leads to neoformation of connective tissue during tissue repair. Such connective tissue activation is also believed to have an important role, in concert with the above-described process of carcinoma cell transformation, in promoting tumor growth and invasion.
The turnover of the connective tissue stroma is controlled by a delicate balance between synthesis of ECM components and their degradation.

A group of proteases of considerable importance for the turnover of ECM components are the matrix metalloproteinases (MMPs). This group of enzymes has the ability to degrade all kinds of ECM proteins, but can also participate in activation or inactivation of a number of bioactive molecules (31). They play a major role in cell adhesion, migration, proliferation and maturation, as well as in the turnover and dynamics of the interstitium. There are currently 23 members of the human MMP family (32), at least 10 of which are secreted. The latter are classified into three different functional subfamilies: the collagenases, e.g. MMP1, which have the ability to degrade fibrillar collagens; the gelatinases, e.g. MMP2, which are involved in degradation of collagen type IV in basal lamina (33); and the stromelysins, e.g. MMP3, which have a broad specificity and degrade many ECM proteins other than fibrillar collagens. MMPs are inhibited by a family of tissue inhibitors of metalloproteinases (TIMPs). The inhibition is produced by the formation of a strong non-covalent complex (34).

In the past decade, another group of proteases, the ADAMTS (a disintegrin and metalloproteinase with thrombospondin type I motifs) families of enzymes, has been found to be capable of cleaving various ECM proteins, and thereby modulating ECM function (35).

The serine protease uPA (the urokinase-type plasminogen activator (uPA), which is expressed by several cell types including fibroblasts (36), is considered to be important for ECM turnover through its ability to convert plasminogen to plasmin, which in turn degrades fibrin and other ECM proteins. uPA also indirectly converts latent matrix metalloproteinases (MMPs) into their active forms (37). The activity of uPA is inhibited by plasminogen activator-1 (PAI-1), which is synthesized by a variety of cells in the connective tissue (38). PAI-1 also protects MMPs from plasmin-mediated activation and ECM proteins from proteolytic degradation (38).

Epithelial-mesenchymal interactions during tissue repair

The initiation of the repair process after tissue damage occurs in three highly integrated and overlapping phases: an inflammatory phase, a phase with proliferation and net production of new ECM, and finally a phase of remodeling of the new tissue to optimize its function. These processes are well described elsewhere (39-41). The process of tissue repair is highly dynamic and complex and involves many cell populations and ECM components. It is tightly regulated with respect to time and compartmentalization by multiple growth factors and cytokines. The macrophage has a central role through its capacity for wound debridement and production of cytokines that mediate recruitment and activation of cells, angiogenesis and regulation of the synthesis of extracellular matrix (42).
The initial inflammatory phase is gradually reduced over time, with decreasing levels of inflammatory cytokines and increasing levels of factors acting as growth factors (43). The precise mechanisms behind this transition are unclear, although it is described as a key feature of any kind of innate type of response (44). By about one week after tissue damage the fibrin rich provisional matrix has become fully invaded by activated fibroblasts geared to production of a collagen-rich matrix, or scar (45). In order to become activated, the fibroblasts have to undergo a phenotypic transformation by differentiating into myofibroblasts. This phenotypic change is mainly stimulated by macrophage-derived TGF-β1 (46, 47). Myofibroblasts express α-SMA and are engaged in extensive cell-matrix adhesions through adherens and gap junctions. These characteristics are important for myofibroblast-driven wound contraction (48). Another function of the myofibroblast is to alter the ECM composition towards a matrix rich in collagen types I-VI and XVIII, glycoproteins and proteoglycans. They also produce proteins capable of modifying the matrix, such as MMPs and TIMPs (48).

During reepithelialization the keratinocytes that migrate and proliferate to cover the injured tissue have a crucial role in reducing the fibrotic response and promoting maturation of the ECM by suppression of further synthesis of ECM components and profibrotic factors by fibroblasts (49, 50). In this context, two synergistic features are worth mentioning. First, the fibroblast expression of connective tissue growth factor/CCN2, a factor that acts in a profibrotic manner, is downregulated by humoral factors released by keratinocytes (50, 51). Second, the expression of both MMP1 and MMP3 in fibroblasts is stimulated by keratinocyte-derived soluble factors (52-54).

Keratinocytes and fibroblasts interact through paracrine signaling to regulate the formation of ECM during tissue repair. Fibroblasts produce keratinocyte growth factor (KGF), a member of the fibroblast growth factor family which binds to a tyrosine kinase receptor exclusively expressed in epithelial cells (55, 56). KGF directs keratinocytes to proliferate and migrate, and the expression of KGF in fibroblasts is stimulated by keratinocyte-derived interleukin-1 (IL-1). Thus, the keratinocytes initiate growth factors in fibroblasts, which in turn stimulate keratinocyte proliferation, creating a double paracrine loop (57-59).

In fibrotic disorders paracrine interactions between epidermal and mesenchymal cells also seem to be of great importance. Fibroblasts from keloid tissue upregulate VEGF expression in keratinocytes more than normal fibroblasts (60). In cocultures, interleukin-18 exerts a significant paracrine control over the expression profile of keloid fibroblasts, with increased synthesis of collagen and other ECM components (61). Recent experiments show that fibroblasts in scleroderma and keloid tissue overexpress KGF, which results in production and secretion of oncostatin M (OSM) by the KGF-stimulated keratinocytes. OSM activates the fibroblasts to increase collagen expression.
through an enhanced uPA-expression, thus indicating a double paracrine activation of fibroblasts leading to fibrosis (62).

Epithelial-mesenchymal interactions in tumors

Solid tumors have two interdependent compartments, the parenchyma with the tumor cells and the stroma in which they are scattered. In epithelial tumors a basal lamina separates the tumor cells from the stroma, although the basal lamina becomes incomplete during tumor invasion (6). The character of the tumor stroma varies between tumors, and also within one defined tumor, but is always formed by ECM components and specific cells such as fibroblasts, myofibroblasts, adipocytes, endothelial cells, pericytes and immune cells (3, 5, 63).

Recent experimental data indicates that the progression of tumors towards a malignant phenotype does not depend solely on the properties of cancer cells themselves but is also highly influenced by features of the tumor stroma (64). Furthermore, interactions between carcinoma cells and fibroblasts, as well as changes in the ECM, have been shown to promote tumor progression. This has been well described elsewhere (65).

The tumor stroma is characterized by the presence of activated fibroblasts with consequent increased deposition of matrix components such as collagen and fibronectin, and also an increased collagen cross-linking, resulting in a dense, fibrotic ECM (66, 67). It is believed that this desmoplastic response is stimulated in part by soluble factors secreted from tumor cells, and that this desmoplasia in turn promotes the growth and invasiveness of tumor cells. For example, malignant oral keratinocytes cells were shown to induce myofibroblast differentiation through the secretion of Transforming Growth Factor β (TGF-β), which in turn promoted tumor cell invasion through secretion of Hepatocyte Growth Factor (HGF) (68).

There is increasing evidence that CCN2 has important roles in tumor progression in upregulating the desmoplastic response. CCN2 may be released from tumor cells as well as from tumor fibroblasts as a response to factors secreted from tumor cells (69, 70). Interestingly, expression of CCN2 in tumor tissue has been correlated with a worse prognosis in various cancers, such as pancreatic, esophageal and hepatocellular carcinomas (71-73).

The particular characteristics that enable individual disseminated tumor cells to interact with the “new” microenvironment and to initiate the changes that lead to a new tumor stroma are collectively grouped into the concept of “the metastatic niche” (74, 75).
Inflammation and tumorigenesis

The tumor stroma regularly exhibits a more or less inflammatory reaction, and there is ubiquitous evidence of a link between inflammation and carcinogenesis. First, several facts suggest that inflammation itself causes cancer (76-79), and persistent chronic inflammation caused by chemical, bacterial or viral agents is a risk factor for cancer (80). In patients with HNSCC, elevated levels of proinflammatory cytokines in serum have been reported, as compared with levels in patients with non-malignant disease (81). On the other hand, there is also evidence that the cancer-induced inflammatory response may act as an antitumor response from the host (82, 83). It is not yet known how the balance between the cancer-promoting and the cancer-inhibiting inflammatory response is regulated (44, 84).

The link between tumorigenesis and chronic inflammation has been an accepted paradigm for decades. At least 20% of all cancer deaths worldwide are linked to underlying infections and inflammation (85), and it is currently accepted that a sustained and potent immune response to a foreign pathogen, self-antigen, or to components of the normal microflora can be the root cause of uncontrolled cancer outgrowth and progression (86). Treatment with non-steroidal anti-inflammatory drugs has been shown to decrease both the incidence and the mortality related to different tumor types such as colorectal cancer (87-89).

The connection between inflammation and cancer is the effect of two schematic pathways: the extrinsic pathway, driven by inflammatory diseases that increase cancer risk; and the intrinsic pathway, driven by genetic alterations that cause, or predispose to inflammation and neoplasia (84). Both pathways converge and support a cancer-related inflammatory microenvironment by activation of transcriptional factors, such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), signal transducer and activator of transcription 3 (STAT3), and hypoxia-inducible factor 1α (HIF1α) in tumor cells (84).

The connection between inflammation and tumorigenesis can be exemplified by chronic hepatitis, which is a common cause of hepatocellular carcinoma (HCC) (90). Here, it has been shown that the upregulation of TNFα in nearby inflammatory and endothelial cells causes an activation of NF-κB in hepatocytes (91). The same group has also shown that anti-TNFα treatment of HCC leads to apoptosis of transformed hepatocytes through a suppression of the NF-κB activation, preventing further progression towards hepatocellular carcinoma (91). These data indicate that inflammation-like signals may drive the progression from premalignant states towards more advanced tumors.
The innate immune system and tumorigenesis

The immune system is a complex and interactive network of biological structures and processes designed to protect the host from disease. The system can schematically be divided into two parts that achieve a layered defense, with increasing specificity and decreasing rapidity. The first part results in an innate response that is immediate and non-specific. The second part results in an adaptive (acquired) response; after the initial encounter with the pathogen a memory of the specific pathogen is created and over a time frame of days to weeks the response is both more vigorous and more specific.

The aim of the evolutionary archaic innate, or non-specific, immune system is to protect the host from infection by other organisms in a non-specific manner. Foreign organisms are recognized in a generic way by virtue of receptors, so-called pattern recognition receptors (PRRs) that recognize molecular patterns that are not shared by the species in question, so-called pathogen-associated molecular patterns (PAMPs), which are exclusively present on microbes. PRRs are also involved in sensing endogenous “danger” signals by recognizing danger-associated molecular patterns (DAMPs) (92). PRRs are expressed on cell surfaces, in intracellular compartments or are secreted into the bloodstream or tissue fluids (93). The principal functions of the PRRs include opsonisation, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory pathways and induction of apoptosis (94). The most well known PRRs are the Toll-like receptors (TLRs). When pathogens are bound to TLRs, both an early-phase and a late-phase NF-κB and MAPK (mitogen-activated protein kinase) activation occur, which results in an induction of proinflammatory cytokines and an inflammatory response (95). The inflammation-associated cytokines are the chief stimulators of the circulating acute-phase proteins produced by hepatocytes. The definition of an acute-phase protein is a protein whose plasma concentration increases or decreases by at least 25 percent during inflammatory conditions (96). Well known acute-phase proteins are C-reactive protein (CRP) and Serum amyloid A (SAP), but members of the complement system are likewise human acute-phase proteins (97). CRP was actually the first innate immunity receptor, PRR, described, and it constitutes, together with SAP, the short pentraxin arm of the pentraxin superfamily (98). CRP and SAP can operate as opsonins when binding to bacterial surfaces (99) and can also bind to C1q, which leads to activation of the classical complement pathway (100).

Pentraxin 3 (PTX3) belongs to the long pentraxins in the pentraxin superfamily and was originally identified as an IL1β-inducible gene in endothelial cells or as a TNF-stimulated gene (TSG)-14 in fibroblasts (101, 102). PTX3 functions as a soluble PRR, mainly operating locally (98), and is produced by a variety of cell types, including endothelial cells, smooth muscle cells,
fibroblasts and adipocytes (101, 103-105). Regarding PTX3 and the complement system, there are data indicating that PTX3 has a regulatory role in the innate immune response. If PTX3 is immobilized, e.g. bound to apoptotic cells, it activates the complement system via C1q, but fluid-phase PTX3 bound to C1q may inhibit activation via a competitive blocking of relevant activation sites (106). Other studies have also suggested a regulatory role for PTX3 in innate immunity (107-109). This dual role of PTX3 in the innate immune system implies that PTX3 protects the tissue from invading pathogens and supports the clearance of damaged material, and in addition it can protect against unwanted complement activation. On the other hand, it may enhance tissue damage during massive injury through complement-mediated mechanisms (108-110).

A link between TLR-receptor signaling and the wound healing response has recently been established (111). There are also indications that TLR activation has dual effects in tumorigenesis, with both anti-tumor and pro-tumor consequences. TLR-ligands can be effective in tumor treatment, but recent studies also suggest that continually activated TLR signaling may contribute to tumor progression (112). The role of TLRs and other PPRs in cancer is still unclear, but studies so far indicate that TLRs play an important part in cancer development (113). In some tumor cells, such as ovarian tumor cells, TLR signaling promotes tumor growth, tumor immune evasion and induces resistance to apoptosis and chemoresistance (114, 115). Recent studies have indicated that TLRs are critical players in the pathogenesis of breast cancer and colorectal cancer (116-118). In prostate cancer, fibroblast growth factors (FGFs) stimulate angiogenesis and tumor growth. PTX3 has been shown to be a natural FGF antagonist and, if overexpressed, an inhibitor of the mitogenic activity in prostate cancer. In biopsies from prostate adenocarcinomas, PTX3 expression is lost in invasive tumor areas (119).

The role of the immune system in inflammation and carcinogenesis is obviously complex, but the microenvironment is nevertheless known to have a major influence. In certain environments the immunoregulatory cells are beneficial to the host, whereas in other settings they may be detrimental and actually support cancer growth. (86).

Interleukin-1 in epithelial-mesenchymal interactions

The interleukin-1 (IL-1) family constitutes a group of cytokines with considerable impact on various physiological functions. For a recent update see (120). There is evidence that this family of molecules plays a role in the control of epithelial as well as fibroblast behavior. Currently the IL-1 family consists of 11 members, including IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra). IL-1α and β are pro-inflammatory cytokines primarily recognized for their capacity to regulate innate and cognate immune responses, and are
produced by a variety of cells participating in the host defense against noxious agents. IL-1α is primarily known as an auto- and paracrine factor, while IL-1β also has an endocrine function; however, they both bind to the same receptor (IL-1R). An important difference is that the IL-1β precursor, in contrast to IL-1α, needs proteolytic processing by caspase-1 in order to become active. IL-1Ra is an endogenous receptor antagonist believed to prevent excessive, tissue-damaging IL-1 mediated effects.

Keratinocytes constitutively synthesize proIL-1α, -β, and IL-1Ra, but these pro-inflammatory cytokines are only activated and released in response to various damaging stimuli. The activation of pro-inflammatory IL-1β from the keratinocytes requires caspase-1 activity, and the activation takes place in innate immune complexes, known as inflammasomes. Thus, keratinocytes are presumably critically involved in the innate immunity of the skin through this mechanism (121). In skin diseases, like atopic dermatitis, IL-1 is part of both the early- and late-phase response. IL-1 expression is also critical in contact sensitivity (122).

As mentioned earlier, IL-1 is also involved in the reepithelialization of wounded skin, where keratinocytes regulate the expression of keratinocyte growth factor (KGF) in fibroblasts through the release of IL-1 (57). Furthermore, keratinocytes have the ability to downregulate the expression of CCN2 in fibroblasts by release of IL-1α (50, 51).

Different roles have been suggested for IL-1 in the process of carcinogenesis; tumor promoting, as well as inhibiting roles have been proposed (123). In cancer patients high local levels of IL-1 usually correlate with tumor invasiveness and bad prognosis (124). Several reports suggest that there is a link between chronic inflammation and tumorigenesis through activation of NF-κB (91, 125-127). In experimental models, the NF-κB of target cells is activated by TNFα, which rescues the cell from apoptosis and enables progression in the malignant process through induced expression of pro-inflammatory cytokines by the activation of NF-κB. The IL-1 pathway involves activation of NF-κB; thus there is an obvious link between NF-κB, IL-1 and tumorigenesis (123).
Present investigation

The overall purpose of this investigation was to compare the effects of oral SCC cells with those of normal oral keratinocytes on fibroblast expression of genes important for ECM composition.

Epithelial cells and cells of mesenchymal origin residing in the connective tissue compartment communicate reciprocally, mainly through the secretion of soluble factors exerting paracrine effects across the basement membrane. This cellular interplay is vital both for embryological organ development as well as for tissue repair during adult life. Moreover, it has been demonstrated that interaction between the malignant epithelium of carcinomas and the supportive tumor stroma is of central importance for tumor progression. The epithelial-mesenchymal interactions of tumors have been particularly implicated in the formation and maintenance of an activated desmoplastic connective tissue matrix that promotes tumor growth and invasion. Based on the above, comparing the interactions occurring in normal tissues with those seen in tumors is highly warranted. However, the number of reports directly comparing normal epithelial cell-fibroblast interactions with carcinoma cell-fibroblast interactions has thus far been limited.

Normal keratinocytes, reflecting the function of epithelia in general, maintain connective tissue homeostasis and counteract expression of genes that favor fibrotic responses (49, 50). In line with this, keratinocytes secrete antifibrotic paracrine factors that decrease the formation of ECM components by fibroblasts as part of the wound healing response.

This mechanism is believed to contribute to the transition from the proliferative phase to the phase of scar tissue maturation. Such a weakened mechanism in SCC would lead to less control of the fibrotic response in fibroblasts and could be an important component in the dysregulation behind the formation of a desmoplastic stroma. This possibility was the origin of the main hypothesis in this thesis, i.e. that the antifibrotic effects exerted by normal keratinocytes are lacking, or at least weakened, in SCC cells. This question was approached through an in vitro experimental setup where epithelial cells and fibroblasts were cocultured, physically separated, in a transwell system allowing for reciprocal paracrine interactions.
Aims

The overall aim of this study was to investigate interactions between fibroblasts and keratinocytes, both benign and malignant, to obtain a better understanding of the communication between these cells in tumors.

The specific aims were to investigate:

- the humoral effects of normal and malignant keratinocytes on fibroblasts with respect to genes involved in extracellular matrix formation and turnover
- any differences between normal and malignant keratinocytes in the above mentioned effects
- the role of IL-1α in the interplay between fibroblasts and normal/malignant keratinocytes
Material and methods

A detailed description is found in the four manuscripts

Cells, cell-isolation and culture

Three different head and neck SCC (UT-SCC-30, UT-SCC-81, UT-SCC-87) cell lines established at the University of Turku were used. The cell lines were established from previously untreated primary tumors of the mobile tongue. The donor of UT-SCC-30 was a 77-year-old female with T3N1M0 grade 1 SCC; UT-SCC-81 was established from a 48-year-old male patient presenting with a T2N0M0 grade 1 SCC; and UT-SCC-87 was established from a 29-year-old female with T3N1M0 grade 1 SCC. None of the donors were smokers. The methods used in establishing and characterizing the cell lines have been described previously (128, 129).

Normal oral keratinocytes (NOK) were obtained from three patients undergoing minor surgery at the Department of Oral and Maxillofacial Surgery: from the gingiva of a 28-year-old male; from the oral mucosa of a 31-year-old male; and from the same location from a 51-year-old male (NOK1, NOK2, NOK3, respectively). All three patients were previously healthy, were without ongoing infections and were non-smoking.

Human primary dermal fibroblasts were obtained from patients undergoing reconstructive breast surgery at the Department of Plastic Surgery. Fibroblasts from one healthy female were used for the experiments.

Skin samples were treated with dispase and epidermis was mechanically separated from the underlying dermis. NOK were isolated as previously described (130). Following mechanical fragmentation, epidermis was treated with trypsin and keratinocytes were propagated on irradiated 3T3 feeder cells in DMEM: HAMs F12 (4:1) supplemented with 10% fetal bovine serum (FBS), and Zn-free insulin, 3,3’,5-triido-D-thyronine, hydrocortisone, cholera toxin, 10 ng/ml EGF, adenine, and gentamicin.

Fibroblasts were isolated from the dermal compartment by treatment with collagenase and subcultured in DMEM with 10% bovine calf serum (BCS) and gentamicin. Subconfluent cells were washed with PBS and detached with trypsin. Cells in passage 1-5 were used for the experiments.
Coculture

Fibroblasts were cultured in collagen gels. Briefly, a cold solution of 1.6 ml collagen type I, 3.1 mg/ml, 0.15 ml (10x) Hank’s balanced salt solution (10xHBSS) and 0.15 ml FBS with fibroblasts (2x10⁵/well), was pH adjusted to pH 7.4 with 5 M NaOH and added to 6-well plates. After polymerization, 2 ml DMEM with 10 % BCS was added to each well. NOK (NOK1, NOK2 or NOK3) or SCC cells (UT-SCC-30, UT-SCC-81 or UT-SCC-87) were seeded in Falcon polyurethane cell culture inserts (4.0 µm pore diameter pre-incubated with a bovine plasma fibronectin / bovine collagen / bovine serum albumin mixture for 2 hours at 37 °C. NOK and SCC cells were seeded in inserts in DMEM: Ham’s F12 (4:1) supplemented with 10% FBS, and insulin 3,3’,5-triido-D-thyronine, hydrocortisone, cholera toxin, EGF, adenine, and gentamicin. After 24 hours the medium was changed in both wells and inserts to 2 ml DMEM/Ham’s F12 (4:1) supplemented with 0.5% FBS in each. Inserts and wells with collagen gels were then combined and propagated as cocultures for an additional 48 hours. As control, 0.15 x 10⁶ fibroblasts were seeded in inserts instead of keratinocytes. Experiments were performed with different seeding concentrations of NOK and SCC cells, and the number of cells after 48 hours was assessed with a cell-counter. The reason for the different seeding-numbers of normal and malignant cells was to compensate for an observed higher proliferation of malignant cells. After titration with different seeding densities, a concentration of 0.30 x 10⁶ for NOK and a concentration of 0.16 x 10⁶ for SCC cells were chosen for the experiments. In this way, the average cell-number at the termination of cocultures was 0.45 x 10⁶ for NOK and 0.43 x 10⁶ for SCC cells, and about 80% cell-density was reached in all samples at the end of the coculture experiments.

Figure 1. Schematic picture of the coculture setup.
RNA-extraction, Real-time PCR and analysis of cytokines

Collagen gels were dissolved in TRIzol reagent (Life Technologies) and RNA was extracted using a modified version of the one-step-phenol-chloroform method (131). The RNA samples were subjected to DNase treatment using the RNase-free DNase set (Qiagen). The integrity and amount of RNA were determined using an Agilent Bioanalyzer 2100 (Agilent Technology, Kista, Sweden).

One microgram total RNA per sample was used as template for synthesis of cDNA using a commercial kit. Two different real-time PCR methods, either TaqMan (Life Technologies) or SsoFast EvaGreen (Bio-Rad), were used for analysis of pro-collagen α1(I), pro-collagen α1(III), fibronectin, connective tissue growth factor (CTGF/CCN2), uPA, PAI-1, MMP1, MMP2, MMP3, MMP9, TIMP1, TIMP2, TIMP3, IL-1β, IL-1Ra, pentraxin 3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All expression values were normalized to the same internal standards, and all data were pooled.

The TaqMan method was performed on a LightCycler (Roche) using primers and fluorescent probes from Applied Biosystems (Foster City, CA) and PCR reagents from Roche (LightCycler FastStart DNA Master Hybridization Probes Kit). Crossing point (Cp) values were calculated by the LightCycler software using the second derivative maximum method. Three cDNA samples for each gene product to be analyzed were diluted 10 times prior to each round of amplification in the LightCycler. Cp values from these samples were subtracted from corresponding undiluted samples (ΔCp). PCR efficiency (E) was then calculated as E=10^(1/ΔCp). Finally, the relative gene expression level (RL) was calculated as E^[Cp ref-Cp test] where Cp ref is the Cp value for a selected chosen reference sample, to which the others are compared. A ratio between the gene of interest RL and the GAPDH RL was calculated for each sample.

The SsoFast EvaGreen method was performed on a LightCycler (7900HT fast real-time PCR system, Applied Biosystems) using reagents from Bio-Rad and gene specific primer sets (sequence available upon request). The average of observed threshold cycle (Ct) values for duplicates was normalized to the average Ct values of the GAPDH gene, the internal standard. Finally the relative gene expression level was calculated using the \(2^{-\Delta\Delta Ct} \) method (132).

Analyses of the concentration of cytokines in coculture media were performed with commercial enzyme-linked immunosorbent assay (ELISA) kits for IL-1-α (Biotrak, Amersham Pharmacia Biotech, Uppsala, Sweden) ab46028, Abcam, Cambridge, UK), IL-1Ra (ab174450, Abcam), and PTX3 (DPTX30, R&D Systems, Inc., Minneapolis, MN).
The recombinant IL-receptor antagonist (rIL-1Ra) anakinra (Kineret®) came from Amgen (Breda, Holland).

**Microarray**

Extraction of RNA from the fibroblasts in the collagen gels was performed as described above. RNA concentration was measured with a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, Palo Alto, CA).

Two micrograms of total RNA from each sample were used to prepare biotinylated fragmented cRNA according to the GeneChip® Expression Analysis Technical Manual (Rev. 5, Affymetrix Inc., Santa Clara, CA). Affymetrix GeneChip® expression arrays (Human Genome U133 Plus 2.0 Array) were hybridized for 16 hours in a 45°C incubator, rotated at 60 rpm. In accordance with the GeneChip® Expression Analysis Technical Manual (Rev. 5, Affymetrix Inc., Santa Clara, CA), the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip® Scanner 3000 7G.

**Ethical approval**

Approval from the local ethics committee (Uppsala University) was obtained (Dnr 2005:332).

**Statistics**

Comparisons between groups were made with paired Student’s t-tests with Bonferroni-Holm (133) correction for multiple tests. P<0.05 was considered a significant difference.
Results

Paper I

We observed that NOK downregulated the expression of mRNA of all three ECM components examined, pro-collagen α1(I), pro-collagen α1(III) and fibronectin, as well as that of CCN2 mRNA (Figure 2). The expression of mRNA of pro-collagen α1(I), fibronectin and CCN2, but not that of pro-collagen α1(III), was downregulated by both UT-SCC-30 and UT-SCC-87.

The expression of pro-collagen α1(I) mRNA in fibroblasts was less downregulated in cocultures with both SCCs than in cocultures with NOK. Considered together, there were statistically significant differences in relative fibroblast gene expression when cocultures with both SCCs were compared with cocultures with NOK for pro-collagen α1(I) and pro-collagen α1(III).

In order to assess any role of IL-1α in the observed keratinocytes / malignant keratinocyte - mediated effects on fibroblast gene expression, we included cocultures with the addition of rIL-1Ra in the experiments. Addition of rIL-1Ra inhibited the effects of NOK, UT-SCC-30 and UT-SCC-87 on the expression of fibroblast mRNA for pro-collagen α1(I), and fibronectin. There was no effect of rIL-1Ra on the expression of pro-collagen α1(III) in cocultures with either NOK or the SCCs. The effect of UT-SCC-30 on fibroblast expression of CCN2, but not the effect of UT-SCC-87 on the same mRNA, was also inhibited by rIL-1Ra. Considered together, however, there was a similar pattern in the effect of rIL-1Ra treatment, with more or less inhibition of the effects exerted by NOK and the SCCs. We concluded that IL-1α secreted from NOK and both investigated SCCs regulate the expression of genes important for ECM structure and turnover in fibroblasts.

There was a higher secretion of IL-1α by UT-SCC-87, but not by UT-SCC-30, compared to NOK.

The concentrations of several cytokines in the coculture medium that are implicated in inflammatory and fibrotic processes, as well as in tumor progression, were analyzed. There were no detectable levels of TNF-α or IL-1β in the culture medium of the control, or in NOK single cultures or cocultures, or in malignant cell single cultures or cocultures.

There was also no detectable active TGF-β in either the mono- or cocultures, which is in accord with previous reports showing that keratinocyte-fibroblast physical cell-cell contact is required for TGF-β activation in
Figure 2. Regulation of gene expression in fibroblasts by normal and malignant keratinocytes. Keratinocytes were cocultured with fibroblasts as described in Materials and methods. Data from real time PCR are presented as gene expression relative to control. Panels A and B, pro-collagen α1(I), panels C and D pro-collagen α1(III), and panels E and F fibronectin. The left panels represent experiments with the squamous cell carcinoma cell line UT-SCC-30, and the right panels represent experiments with the cell line UT-SCC-87. F: fibroblasts only (control; number of experiments, n=13); NOK: normal oral keratinocytes in coculture with fibroblasts (n=10); SCC30 or SCC87: malignant keratinocytes (UT-SCC-30 or UT-SCC-87) in coculture with fibroblasts (n=8 and 10, respectively). The two right outermost columns in each graph represent experiments with addition of 250 µg/ml rIL-1Ra (n=8, 8, and 5, respectively). Mean ± SEM. Paired t-test. *, **, and *** statistically significant at \( p \leq 0.05 \), \( p \leq 0.01 \) and \( p \leq 0.001 \), respectively.

keratinocyte-fibroblast cocultures.
IL-1α was detected both in cocultures and in monocultures with NOK and both SCCs, while, as shown previously, there was no detectable IL-1α in fibroblast monocultures (data not shown). The concentrations of IL-1α in NOK and in SCC monocultures were not different from corresponding cocultures with fibroblasts (data not shown). The IL-1α concentration range was 50 -300 pg/ml, which is in agreement with previously published results. IL-1α levels were similar in cocultures with UT-SCC-30 and in those with NOK. However, IL-1α levels were about twice as high in cocultures with UT-SCC-87 than in those with NOKs.

The addition of rIL-1Ra further upregulated the secretion of IL-1α in cocultures with NOK, but not in cocultures with UT-SCC-30 or UT-SCC-87.

Paper II

The second paper focused on regulation of the system of enzymes involved in ECM turnover and growth factor activation. mRNA obtained from the 10 consecutive experiments presented in paper I was analyzed further for fibroblast expression of uPA, PAI-1, MMPs 1-3 and TIMPs 1-3. The effect of IL-1α was deduced from samples treated with rIL-1Ra. We found that SCC cells and NOKs differentially regulated the expression of plasmin regulators uPA and PAI-1 in fibroblasts (Figure 3). While SCC cells did not affect the expression of uPA and PAI-1, both genes were upregulated by NOKs. The expression of MMP1 and MMP3 was strongly upregulated by both normal and malignant keratinocytes. MMP1 was, however, less upregulated by SCC cells compared to NOKs. There was no effect of coculture with either SCC cells or NOKs on the expression of MMP2 or TIMP1 mRNA levels. Both SCC cells and NOKs, however, led to a downregulation of the expression of TIMP2 and TIMP3.

Co-treatment with Anakinra had no effect on the increased expression of uPA by fibroblasts in cocultures with NOK but resulted in a further increased expression of PAI-1. Finally, the inhibitory effect of SCC cells and NOKs on the expression of TIMP2 and TIMP3 was partially reversed, with statistical significance for the effects of SCC cells on TIMP2 and the effects of NOKs on TIMP3.

Paper III

There is only limited data on the effects on overall gene expression in fibroblasts exposed to the plethora of humoral products released from epithelial
cells in an environment with restricted physical contact. We hypothesized that there would be differences in the gene expression pattern in fibroblasts exposed to SSC cells compared to those exposed to NOK. We also suspected that such differences would provide a lead to possible early mechanisms in the differentiation of resting fibroblasts into CAF-like phenotypes. Using the same coculture systems as in previous papers, our approach was to study the global gene expression pattern in fibroblasts exposed to NOK or to SCC using Affymetrix™ gene expression arrays, and subsequent functional annotation and cluster analysis using DAVID online analysis and gene set enrichment analysis using the GSEA software.

We found that a total of 82 transcript IDs were differentially expressed, with a > 2-fold, significant (p<0.05) difference in expression in fibroblasts cocultured with SCC as compared to cocultures with NOK (Figure 2).

Fifty-two of the 82 transcript IDs were upregulated, i.e. with a higher expression in SSC cocultures than in NOK cocultures, and 30 were downregulated. Functional DAVID analysis demonstrated an enrichment of collagen related genes. GSEA analysis demonstrated similarities with publically available gene sets which are characterized by a nonspecific, innate–type of response with activation of both interferon pathways and connective tissue turnover.

Figure 3. Regulation of gene expression in fibroblasts by normal (NOK) and malignant keratinocytes (SCC-87). Keratinocytes were cocultured with fibroblasts for 48 h as described in Materials and methods. Fibroblast gene expression was measured with real-time PCR. Data are presented as gene expression relative to control. A: Urokinase-type plasminogen activator, and B: plasminogen activator inhibitor 1.

F: fibroblasts only (control; number of experiments, n = 10). The two right outermost columns in each graph represent experiments with addition of 250 µg/ml of the interleukin-1 (IL1) receptor antagonist rIL-1Ra. Data are mean values ± SEM. Paired t-tests. Statistically significant at *p<0.05, **p<0.01 and ***p <0.001, respectively.
Figure 4. Heat map and hierarchical clustering of the 82 regulated fibroblast genes. Three experimental groups were run in triplicates. SSC = Squamous cell carcinoma cells in insert; NOK = normal oral keratinocytes in insert; F= fibroblasts only. The heat maps are normalized to the mean of the “F-groups”.

Paper IV

Based on findings in the previous papers we wanted to further analyze the ability of keratinocytes to induce an innate-type immune response, which we hypothesized to be mediated by release of IL-1α from the keratinocyte population. Since IL-1α is secreted in high concentrations in both NOK and SCC cultures, further analysis was warranted to see if there was indeed a differential activation of factors in the innate immune response. First, we confirmed that the secretion of IL-1α was twice as high from SCC as compared to
NOK. In accordance with our previous results, fibroblasts did not contribute at all to IL-1α production, and further analysis revealed a gradient within the coculture system with a lower concentration of IL-1α seen in the fibroblast compartment. The net biologic activity of IL-1α in a system is dependent on the amount of concomitant release of IL-1Ra. Interestingly, there was a considerable difference between cell types, where SCC produced very little IL-1Ra compared to NOK, resulting in a considerably higher IL-1α / IL-1Ra quotient in SCC cocultures.

Based on the gene microarray results from paper III, we chose the mRNA expression and protein release of pentraxin 3 (PTX3) as indicators of an innate-type immune response. NOK from three different donors and three different SCC lines were used in the study. PTX3 was upregulated at both the mRNA and protein level in fibroblasts, in both NOK and SCC cocultures (Figure 5). Despite the above described difference in the IL-1α / IL-1Ra quotient, we did not observe any difference in the extent of PTX3 protein secretion between cocultures with NOK and SCC. However, PTX3 mRNA upregulation was more pronounced in SCC cocultures, which is in line with the differential expression observed in the gene microarray analysis in paper III.

We also observed that coculture of fibroblasts with both NOK and SCC cells resulted in a strong upregulation of mRNA levels for both IL-1β and for its inhibitor IL-1Ra in well fibroblasts (Figure 5C and D). This response was abrogated by the addition of rIL-1Ra to the coculture.

Lastly, we wanted to investigate the fibroblast expression of genes involved both in ECM turnover and the inflammatory process. To this end mRNA levels of MMPs 2, 3 and 9 in fibroblasts were analyzed. All three proteinases were upregulated in our system, MMP3 and 9 in both NOK and SCC cocultures and MMP2 in two out of three SCC cocultures. All effects on MMP3 and 9 were completely reversed by IL-1α inhibition, and the effect on MMP2 was partially reversed. Thus, the early fibroblast response to IL-1α secreted from both normal and malignant keratinocytes in our model entails factors involved in the highly conserved innate-immune response in inflammation and in ECM metabolism.
Figure 5. Effect of rIL-1Ra on the concentration of PTX3 (A) and on the expression of genes for PTX3 (B), IL1-1β (C) and IL-1Ra (D) in fibroblasts regulated by normal and malignant keratinocytes. In A data are given as ng/ml in medium and in B,C and D as gene expression relative to control. The concentration of PTX3 was analyzed with ELISA in medium from the well, i.e. the fibroblast compartment and the gene expression with real-time PCR. SCC30, SCC81 and SCC87 denote different tumor cell lines; NOK1-3 indicates different normal keratinocytes and F indicate a control with fibroblasts in the insert. In separate cultures IL-1α was inhibited by the addition of 250 μg/ml rIL-1 Ra. Mean ± SEM.
Discussion

In this thesis we present differences in the way NOK and SCC cells from the oral cavity communicate with fibroblasts with respect to the regulation of ECM composition and turnover and with respect to the activation of an innate-type response. The first main finding was that SCC cells were deficient in their anti-fibrotic, ECM downregulating capability compared to NOK and that many of the effects of keratinocytes on fibroblasts in our system can be ascribed to keratinocyte-derived IL-1α. Secondly, we found that SCC cells lacked the ability of NOK to upregulate the serine protease uPA and the inhibitor PAI-1 through the secretion of soluble factors. Thirdly, an analysis of overall gene expression also revealed an activation of an innate-type response in fibroblasts. Lastly, we demonstrated that IL-1α from NOK and SCC upregulated the expression of several factors involved in the innate immune response, inflammation and ECM turnover. At this stage we suggest that these may be fundamental differences that contribute to the formation of an aberrant inflammatory, desmoplastic and tumor promoting stroma.

The research group has previously investigated effects of keratinocytes on fibroblasts in cocultures with fibroblasts in monolayers (50, 134). Here we used a model with fibroblasts in non-contracting collagen gels. Fibroblasts in gels have a lower, more controlled, metabolic and proliferative activity compared to fibroblasts in monolayer culture (135). Thus, this model should constitute a system well adapted to analyze both stimulatory and inhibitory effects on gene expression. Further, non-contracted gels will contain cells with important phenotypic similarities to the myofibroblasts of tissue repair, and to the cancer associated fibroblasts of tumors.

A semipermeable membrane separated keratinocytes from fibroblasts and precluded any direct cell-cell interactions. Thus, only paracrine signaling through the secretion of soluble factors was possible between the two different cell types. In wound healing and in tumors, paracrine signaling occurs concomitantly with direct cell-cell interactions in areas devoid of an intact basement membrane and, in particular, at leading epithelial edges with close keratinocyte-fibroblast juxtaposition (136). However, by physically separating the two cell types into different compartments, pure fibroblast mRNA isolation was greatly facilitated, and it also allowed us to dissect out the paracrine component in the keratinocyte-fibroblast interplay. The keratinocytes were seeded in a separate insert on a type I collagen-fibronectin substrate, which promotes keratinocyte attachment and survival. The seeding
density of NOK was chosen to produce about 80-90 % confluence at the end of the 48-hour coculture period (data not shown). This experimental design maintained the NOK in an activated migratory and proliferative phenotype, corresponding to the process of re-reepithelialization, throughout the experiment. The average seeding density was lower for the SCCs and this produced an equal average number of cells at termination of cocultures for the SCCs and NOKs – a measure taken to equalize overtime the total number of cells participating in the epithelial - mesenchymal interplay. We used a medium that contained only 0.5 % normal calf serum. This concentration was chosen to maximally reduce the dosage of exogenous growth factors while maintaining a milieu that allows cell viability. Another important aspect is that serum contains components normally not present in healthy tissues in vivo, and reflects a wound-like environment (137). This strategy optimizes the possibility of revealing growth stimulatory and inhibitory effects due to humoral signaling from cocultured cell types. Considered together, this model allowed us to explore fibroblast responses to keratinocyte-derived factors in a wound healing-like scenario and make comparisons with the scenario in a malignant process.

In paper I the well-known antifibrotic properties of keratinocytes were confirmed with a NOK-mediated downregulation of the ECM components pro-collagen α1(I), pro-collagen α1(III), fibronectin and CCN2. Similar effects were obtained in cocultures with the two SCCs, UT-SCC-30 and UT-SCC-87, although they were less pronounced than for NOK. The difference could either be attributed to synergistic effects of other factors acting in concert with IL-1α in NOK cocultures, but less so in SSC, and/or to an overweight of factors that act in a profibrotic manner in cocultures with the SCCs. However, since both CCN2 and fibronectin were similarly downregulated in both NOK and SCCs, it seems that there are more redundant mechanisms behind the downregulation of pro-collagens α1(I) and α1(III) in cocultures with NOK, rather than the presence of profibrotic factors secreted by the SCCs.

In paper I, cocultures with both NOK and SCCs had the potential to downregulate the expression of all investigated genes. However, inhibition of IL-1α caused different degrees of reversal with respect to the different genes and the different cell types. The downregulation caused by UT-SCC-30 was fully reversed for all downregulated genes, while that caused by UT-SCC-87 was only partially reversed. One possible explanation could be that UT-SCC-87 released much more IL-1α than UT-SCC-30. Further analysis of the IL-1α system presented in paper IV revealed that the quotient of IL-1α to endogenous IL-1Ra was generally much higher in SCC cocultures. This implies that the theoretical net IL-1α activity is higher in SCC cocultures and adds support to the concept that other IL-1 agonistic and antifibrotic factors are secreted by NOK.
A not yet exploited observation seen in paper I was that SSC cells do not autoregulate their IL-1α production, in contrast to the observation regarding keratinocytes (138). This interpretation is based on the observation that addition of rIL-1Ra increased the concentration of IL-1α in cocultures with NOK but not in cocultures with SCC. This suggests a higher degree of independence of SCCs with respect to the concentration of IL-1 in the surrounding microenvironment.

The observation that CCN2 was downregulated in cocultures with both UT-SCC-30 and UT-SCC-87 contradicts other reports on induction of CCN2 expression in the stroma of tumors. As TGF-β is the main inducer of CCN2 expression and ECM synthesis in tissue repair as well as in the tumor microenvironment, the absence of CCN2 upregulation in both SSC tumor cocultures studied may be due to lack of malignant cell-fibroblast cell-cell contacts, or absence of other TGF-β activating mechanisms in our coculture system.

In paper II we further analyzed the mRNA obtained from the experiments presented in paper I. The main finding was an observed differential regulation of the two plasmin regulators, uPA and PAI-1, which have been shown to be highly important during wound healing and in tumor progression. Several of the effects observed were, again, typical, well-known responses to IL-1α, such as upregulation of MMP1 and MMP3. The pattern of keratinocyte-mediated regulation of MMP1 and 3 and also MMP2 expression was later confirmed in paper IV. All observed significant effects except the regulation of the plasmin regulators uPA and PAI-1 were more or less reversed by IL-1α inhibition.

The expression of both uPA and PAI-1 was upregulated by NOK and not affected by SCC cells. Moreover, PAI-1 expression was further accentuated in NOK cocultures and upregulated in SCC cocultures by IL-1 inhibition, suggesting that keratinocyte-derived IL-1 downregulates its expression, and that this mechanism is counter-regulated by other factors secreted in cocultures with keratinocytes. This PAI-1 upregulating, IL-1α antagonistic mechanism appeared to be more pronounced in cocultures with NOK, since total IL-1α inhibition, as achieved by a 250 µg/ml concentration of Kineret®, produced stronger PAI-1 upregulation in cocultures with NOK than SCC. Considered together, these data suggest that the malignant keratinocytes used in this investigation were deficient in their ability to upregulate the expression of both uPA and PAI-1 in fibroblasts through paracrine mechanisms. Pertaining to this, while the expressions of uPA and PAI-1 have been shown to be elevated in oral squamous cell carcinoma tissue, it has also been demonstrated that cell-cell contact is required for squamous cell carcinoma cells and pancreatic carcinoma cells to induce uPA in fibroblasts (139-141).

As commented on above, the basement membrane normally separates the epithelium and underlying connective tissue compartment, which is why
keratinocytes and fibroblasts mainly communicate through soluble factors. This normal tissue architecture is disrupted by tissue injury and by malignant processes, allowing for an increase in epithelial – mesenchymal communication through direct cell-cell contacts. This is particularly true for malignant tumors, characterized by disordered tissue architecture with a mixture of carcinoma cells and stromal cells in close apposition. In view of the above, the lack of upregulation of uPA and PAI-1 in cocultures with SCC cells could represent a phenotypic alteration that is in accord with a coexistence of cancer cells and fibroblasts in close proximity. Such fibroblast-carcinoma cell juxtapositioning has been shown to play a role in cancer cell invasion and migration. It is possible that abundant direct cell-cell interactions between fibroblasts and cancer cells in the tumor milieu could also be important for the buildup of a tumor-supporting stroma.

There was an IL-1α-mediated downregulation of the MMP inhibitors TIMP2 and 3 in cocultures with NOK and SCC cells, while the expression of TIMP1 was unaffected. The role of TIMPs in tumor progression has been shown to be highly complex. TIMPs have been found to be inhibitory to invasion, metastasis and angiogenesis (142). Considered together, these data support the notion that keratinocytes have the capacity to activate an increase in fibroblast-mediated ECM turnover through the secretion of IL-1α, both in wound healing as well as in the tumor environment.

In paper III, overall differences in wide range gene expression patterns were investigated using a gene microarray-based approach. The analysis produced a list of 82 differentially expressed transcript IDs whereof 52 were upregulated and 30 were downregulated (> 2-fold, p<0.05) in fibroblasts cocultured with SCC as compared to NOK. Subsequent functional annotation and cluster analysis revealed enrichment of collagen related genes. Gene set enrichment analysis demonstrated similarities with gene sets, reflecting a nonspecific, innate–type response with activation of both interferon pathways and connective tissue turnover. Thus in addition to supporting previous data on differential expression of genes for connective tissue components, the analysis pointed at differences between NOK and SCC concerning their ability to activate non-specific innate immune responses in fibroblasts. Examples of differentially expressed genes that have previously been linked specifically to a malignant stromal phenotype are chemokine (C-X-C motif) ligand 12 and tumor necrosis factor alpha-induced protein 6, TNFAIP6, a member of the hyaluronan-binding protein family. Differentially expressed genes linked to inflammation, e.g. IL-11 and IL-33, and innate immunity (pentraxin 3; PTX3) are known responders to pro-inflammatory cytokines, including IL-1.

In paper IV in this thesis the effects of normal and malignant keratinocytes on the expression of factors involved in innate immunity, inflammation and ECM catabolism were investigated further. The gene microarray analysis in paper III had pointed towards a differential regulation of factors in-
volved in innate immunity, most notably PTX3. In order to corroborate this finding we analyzed mRNA as well as protein levels for PTX3 in cocultures with three different NOK and three different SCC lines. It was demonstrated that both NOK and SCC upregulate mRNA and protein expression for PTX3 through the secretion of IL-1α. PTX3 protein levels were upregulated to an equal extent. The upregulation of PTX3 mRNA was, however, more pronounced in SCC cocultures. This was in line with the gene microarray data, demonstrating a differential effect on PTX3 gene expression. Analysis of IL-1α and IL-1Ra concentrations revealed a much higher IL-1α / IL-1Ra quotient in malignant cocultures, suggestive of an increased net IL-1α activity in SCC cocultures compared to NOK cocultures. While this did not translate into higher levels of secreted PTX3 protein, it could possibly explain the observed differential regulation of PTX3 mRNA. Thus, increased expression of PTX3 mRNA in SCC cocultures was not followed by a corresponding difference in the level of secreted PTX3 protein. Tentative explanations for this discrepancy in relative PTX3 mRNA and protein levels could be related to the presence of other factors, activators of PTX3 translation and/or release, co-activated by IL-1α in cocultures with NOK.

In paper IV we also confirmed an upregulation of mRNAs for MMPs 2, 3 and 9 by an IL-1α-dependent mechanism. These effects were equally pronounced in cocultures with NOK and SCC.

A certain limitation of the studies conducted so far is that they are based on only a few cell lines. This is an important point, as the development of any future anti tumor therapy has to be based on general mechanisms. However, it is known that there is a considerable phenotypic variability among cells obtained from oral cancers, which is why biologic observations that can truly be said to represent all SCCs must rely on a large number of specimens. Nevertheless, the observations made by us still represent a specific behavior of three unique tumors, the representativity of which needs more extensive validation in a larger population.
Conclusions

NOK and SCC regulate fibroblast expression of genes involved in ECM structure, turnover and innate immunity. The downregulation of procollagens α1(I) and α1(III) was more pronounced in cocultures with NOK, while the expression of CCN2 and fibronectin was downregulated by both NOK and the SCCs to a similar extent. A recombinant interleukin-1 receptor antagonist reversed many of the observed effects, suggesting involvement of IL-1α in cocultures with NOK as well as with SCCs. The observed effects on fibroblasts suggest that NOK are more antifibrotic compared to UT-SCC-30 and UT-SCC-87.

The expression of uPA and PAI-1 was upregulated in cocultures with NOK cells but not with SCCs, while both NOK cells and SCCs regulated MMPs 1 and 3 and TIMPs 2 and 3 to a similar extent and left MMP2 and TIMP1 largely unaffected. Thus, NOK and SCC regulate fibroblast expression of genes involved in tumor stroma turnover differentially in vitro.

Wide range gene expression analysis using a gene microarray based approach revealed differential effects on genes for structural ECM components and for genes involved in innate immunity.

The expression of mRNA for the pattern recognition receptor PTX3, and secretion of PTX3 protein into coculture media, was upregulated by IL-1α from NOK and SCC. The upregulation of PTX3 mRNA was more pronounced in SCC cocultures; however, there was no difference in the extent of PTX3 protein secretion between NOK and SCC cocultures.
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