Normal Oral Keratinocytes and Head and Neck Squamous Carcinoma Cells Induce an Innate Response of Fibroblasts

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Abstract. *Background: Tumor stroma is similar to the connective tissue of chronic inflammation. The extracellular matrix of tumors is formed by cancer-associated fibroblasts that also modulate the inflammatory response. Materials and Methods: We studied the ability of oral keratinocytes (NOK) and oral squamous cell carcinoma cells (SCC) to induce an innate immune response in fibroblasts. Co-cultures with fibroblasts in collagen gels and keratinocytes in inserts were used. Pentraxin 3 (PTX3) was used as an indicator of an innate immune response. Results: SCC and NOK up-regulated fibroblast mRNA expression and protein release of PTX3. mRNA levels were more pronounced in cultures with malignant cells. The induction of PTX3 was abrogated by an interleukin-1 receptor antagonist Conclusion: Keratinocytes have the capacity to induce an interleukin-1-dependent innate immune response by fibroblasts in vitro. This could be important for subsequent fibroblast modulation of the inflammatory reaction in non-malignant and malignant disease processes.*

The stromal compartment of tumors plays a vital role in tumor progression. The notion of an aberrant microenvironment with activated connective tissue cells and a modified extracellular matrix (ECM) that sets the stage for malignant cells to proliferate, migrate and invade is now widely accepted (1-3). This aberrant, activated connective tissue microenvironment may form in areas of chronic inflammation, elicited by various tissue-damaging stimuli, and is further modified by interactions between malignant and stromal cells.

Ever since the time of Virchow, inflammation has been recognized as a risk factor for the establishment of uncontrolled malignant growth (4-7). Chronic inflammation drives transformation of the ECM towards a fibrotic, scar-like milieu. There are ample examples of carcinomas arising in tissues marked by a chronic inflammatory state that results in tissue fibrosis.

Cancer-associated fibroblasts (CAFs) are major cellular constituents of the tumor stroma. CAFs share many similarities with the myofibroblasts of healing wounds and their metabolic activity is, to a large extent, geared towards the production of ECM, often resulting in desmoplasia (8, 9). These cells interact with malignant cells and infiltrating leukocytes through paracrine factors, and through direct cell–cell contacts, to promote stromal modification, as well as tumor-cell migration and invasion. The mechanisms of this interplay are only partially known.

We previously described several paracrine effects of keratinocytes on fibroblasts and identified interleukin-1-alpha (IL1α) as a major soluble factor in this interplay (10-13). We have used an *in vitro* co-culture model system to elucidate differential effects between malignant keratinocytes and normal keratinocytes on fibroblast gene expression. Genes important for the turnover of ECM are differentially regulated in this system so that malignant keratinocytes are lacking, or possess weaker anti-fibrotic mechanisms than normal keratinocytes. Using a gene-expression microarray approach, we identified activation of a group of genes involved in the innate immune system, mainly represented by differential activation of the gene for pentraxin 3 (*PTX3*), but also of other genes related to an inflammatory response, *e.g.* chemokine (C-X-C motif) ligand 12 and tumor necrosis factor alpha-induced protein 6 (14).

PTX3 is a member of the pentraxin superfamily which also includes C-reactive protein and serum amyloid P. Those are well known acute-phase proteins and belong to the short pentraxins (15), while PTX3, a 45-kDa glycoprotein, belongs to the long pentraxins (16). PTX3 is highly conserved in evolution among many species (17) and is produced by a large number of cell types and tissues, in particular by innate immunity cells, *e.g.* mononuclear phagocytes, dendritic cells, fibroblasts, and endothelial cells (15) in response to primary inflammatory signals (*e.g.* tumor necrosis factor-alpha (TNFα), IL1β) and Toll-like receptor (TLR) engagement (18).

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It plays a protective role by interacting with the complement system and facilitating the clearance of foreign material from the circulation. IL1α is a well-known and strong inducer of pentraxin. However, the role of pentraxin in the dynamic, multifactorial paracrine interplay between keratinocytes and fibroblasts has not been previously reported.

In the present study, we wanted to further investigate the hypothesis that the effect of both malignant and normal keratinocytes on fibroblasts has several characteristics of an innate response. To this end, we measured the effects of keratinocytes on the expression and release of PTX3 by fibroblasts in three-dimensional keratinocyte-fibroblast co-cultures. As IL1 is one of the primary inflammatory signals, and together with IL6 it is the main regulator of the production of acute-phase proteins (19), its role in the present observation was also assessed.

Materials and Methods

Cells and reagents. Approval from the local Ethics Committee (Uppsala University) was obtained (Dnr 2005:332). This approval entailed written informed consent and written patient information. Three different head and neck squamous cell carcinoma (SCC) (UT-SCC-30, UT-SCC-81, UT-SCC-87) cell lines established at the University of Turku were used in this study. The cell lines were established from previously untreated primary tumor 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 the donor of UT-SCC-87 was a 29-year-old female with T3N1M0 grade 1 SCC. None of the donors were smokers. The methods used to establish and characterize the cell lines have been described previously (20, 21)

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). All three patients were previously healthy without ongoing infections and were non-smokers. 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.

The recombinant IL-receptor antagonist (rIL1Ra) anakinra (Kineret®) came from Amgen (Breda, Holland).

Cell isolation and culture. Mucosal samples were treated with dispase and the epithelium was mechanically separated from the underlying *lamina propria*. NOKs were isolated as previously described (22). Following mechanical fragmentation, the epithelial layer was treated with trypsin and keratinocytes were propagated on irradiated 3T3 feeder cells in Dulbecco's Modified Eagle's medium (DMEM):HAMs F12 (4:1) supplemented with 5 μg/ml Zn-free insulin (Sigma Chemical Co., St. Louis, MO, USA), 2 nM 3,3',5-triido-D-thyronine (Sigma), 0.4 μg/ml hydrocortisone (Sigma), 0.1 nM cholera toxin (Sigma), 10 ng/ml epidermal growth factor (EGF) (Austral Biologicals, San Ramon, CA, USA), 24 μg/ml adenine, 10% fetal bovine serum (HyClone, Logan, UT, USA), and 50 μg/ml gentamicin. NOKs in passage 2-5 were used. Fibroblasts were isolated from the dermal compartment by treatment with collagenase and subcultured in DMEM with 10% bovine calf serum (HyClone) and 50 μg/ml gentamicin. Subconfluent cells were washed with phosphate-buffered saline (PBS) and detached with trypsin. Cells in passage 1-5 were used for the experiments.

Co-culture. Fibroblasts were cultured in collagen gels. Briefly, a cold solution of 1.6 ml collagen type I (Vitrogen, Cohesion, Palo Alto, CA, USA), 0.15 ml (10×) Hank's balanced salt solution and 0.15 ml fetal bovine serum with or without fibroblasts (2×10^5/well) was adjusted to pH 7.4 with 5 M NaOH and added to 6-well plates. After polymerization at 37˚C, 2 ml DMEM with 10% bovine calf serum (HyClone) was added to each well. NOKs, or malignant keratinocytes at passage 16 or 17, were seeded in Falcon polyurethane cell culture inserts (4.0 μm pore diameter) pre-incubated with a mixture of 10 μg/ml bovine plasma fibronectin (Gibco BRL/Life Technology, Paisley, UK), 30 μg/ml bovine collagen (Vitrogen) and 10 μg/ml bovine serum albumin (Sigma) for 2 h at 37˚C. NOK and malignant oral keratinocytes were seeded in inserts, both in DMEM: HAM's F12 (4:1) supplemented with 5 μg/ml Zn-free insulin (Sigma Chemical Co.), 2 nM 3,3',5-triido-D-thyronine (Sigma), 0.4 μg/ml hydrocortisone (Sigma), 0.1 nM cholera toxin (Sigma), 10 ng/ml EGF (Austral Biologicals), 24 μg/ml adenine, 10% fetal bovine serum (HyClone), and 50 μg/ml gentamicin. After 24 h, the medium was changed in wells and in inserts to 2 ml DMEM/Ham's F12 (4:1) supplemented with 0.5% fetal calf serum in each. Inserts and wells with collagen gels were combined and propagated as co-cultures for an additional 48 h. As control, 1.5×10^5 fibroblasts were seeded in inserts instead of keratinocytes. Experiments were performed with different seeding densities of NOK and malignant keratinocytes, and the number of cells after 48 h was assessed with a cell-counter (Z2 Cell and Particle Counter; Beckman Coulter AB, Pasadena, CA, USA). The reason for the different seeding numbers for normal and malignant cells was to compensate for observed higher proliferation of malignant cells. After titration with different seeding densities, 3.0×10^5 for NOK and 1.6×10^5 for malignant cells were chosen for the experiments. In this way, the average cell number at the termination of co-cultures was 0.45×10^6 for NOK and 0.43×10^6 for malignant cells, and a cell-density of about 80% was reached in all samples at the end of the co-culture experiments.

RNA extraction. Collagen gels with cells were dissolved and RNA was extracted using a modified version of the one-step phenol–chloroform method (23). Shortly thereafter, gels were dissolved in TRIzol (Thermo Fisher, Waltham, MA, USA) under extensive vortexing for 60 s, and then mixed with chloroform (1:1 v/v, TRIzol:chloroform). After incubation for 5 min at room temperature, samples were centrifuged at 14,000 × *g* for 15 min at 4˚C. The aqueous RNA phase was gently mixed with 80% ethanol (1:1v/v) in new tubes. Samples were then used for completion of the RNA extraction using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The samples were subjected to DNase treatment using an RNase-free DNase set (Qiagen). The integrity and the amount of RNA were determined using an Agilent Bioanalyzer 2100 (Agilent Technology, Kista, Sweden).

Real-time polymerase chain reaction (PCR). One microgram total RNA per sample was used as template for synthesis of cDNA using a Reverse Transcription System from Promega (Madison, WI, USA). Quantitative real-time PCR was used for analysis of *IL1B, IL1RA*,

matrix metalloproteinase *(MMP)2, MMP3, MMP9*, and *PTX3* (primers from Sigma-Aldrich, St. Louis, MO, USA). The real-time PCR method used was the SsoFast EvaGreen (Bio-Rad, Hercules, CA, USA) and it was performed on a LightCycler (7900HT fast real-time PCR-system; (Applied Biosystems, Waltham, MA, USA) using reagents from Bio-Rad and gene-specific primer sets (sequences available upon request). The average of observed threshold cycle (Ct) values for duplicates was normalized to average Ct values for the glyceraldehyde 3-phospate dehydrogenase (*GAPDH*) gene, the internal standard. Finally the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (20).

Enzyme-linked immunosorbent assay (ELISA). Analysis of the concentration of cytokines in co-culture media was performed with commercial ELISA kits for IL1α, IL1Ra (ab46028 (sensitivity<10 pg/ml, range 31.2-1000pg/ml) and ab174450 (sensitivity 62pg/ml, range 120-7500pg/ml); Abcam, Cambridge, UK) and PTX3 (DPTX30 (mean minimum detectable dose 0.025 ng/ml); R&D Systems, Inc., Minneapolis, MN, USA).

Statistical analysis. Comparisons between groups were assessed with a paired Student's *t*-test with Bonferroni correction for multiple tests, and $p \leq 0.05$ was considered a significant difference.

Results

Production of IL1α and IL1Ra in NOK and SCC co-cultures. There was no overall difference in IL1α concentration in inserts with different SCC lines compared to corresponding inserts with the NOK lines (Figure 1A). There were, however, differences between the different SCC lines in that SCC87, as previously reported (12), produced more IL1α than corresponding NOKs (not shown). There was a substantial concentration gradient over the membrane that separated the two cell compartments, with a lower concentration of IL1α in the fibroblast compartment. Furthermore, there was virtually no IL1α in fibroblast–fibroblast co-cultures. In separate experiments, inserts were grown without fibroblasts in the well. The presence or absence of fibroblasts did not affect the concentration of IL1α in the inserts (data not shown), precluding a significant autoregulatory feedback between the cell types that affected the release of IL1α.

In order to describe the net activity of IL1α in the system, we went on to analyze release of the endogenous inhibitor IL1Ra. There was a considerable difference between cell types, where SCC produced less IL1Ra compared to NOK (Figure 1B). The quotient IL1α/IL1Ra was, therefore, significantly higher in SCC (Figure 1C), implying a higher net IL1 agonistic mechanism in these cells. There were no detectable levels of IL1β in the co-culture medium as measured by ELISA (not shown).

Keratinocytes induce an innate immune response in fibroblasts. Based on the results of an array-based expression analysis showing that fibroblasts in co-culture with NOK or SCC presented wide changes in their gene expression, partly

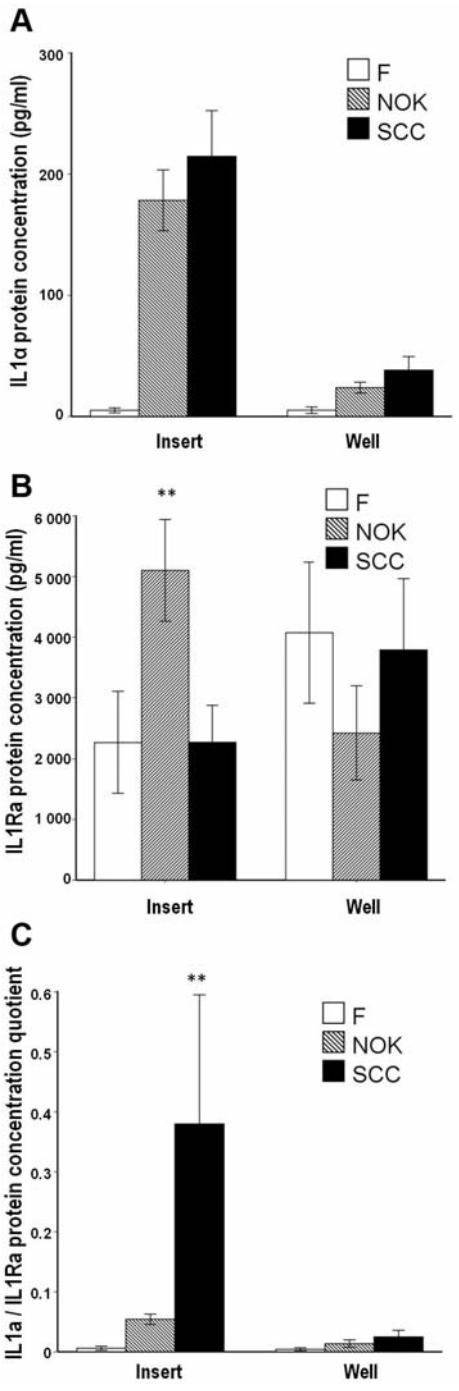

Figure 1. *A: The concentration of interleukin-1α (IL1α) was analyzed with enzyme-linked immunosorbent method in medium from both the insert, i.e. the keratinocyte compartment, and the well, i.e. the fibroblast compartment. Squamous cell carcinoma (SCC) represents all SCC cell lines combined, normal oral keratinocytes (NOK) represents all NOK cell lines, and F represents experiments with fibroblasts in the insert. N was between 34 and 65 in each subgroup. B: The concentration of IL1Ra was similarly analyzed. N is between 28 and 46 in each subgroup. C: The quotient between the IL1α concentration and the corresponding ILRa concentration in each compartment given. N is between 10 and 33 in each subgroup. Data are the mean±SEM. **p≤0.05.*

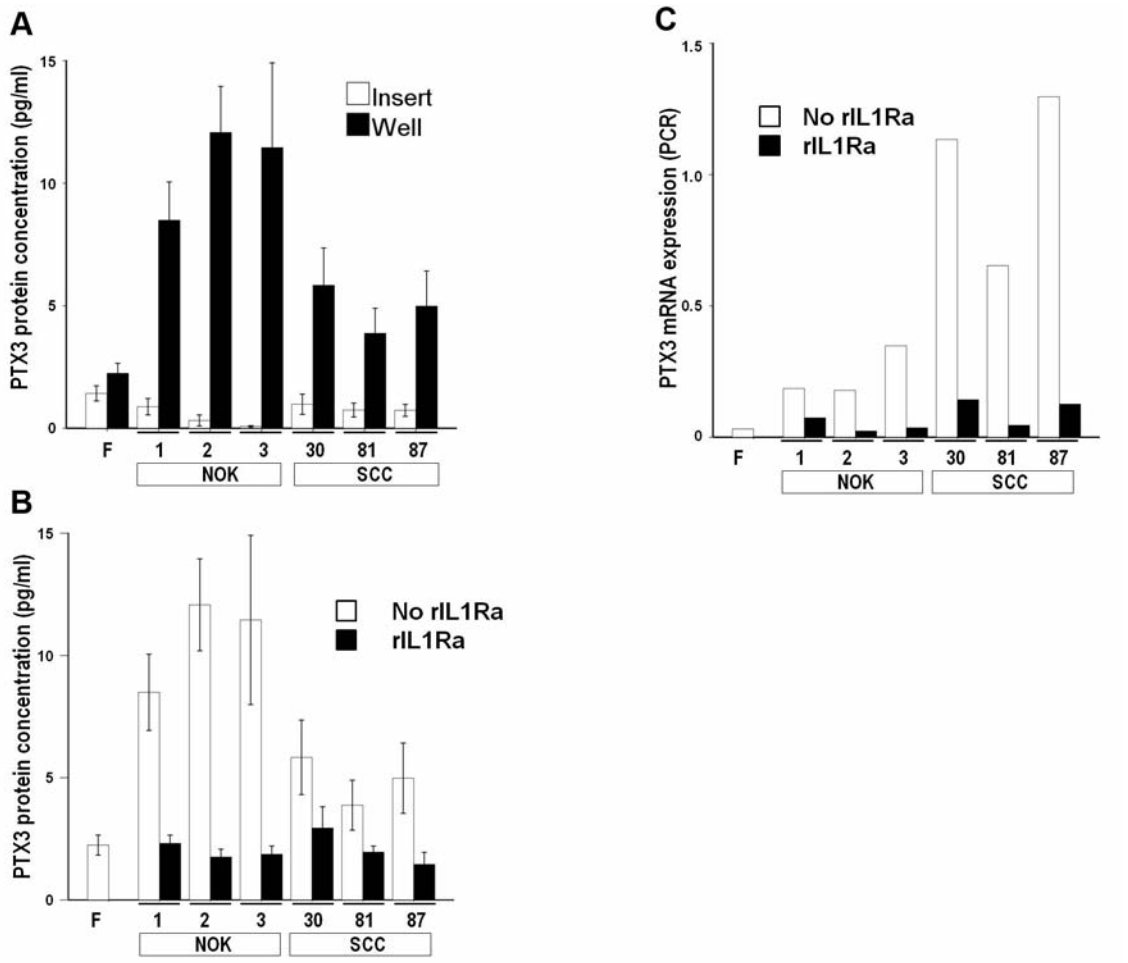

Figure 2. *A: The concentration of pentraxin 3 (PTX3) was analyzed with enzyme-linked immunosorbent method (ELISA) in medium from both the insert, i.e. the keratinocyte compartment, and the well, i.e. the fibroblast compartment. The number of compartments analyzed in each group ranged from 12 to 35. B: The concentration of PTX3 was analyzed with ELISA in medium from the well as in Figure 2A. In separate cultures interleukin-1-alpha (IL1α) was inhibited by the addition of 250 μg/ml recombinant interleukin-1 receptor antagonist (rIL1Ra). The number of compartments analyzed in the three groups ranged from 14 to 30. C: Expression of the PTX3 gene in the fibroblast compartment from seven different cultures measured with real-time polymerase chain reaction after 48 h of co-culture. Data are presented as gene expression relative to control. Experiments were performed with and without the addition of 250 μg/ml rIL1Ra to the medium. SCC30, SCC81 and SCC87 denote different tumor cell lines; NOK1-3 indicates different normal keratinocytes and F indicate fibroblasts in the insert. Data are the mean±SEM.*

in a manner similar to that in an innate response (14), we chose to assess this in more detail. To this end, we utilized PTX3 protein secretion as an indicator of overall innate response activation. We observed high concentrations of PTX3 in the media of co-cultures with NOK from three different donors and three different SCC tumor cell lines, as assayed by ELISA (Figure 2A). PTX3 was present both in media from the insert (stimulator cell), albeit at very low concentrations, and at high concentrations in media from the wells (fibroblasts). There was no PTX3 production in SCC or NOK co-cultures without target fibroblasts (data not shown), demonstrating that fibroblasts produced the protein. The possibility that the fibroblasts stimulated the epithelial cells to produce PTX3 in the co-cultures is less likely, since the insert medium was very low in PTX3.

Furthermore, co-culture of fibroblasts with NOK and SCC cells resulted in a strong up-regulation of mRNA levels for PTX3, and more so in the three SCC cell lines than in the three NOK cell lines (Figure 2C). There was no mRNA expression for PTX3 in SCC cell lines or NOK cell lines (data not shown). Taken together, the results show that NOK and SCC up-regulated PTX3 secretion from fibroblasts, but did not contribute themselves to the total concentration of PTX3 in the system. There was no difference in the extent of PTX3 secretion between NOK and SCC, despite the above-described higher IL1α/IL1Ra quotient in SCC co-cultures.

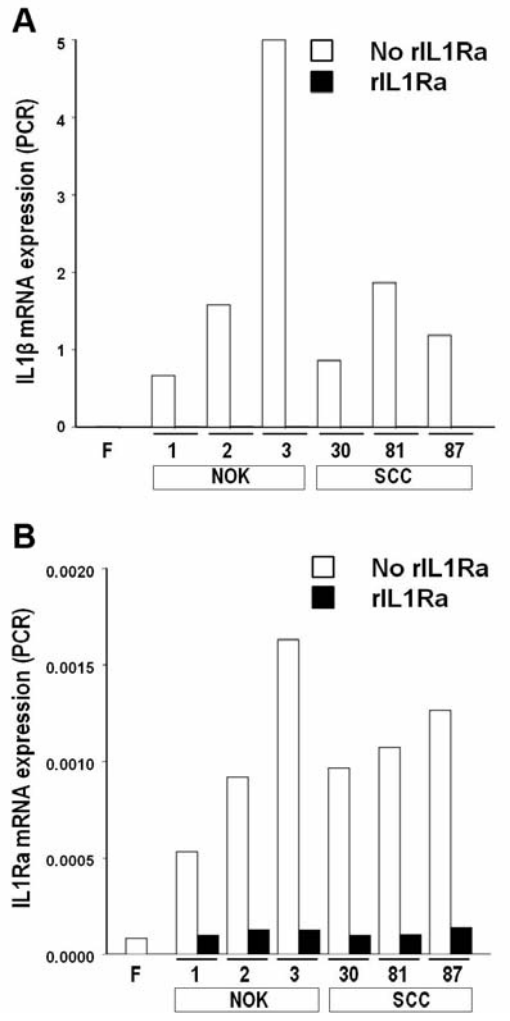

Figure 3. *Effect of recombinant interleukin-1 receptor antagonist (rIL1Ra) on the expression of fibroblast genes regulated by normal and malignant keratinocytes. Fibroblast gene expression was measured with real-time polymerase chain reaction. A: interleukin-1 beta (IL1B), and B: interleukin-1 receptor antagonist (IL1RA). Experiments were performed with and without the addition of 250 μg/ml rIL1Ra to the me-dium. SCC30, SCC81 and SCC87 denote different tumor cell lines; NOK1-3 indicates different normal keratinocytes and F indicate fibroblasts in the insert. Data are from seven different cultures and gene expression is reported relative to that of the control. Data are the mean±SEM.*

Finally, a key feature of an innate response is its ability to enforce itself by virtue of autocrine loops. Here, local production of IL1 participates in its own autocrine loop (24). We observed that co-culture of fibroblasts with both NOK and SCC cells resulted in a strong up-regulation of mRNA levels for *IL1β* in wells with fibroblasts (Figure 3A). A similar response was observed for up-regulation of IL1Ra in these cells (Figure 3B). However, as stated above, there was no secretion of IL1β into the medium (not shown).

The innate-like response by fibroblasts is regulated by IL1α. As cytokines such as IL1β and TNFα have been reported to cause synthesis and release of PTX3 in various cell types, we hypothesized that IL1α released from the epithelial cell compartment would be a probable inducer of this response. This would be analogous to the effects of epithelial cell-derived IL1α observed in previous work by this group (11, 12). To assess this possibility, we added the IL1Ra antagonist anakinra (Kineret®) to the co-cultures (Figure 2B and C). The antagonist abolished the PTX3 response at the mRNA, as well as the protein level in all cultures, showing that IL1 produced by NOK and SCC is the main stimulator of PTX3. Furthermore, the up-regulation of both IL1β and IL1Ra was abrogated by the addition of IL1Ra antagonist, further indicating that their induction in co-cultures forms part of a reciprocally regulated IL1 system (Figure 3A and B).

Discussion

This study demonstrated that three tumorigenic oral SCC cell lines and three different NOK cell populations activate fibroblasts in an 'innate' immune response manner. This reflects the ability of fibroblasts to contribute to the innate immune response upon activation by epithelial cells both in tissue repair and in malignant processes.

There was a strong fibroblastic response in co-cultures with only humoral contact in the form of PTX3 expression and release into the medium. This observation links the fibroblast innate type of response to intercellular signaling between epithelium and stroma. Considered from a general perspective this supports the concept that interactions between keratinocytes and fibroblasts modulate an inflammatory response in a wider context that is not limited to genes related to ECM composition. Previous work from our group focused on the effects of NOK and SCC on the expression of genes involved in ECM structure, production and turnover in fibroblasts. In an experimental set-up identical to that used here, we identified differential effects on several ECM-related genes (12, 13). Fibroblasts appeared to be less anti-fibrotic, manifested as less pronounced suppression of collagen types I and III expression in co-cultures with SCC. In a subsequent article, a differential regulation of protease modulators urokinase plasminogen activator and plasminogen activator inhibitor 1 was described, while a previously well-known up-regulation of MMP1 and -3 by both SCC and NOK was confirmed (13). Some of the effects on fibroblast gene expression observed in those earlier studies were totally or partially related to NOK- or SCC-derived IL1α. Here, we similarly identified IL1α as the keratinocyte-derived factor inducing *PTX3* in fibroblasts. The fibroblast population in the stroma is traditionally thought of as mainly structural cells that produce ECM. Lately it has become clear that

these cells also play a key role in directing the immune and inflammatory response. The fibroblast population constitutes a highly heterogeneous group of distinctly differentiated cell types that exhibit a considerable topographic diversity (25). Several studies imply that fibroblasts are able to modify their regional identity under the influence of inflammation (26-29). It was recently observed that modulated fibroblasts play an important role in the local inflammatory process both in tumor tissue and in non-malignant conditions (30). In line with this, a recent study from our group demonstrated keratinocyte-mediated activation of genes involved in the acute innate immune response in fibroblasts (14).

PTX3 was identified in the early 1990s in human endothelial cells and fibroblasts as TNFα- or IL1β-inducible mRNA and protein, respectively (15). It is a pattern-recognition molecule that is present in the blood and body fluids, and shares the ability to recognize pathogens and promote their disposal. In the present study, we were able to examine PTX3 secretion from fibroblasts in a co-culture system designed for paracrine reciprocal interplay with keratinocytes, entailing complex mechanisms with multiple factors involved. PTX3 release was completely dependent on IL1α from keratinocytes. We were not able to detect any difference in the extent of PTX3 protein secretion. However, in accordance with our previous results from the gene-expression microarray analyses (14), we did observe a differential regulation of *PTX3* mRNA, with a more pronounced up-regulation in SCC co-cultures. Thus, while the net PTX3 secretion was equal in NOK and SCC co-cultures, fibroblasts in co-culture with SCCs responded with a marked transcriptional activation. This implies a differential regulation entailing differences in the regulation of *PTX3* translation and release. It appears that while the higher IL1α/IL1Ra quotient in SCC co-cultures did not translate into increased net PTX3 production, it was associated with a more pronounced response at the mRNA level. At the same time, addition of rILRa caused complete reversal of *PTX3* activation of both mRNA and protein levels. This phenomenon could possibly be explained by the presence of other factors, activators of *PTX3* translation or release, co-activated by IL1α in co-cultures with NOK. Pertaining to this, we previously showed that changes in fibroblast gene expression in co-cultures with NOKs indicate a more complex and multifactorial, regulatory interplay as compared to co-cultures with SCCs, where unopposed effects directly linked to IL1α seem to predominate.

Here, the secretion of PTX3 responded directly to epithelial cell-derived IL1α, without differences in secreted levels between co-culture with SCC and NOK, and without evident modulation by other factors in the system. In wound healing, the secretion of IL1α is turned off once the basement membrane has been restored and a neo-epidermis has been established (31). However, in tumors with chronic inflammation driven by tumor cell-derived IL1α, continuous PTX3 release from CAFs could play a role in a modification of the stroma that supports tumor progression.

In conclusion, malignant and NOKs stimulated fibroblasts to express factors of the innate immune response and inflammation in an *in vitro* co-culture system *via* an IL1α-dependent paracrine mechanism. The secretion of PTX3 by fibroblasts was strongly up-regulated by both normal and malignant keratinocytes. However, the up-regulation of *PTX3* mRNA in fibroblasts was more pronounced in co-cultures with malignant keratinocytes. These findings may have important implications for the fibroblast-mediated immune response in the microenvironment of tumors.

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