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ORIGINAL PAPER

Head and neck squamous cancer stromal fibroblasts produce growth factors influencing phenotype of normal human keratinocytes

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Abstract Epithelial–mesenchymal interaction between stromal fibroblasts and cancer cells influences the functional properties of tumor epithelium, including the tumor progression and spread. We compared fibroblasts prepared from stroma of squamous cell carcinoma and normal dermal fibroblasts concerning their biological activity toward normal keratinocytes assessed by immunocytochemistry and profiling of gene activation for growth factors/cytokines by microarray chip technology. IGF-2 and BMP-4 were determined as candidate factors responsible for tumor-associated fibroblast activity that influences normal epithelia. This effect was confirmed by addition of recombinant IGF-2 and BMP4, respectively, to the culture medium. This hypothesis was also verified by inhibition experiments where blocking antibodies were employed in the medium conditioned by cancer-associated fibroblast. Presence of these growth factors was also detected in tumor samples.

Keywords Cancer microenvironment · BMP-4 · IGF-2 · Epithelial–mesenchymal interaction · Cancer-associated fibroblasts

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Introduction

Malignant tumors of epithelial origin are composed of the malignant epithelial cells and from the stroma that includes fibroblasts producing extracellular matrix, vessels providing oxygen and nutrients from infiltrating leukocytes. The stroma constitutes the microenvironment suitable for tumor growth and spread through organism (for review see De Wever and Mareel 2003; Lorusso and Rüegg 2008). The cancer stem cell hypothesis (for review see Sell 2004) implies the imperative idea on tumor microenvironment as the niche for malignant stem cells (for review see Li and Neaves 2006). Observations from various types of carcinomas indicate the remarkable role of stromal fibroblasts for tumor progression and metastasis (Orimo et al. 2005; Kaminski et al. 2006; Koukourakis et al. 2006; Hawsawi et al. 2008; Hwang et al. 2008).

We observed that fibroblasts prepared from basal cell carcinoma are able to influence the phenotype of normal

human keratinocytes. The affected keratinocytes consequently expressed keratin 19, an intermediate filament present in epidermal stem cells and in keratinocytes from the basal cell carcinoma itself (Lacina et al. 2007b). When we cocultured the normal keratinocytes with the stromal fibroblasts from the squamous cell carcinoma, the phenotypic changes of keratinocytes were more pronounced in comparison with the effect of stromal fibroblasts from the basal cell carcinoma. In this case, the keratinocytes express not only keratin 19 but also keratin 8 (Lacina et al. 2007a). Expression of this keratin in squamous epithelium is typical for the prenatal development (Troy and Turkmen 2005). Under the pathological conditions in squamous cell carcinoma, the expression of keratin 8 is associated with very poor prognosis of the patient (Casanova et al. 2004; Gires et al. 2004). Surprisingly, keratinocytes cocultured with squamous cell carcinoma-derived fibroblasts also express vimentin and transcription factor snail (SNAI1) that is considered as a marker of epithelial–mesenchymal transition (Lacina et al. 2007a). This observation can be of the clinical importance as the above-mentioned process is tightly connected with the spreading of malignant cells in the organism (for review see Thiery and Sleeman 2006; Potenta et al. 2008). Interestingly, the described changes of keratinocyte phenotype were observed in the directly cocultured cells as well as in a system, where both populations, i.e., stromal fibroblasts and normal epithelium, were separated by microporous membrane avoiding direct contacts of both cell populations (Lacina et al. 2007a). This observation indicates an emerging role of soluble molecules such as growth factors and cytokines produced by stromal cells on the functional phenotype of keratinocytes. To understand the phenomenon, we compared the transcriptoma of normal fibroblasts and squamous cell carcinoma-associated fibroblasts using microchip array technology. In particular, we screened candidate growth factors/cytokines potentially influencing the normal keratinocytes. Selected candidate molecules were visualized in cultured fibroblasts and stromal cells as well as in sections from the normal and cancer tissue by immunohistochemistry. Putative biological activity of these molecules was tested on normal keratinocytes by addition of these substances to the culture medium and vice versa by their inactivation by blocking antibodies in the medium conditioned by cancer-associated fibroblasts.

Materials and methods

Tumor samples

Ten squamous cell carcinomas of the head and neck (3 from root of tongue, 4 from tonsil, 3 from larynx) and three pieces of normal tissue (2 from tonsil and 1 from tongue) were obtained from Department of Otorhinolaryngology, Head and Neck Surgery and Department of Stomatology (Charles University, First Faculty of Medicine, Prague) with consent of patients and the Local Ethical Committee according to the principles of Helsinki Declaration. All donors of tumors were males over 40 years of age with smoking history. According to clinical classification, all tumor samples were of stage T3 or T4 with regional lymph node metastases (stage N2), without distant organ metastases (stage M0) and of histological differentiation grade 3 from pathological point of view. Tumor samples were frozen in liquid nitrogen using Tissue-Tek (Sakkura, Zoeterwoude, The Netherlands) as a cryoprotective agent. Frozen sections (5 μm) were prepared using cryostat (Cryocut-E, Reichert-Jung, Vienna, Austria). These sections were used for immunohistochemical analysis.

Two tumors of tongue (T3, N2, M0) were also used for preparation of stromal fibroblasts for further characterization by DNA microarrays, qRT-PCR, immunohistochemistry and analysis of cytokines produced to cultivation media by ELISA. Afterward, these stromal cells were used for cocultivation experiments with normal keratinocytes to evaluate their influence on biological activity. 3T3 mouse fibroblasts and/or normal dermal fibroblasts were used as reference for comparison.

Cultivation of 3T3

The commercially available 3T3 murine embryonic fibroblasts were propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Biochrom, Berlin, Germany) at 37°C and 5% CO_2. The subculture was performed three times a week using mixture of 0.25% trypsin and 0.02% EDTA 1:1 (Biochrom, Berlin, Germany). These cells were included in the study, since they have been used as a gold-standard feeder cells for cultivation of keratinocytes for more than 30 years (Rheinwald and Green 1975), and the keratinocytes cocultured with these feeder elements are extremely well characterized.

Cultivation of human interfollicular keratinocytes (IFK) and human dermal fibroblasts (HF)

Samples of human skin from healthy volunteers (2 donors) were obtained from the Department of Aesthetic Surgery (Charles University, Third Faculty of Medicine in Prague) according to ethical principles described above. Small pieces of full thickness skin samples were enzymatically treated with 0.25% trypsin at 4°C overnight, epidermis was pealed off and the suspension of

interfollicular keratinocytes was obtained by trituration. These keratinocytes were multiplied on mitomycin (Sigma, Praha, Czech Republic)-treated 3T3 layer using the keratinocyte culture medium (DMEM) and F12 medium 3:1 (Biochrom, Berlin, Germany) with 10% fetal bovine serum and enriched with insulin, choleratoxin, hydrocortisone and epidermal growth factor (Sigma, Praha, Czech Republic), according to Rheinwald and Green (1975). The remaining dermis after removal of epidermis was minced into small pieces which were seeded on cultivation dish and gently covered with DMEM. After several days the migrating fibroblasts were harvested by trypsinization and subcultured in DMEM with 10% fetal bovine serum at 37°C and 5% CO_2. For further use, both types of cells were frozen in aliquots in 10% dimethyl sulfoxide (Sigma, Praha, Czech Republic) and stored in liquid nitrogen.

Cocultivation of SCCF, 3T3 and HF with normal or malignant keratinocytes

The tumor samples of squamous cell carcinoma from the root of tongue were obtained as described above. SCCF were prepared according to modified method of Grando et al. (1996) as described in details in Lacina et al. (2007a, b). Fibroblasts migrating from small pieces of tumor biopsy were harvested and expanded in DMEM with 10% fetal bovine serum (Biochrom, Berlin, Germany) at 37°C and 5% CO_2. We monitored impact of 3T3, normal dermal fibroblasts or fibroblasts prepared from the tumor stroma on keratinocytes and the induced changes of their phenotype. All types of fibroblasts, it means 3T3, normal dermal fibroblasts or fibroblasts harvested from the tumor stroma were seeded on glass coverslips at a density of 2,500 cells/cm^2 and cultured for 24 h. The suspension of interfollicular keratinocytes (20,000 cells/cm^2) was added to the fibroblasts and cultured in the keratinocyte culture medium for 6 days. The impact of the medium conditioned by SCCF (enriched by their soluble products) was tested on keratinocytes cultivated directly with 3T3 cells and HF, respectively. Since, based on results of microarray analysis, BMP-4 and IGF-2 had been selected as factors participating in the control of phenotype of keratinocytes cocultured with cancer stroma-associated fibroblasts, we evaluated the keratinocyte phenotypic pattern after their introduction to cultivation system. BMP-4 (R&D Systems, Minneapolis, MN, USA) in concentration recommended by supplier, i.e., 30 ng/ml) and IGF-2 (R&D Systems, Minneapolis, MN, USA in concentration recommended by supplier, i.e., 5 ng/ml) were added to the standard DMEM medium. To verify the obtained results, the keratinocytes were also cultured in the SCCF-conditioned medium in the presence of blocking goat antibodies against BMP-4 (MAB-757) (R&D Systems, Minneapolis, MN, USA in concentration recommended by supplier, i.e., 3 μg/ml) and mouse IGF-2 (AF-292-NA) (R&D Systems, Minneapolis, MN, USA in concentration recommended by supplier, i.e., 20 μg/ml). The irrelevant antibodies (Dako, Glostrup, Denmark, clones 10E3 E0432) were employed to exclude the non-specific effect of antibodies against both cytokines, for example by binding to Fc receptors.

Phenotypic characterization of HF, SCCF and keratinocytes cultured under the influence of fibroblasts by immunocytochemistry

Cultured cells and sections from tumor and normal tissue were washed by phosphate buffered saline (PBS at pH 7.3) and fixed with 2% paraformaldehyde in PBS at the same pH and permeabilized with Triton X-100 in case of cultured cells (Sigma-Aldrich, Prague, Czech Republic). Pankeratin was detected by rabbit polyclonal antibody (Abcam, Cambridge, UK) and keratin 8, keratin 19 and vimentin by mouse monoclonal antibodies from DAKO (Glostrup, Denmark). Goat anti-rabbit IGF-2 (AF-292-NA) and BMP-4 mouse antibodies (MAB-757) (R&D Systems, Minneapolis, MN, USA) were used for the detection of both mentioned cytokines. CD34 (QBEnd 10), CD68 (KP1) and smooth muscle actin/1A4) were detected by monoclonal antibodies (DAKO, Glostrup, Denmark) and CD45 (BRA-55) by monoclonal antibody (Sigma-Aldrich, Prague, Czech Republic) in characterization of stromal fibroblasts only. Swine anti-rabbit or anti-mouse antibody labeled by FITC (SwAR-FITC, SwAM-FITC, AlSeVa, Prague, Czech Republic) and goat anti-mouse antibody labeled by TRITC (Sigma-Aldrich, Prague, Czech Republic) were used as second step antibodies. All antibodies were diluted as recommended by suppliers. Simultaneous staining at single cell level was performed as described in (Froňková et al. 1999). Control of the reaction specificity was made by the omission of the first step antibody or by its replacement by antibody not occurring in studied cells to exclude the false positivity by the binding of antibodies via Fc fragments to Fc receptors. DNA of cell nuclei was visualized using DAPI (Sigma-Aldrich, Prague, Czech Republic). The cells were mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA). Specimens were inspected using fluorescence microscope Nikon Eclipse 90i equipped by filterblocks specific for FITC, TRITC and DAPI, by high resolution cooled Vosskühler Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany) and a computer-assisted image analyzer (LUCIA 5.10) (Laboratory Imaging, Prague, Czech Republic). Student's non-paired *t* test was used to assess significance levels.

RNA analysis

RNA for analysis was harvested from subconfluent HF and SCCF cultured for 28 days. Two parallel cultivations (10-cm^2 culture dish) for each type of cells were made. In the stage of subconfluency the culture medium was removed and 360 µl of RLT lysis buffer (QIAGEN Inc., Valencia, CA, USA) was added. The cells were homogenized, transferred to a microcentrifuge tube and quickly frozen in liquid nitrogen. The samples were stored at −80°C. Total RNA was isolated by RNeasy Micro Kit (QIAGEN Inc., Valencia, CA, USA), according to the procedure for animal cells. Quantity of the RNA was measured on NanoDrop ND-1000 (NanoDrop Technologies LLC, Wilmington, DE, USA). RNA integrity was assessed on Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). All RNA samples had RIN above 9.

Illumina Human-6 v 2 Expression BeadChip (Illumina Inc., San Diego, USA) was used for the microarray analysis following the standard protocol (150 ng of total RNA was amplified with Illumina TotalPrep RNA Amplification Kit (Ambion Inc., Austin, TE, USA) and 1.5 µg of amplified RNA was hybridized on the chip according to the manufacturer procedure). All subsequent analyses were done on two biological replicates.

Microarray analysis

The raw data (TIFF image files) was analyzed using *beadarray* package (Dunning et al. 2007) of the *BioConductor* within the *R* environment (R Development Core Team 2007). All hybridizations passed quality control. The data were background corrected and normalized with the probe level quantile method. The probes with intensity level lower than 95-percentile of negative controls of the BeadChip in all samples were disregarded before detection of differential expression. Differential expression was performed with the *limma* package (Smyth 2005) on intensities that were variance-stabilized by logarithmic transformation. Annotation of differentially expressed transcripts was done with package *biomaRt* (Durinck et al. 2005) against the Ensemble database (version 47). Only transcripts with false discovery rate smaller than 0.05 and fold change smaller than 0.5 or higher than 2 were reported and used in the downstream analysis. The data were deposited to the ScanExpress database under accession number E-MTAB-97. The analysis was targeted mainly on genes involved in extracellular signaling.

Quantitative real-time PCR

RNA was isolated as described in RNA analysis. Reverse transcription was performed by QuantiTect® Reverse Transcription Kit (QIAGEN Inc., Valencia, CA, USA) according to manufacturer's instruction (starting with 50 ng/µl RNA). Final cDNA was diluted five times and 2 µl aliquot was used as a template for quantitative real-time PCR (qRT-PCR). qRT-PCR was performed on LightCycler 2.0 System using LightCycler® 480 DNA SYBR Green I Master kit (Roche Diagnostics GmbH, Germany). PCR reactions (5 µl) were run according the standard manufacturer's protocol cycled 60 times. Target genes: bone morphogenetic protein 4 (BMP-4, ENSG00000125378, TCCACAGCACTGGTCTTGAG, TGGGATGTTCTCCAGATGTTCT), insulin-like growth factor 2 (IGF-2, ENSG00000167244, CGAGCCTTCTGCTGAGCTAC, AGGCCAAGAAGGTGAGAAGC) and vimentin (VIM, ENSG00000026025, AAAGTGTGGCTGCCAAGAAC, AGCCTCAGAGAGGTCAGCAA), and housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ENSG00000111640, GCTCTCTGCTCCTCCTGTTC, ACGACCAAATCCGTTGACTC), ribosomal protein S9 (RPS9, ENSG00000170889, CTGACGCTTGATGAGAAGGAC, CAGCTTCATCTTGCCCTCAT) and TATA box binding protein (TBP, ENSG00000112592, GAACATCATGGATCAGAACAACA, ATAGGGATTCCGGGAGTCAT) were measured under same conditions from the same cDNA. Results were analyzed by LightCycler software and crossing point values were further determined using the *R* environment (R Development Core Team 2007). Every examined cell culture was performed in eight replicates (2 biological, 2 RT and 2 technical replicates). Stability measurement of housekeeping genes and normalization to housekeeping genes was carried out using method of Vandesompele et al. (2002). Statistic significance of changes in mRNA level of target genes between different samples was calculated by *t* test.

ELISA detection of BMP-4 and IGF-2

Cultivation media were collected from dermal fibroblasts and SCCF cultured for 24 h and for 1 week under the same conditions as in investigation of their transcriptoma and qRT-PCR analysis. ELISA kit for detection of BMP-4 was purchased from R&D Systems Europe (Abingdon, UK) and for detection of active IGF-2 from Beckmann Coulter (Miami, FL, USA). Specimens were prepared according to the supplier instructions and measured in 96-well plate at 570 nm on Universal Microplate Reader EL 800 (BIOTEK Instruments, Winooski, VT, USA). The obtained data were compared with the data from media harvested from

cocultures of dermal fibroblasts or SCCF with normal keratinocytes and SCCF cocultured with cells of FaDu line prepared from squamous cell carcinoma of hypopharynx cultured as described (Lacina et al. 2006) in order to evaluate the possible effect of epithelial cells on production of both cytokines.

Results

Characterization of SCCF

The SCCF uniformly exhibited presence of vimentin. No keratins were detected by immunohistochemistry in these cells (Fig. 1a). Isolated cells were immunohistochemically negative for other markers, i.e., CD34 (endothelial cells precursors), CD45 (leukocytes), CD68 (macrophages) and smooth muscle actin (SMA, myofibroblasts). Therefore, these cells shall be considered as fibroblasts. While there were no SMA detected by immunohistochemistry, smooth muscle actins ACTA2 and ACTG2 were detected at mRNA level. Moreover, these transcripts showed different transcriptional activities in SCCF and HF (ACTA2: mean logarithmic expression in HF 14.4, mean in SCCF 13, $P = 7 \times 10^{-8}$; ACTG2: HF 7.3, SCCF 11.5, $P = 4 \times 10^{-9}$).

Characterization of keratinocytes cultured with SCCF

The keratinocyte colonies cultured under the influence of normal human fibroblasts and SCCF differed significantly. SCCF-induced keratinocytes formed irregularly shaped colonies frequently bridged by elongated epithelial cells (Fig. 2a, b). Concerning the phenotype of keratinocytes cocultured with SCCF, they express pankeratin (Fig. 2a, b) as implied by their nature.

The activity of cultured SCCF was tested by phenotype analysis of cocultured normal keratinocytes. Here, cocultivation of normal keratinocytes with HF or 3T3 cells was used as a reference (Fig. 1e, j; Table 1). The results demonstrated that the cells prepared from the tumor stroma induced a remarkable expression of keratin 8 (Fig. 1b) and keratin 19 (Fig. 1c) in originally almost negative normal interfollicular keratinocytes in comparison to keratinocytes cultured with normal HF or 3T3 cells (Table 1). We frequently detected fibroblast-like cells that were positive for keratins as well as for vimentin in coculture of normal keratinocytes with SCCF (Fig. 1d). No similar phenotype (concerning expression of keratins 8 a 19 and vimentin) was observed in cocultures with normal human dermal fibroblast (HF) or 3T3 cells (Fig. 1j; Table 1), up to very rare events.

Characterization of SCCF using RNA microarrays, qRT-PCR, immunocytochemistry and ELISA

We observed in our previous studies that the SCCF exhibited significant biological activity on normal keratinocytes even after separation of both populations by microporous membrane in transwell system. We hypothesized that soluble growth factors and cytokines secreted into the medium by fibroblasts play major role in the activity. We have subsequently focused the screening of differences in transcription activity on genes encoding growth factors and cytokines. SCCF differed significantly from HF in 560 transcripts (false discovery rate <0.05 and fold change 2 or more), 13 of them were cytokines (Fig. 3a, b). Based on literature data, we selected two transcripts, IGF-2 and BMP-4, as the most suitable candidate molecules influencing the keratinocyte phenotype. Presence of mRNA of both the factors was also verified by qRT-PCR, and results demonstrated a significant upregulation of mRNA for both these proteins (Fig. 4a). This result was also confirmed by quantitative immunocytochemistry (Fig. 4b–g). Since only the proteins that are secreted to medium are able to influence the phenotype of cocultured keratinocytes, we tested presence of both IGF-2 and BMP-4 in the cultivation medium where HF and SCCF were cultured by ELISA. Surprisingly, we detected IGF-2 only (Fig. 4h).

Effect of IGF-2 and BMP-4 on phenotype of cultured keratinocytes

To confirm that the biological activity of SCCF to the cocultured keratinocytes is attributable to these growth factors, we have enriched the standard cultivation medium (DMEM) by recombinant IGF-2 and BMP-4. Although the production of the latter protein to the medium has not been confirmed, it can not be excluded that this protein is produced to the medium under different experimental condition or in vivo. Both bioactive substances were employed alone or in a mixture. The phenotype of normal keratinocytes grown in standard DMEM ("SCCF unconditioned") and the phenotype of keratinocytes grown in the SCCF enriched medium were compared. The appearance of colonies cultured in presence of either IGF-2 or BMP-4 alone, and also in combination, was identical to the colonies in which keratinocytes were cocultured with SCCF (Fig. 2b–d). We observed a very low effect of both added factors on the expression of keratin 8 (on the contrary to the SCCF conditioned medium), particularly in 3T3-cocultured keratinocytes (Table 1). However, the number of keratinocytes exhibiting the signal for keratin 19 and for keratins coexpressed with vimentin was comparable with the

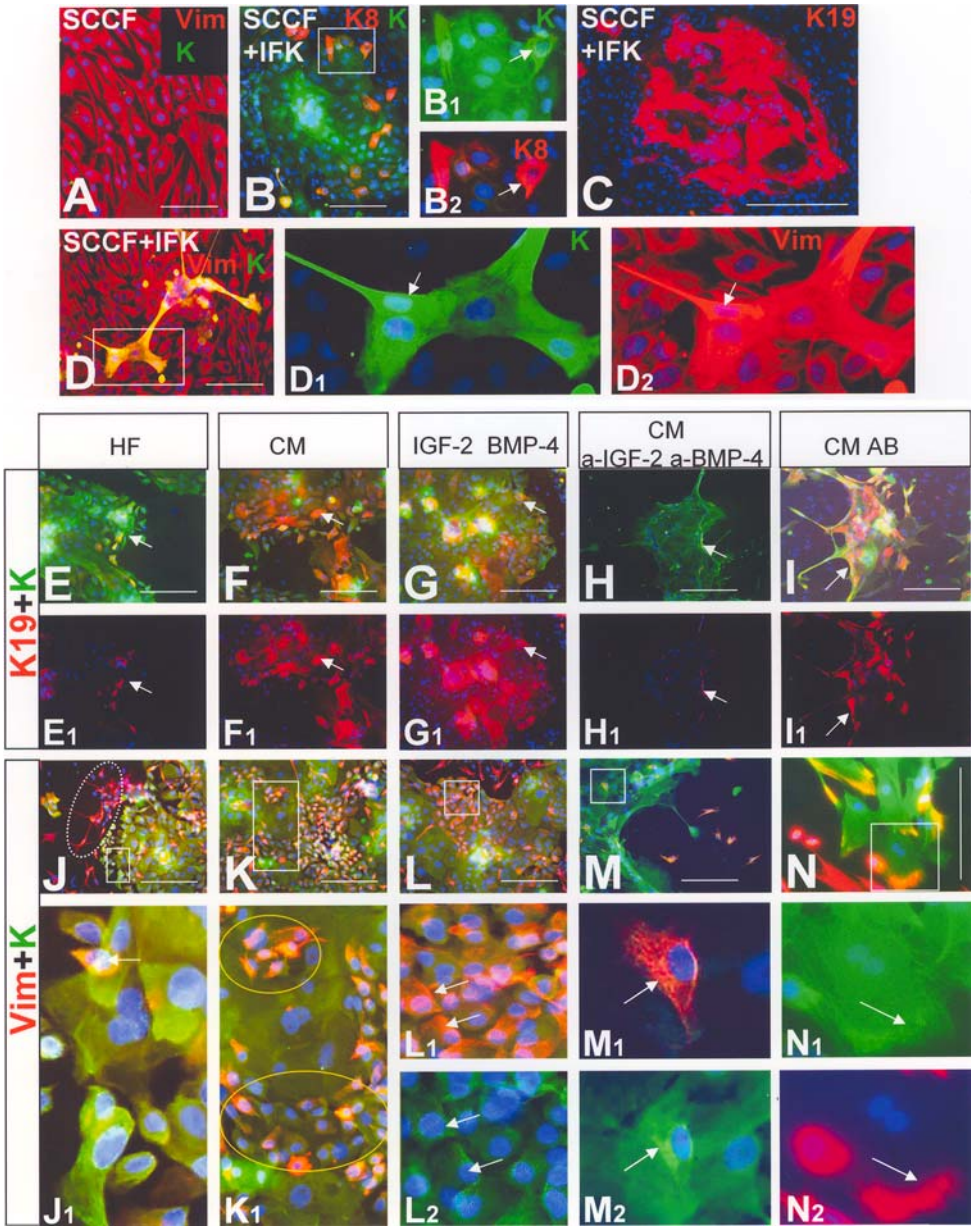

Fig. 1 All SCCF express vimentin and no keratins (**a**). They also induce expression of keratin 8 (**b**) and keratin 19 (**c**) in cocultured normal interfollicular keratinocytes (IFK). Some cocultured keratinocytes are of the fibroblast-like morphology and coexpress both the keratins and vimentin (**d**). Very rare normal keratinocytes cocultured with normal human fibroblasts (HF) (**e**, **j**) are positive for keratin 19 (**e** *E1* the same cell is marked by *white arrow*) and for vimentin (**j**). Group of vimentin-positive cells *encircled* by *white dashed ellipse* are human fibroblasts (**j**). The periphery of colony in white quadrangle (**j**) is enlarged (*J1*) and only one cell is positive for both keratins and vimentin (*arrow*). Application of the SCCF-conditioned medium (CM) to coculture of normal keratinocytes with normal dermal fibroblasts (**f**, **k**) stimulates expression of keratin 19 (**f** *F1* the same cell is marked by *white arrow*) and of vimentin in keratinocytes (**k**). Groups of cells with double positivity for keratins and vimentin in white quadrangle are enlarged (*K1*) where they are *encircled* by *yellow ellipses*. Application of IGF-2 and BMP-4 to coculture of normal keratinocytes and normal fibroblasts (**g**, **l**) stimulates expression of keratin 19 (**g** *G1* the same cell is marked by *white arrow*) and of vimentin. Group of double-positive cells in *white quadrangle* is enlarged (*L1*, *L2*) and presented in double signal mode (*L1*) or in channel where only keratin is visible (*L2*). The distinct cells are marked by *white arrows*. Application of specific anti-IGF-2 and anti-BMP-4 antibodies to culture to which the SCCF-conditioned medium was added (**h**, **m**) minimizes expression of both keratin 19 (**h**) and coexpression of vimentin and keratins (**m**). When an irrelevant antibody replaces the specific antibodies (**i**, **n** *CM AB*), the numerous keratin 19 positive cells (**i** I1) and numerous cells with double positivity for keratins and vimentin (**n** *N1*, *N2*) are observed. Enlarged cell in *white quadrangle* demonstrates a high positivity for vimentin in the periphery of the cell where also keratins are observed (*N1*, *N2*; *white arrow*). Nuclei are counterstained by DAPI (*blue signal*). *Bar* 50 μm

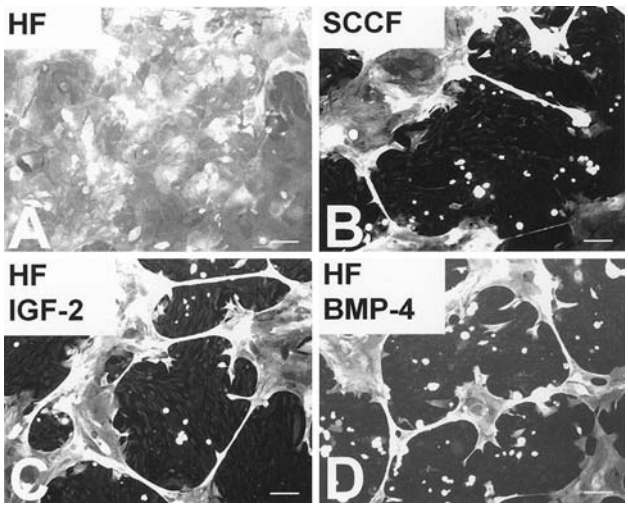

Fig. 2 Colonies of keratinocytes cocultured with normal fibroblasts (**a** HF), fibroblasts prepared from squamous cell carcinoma (**b** SCCF), normal fibroblasts with IGF-2 (**c**) and normal fibroblasts with BMP-4 (**d**) differ significantly. Detection of pankeratin; *bar* 30 μm

situation observed in the experiment where the SCCF or the SCCF -conditioned medium was used (Fig. 1e–g, j, k; Table 1). This result was corroborated by inhibition experiment in which the blocking antibodies were used (Fig. 1h, m; Table 1). It should be noted here that when both antibodies *i.e.* anti-BMP-4 and anti-IGF-2 were used separately the inhibitory effect was only partial in comparison with simultaneous application of both antibodies. The simultaneous inhibition of activity of both growth factors in the SCCF-conditioned medium resulted in total phenotype reversal of keratinocytes. A control non-immune antibody had practically no effect on phenotype of keratinocytes influenced by the SCCF-conditioned medium (Fig. 1i, n; Table 1).

In vivo immunohistochemical detection of IGF-2 and BMP-4 in tumor tissue

The possible biological participation of both growth factors was also evaluated by their detection in frozen sections from squamous cell carcinomas of the head and neck. While practically no signal was detected in normal tissues, a very strong signal for BMP-4 was observed in malignant epithelia and a moderate signal in tumor stromata (Fig. 5a, b). IGF-2 was specifically detected in tumor stromata. The malignant epithelia and normal tissues were negative (Fig. 5c, d). This observation was uniform across all studied tumors.

Table 1 Influence of culture condition on expression of keratin 8, keratin 19 and vimentin in normal human keratinocytes

Marker	No cytokine	No cytokine		BMP-4		IGF-2		BMP-4 +IGF-2		CM		CM + spec AB		CM + irrelevant AB	
	SCCF	3T3	HF	3T3	HF	3T3	HF	3T3	HF	3T3	HF	3T3	HF	3T3	HF
K8 (%)	>20	<5	<5	<5	>10	<5	>15	<5	>10	>10	>20	<5	<5	>15	>15
K19 (%)	>45	<5	<5	>30	>30	>30	>40	>30	>40	>30	>40	<5	<5	>30	>40
Vim (%)	>45	<5	<5	>25	>15	>30	>20	>30	>25	>40	>30	<5	<5	>30	>30

SCCF squamous cell carcinoma fibroblasts, *3T3* 3T3 mouse fibroblasts, *HF* normal human dermal fibroblasts, *CM* conditioned medium, *CM+ spec AB* conditioned medium and specific antibodies; *CM+ irrelevant AB* conditioned medium and antibody not recognizing IGF-2 and/or BMP-4

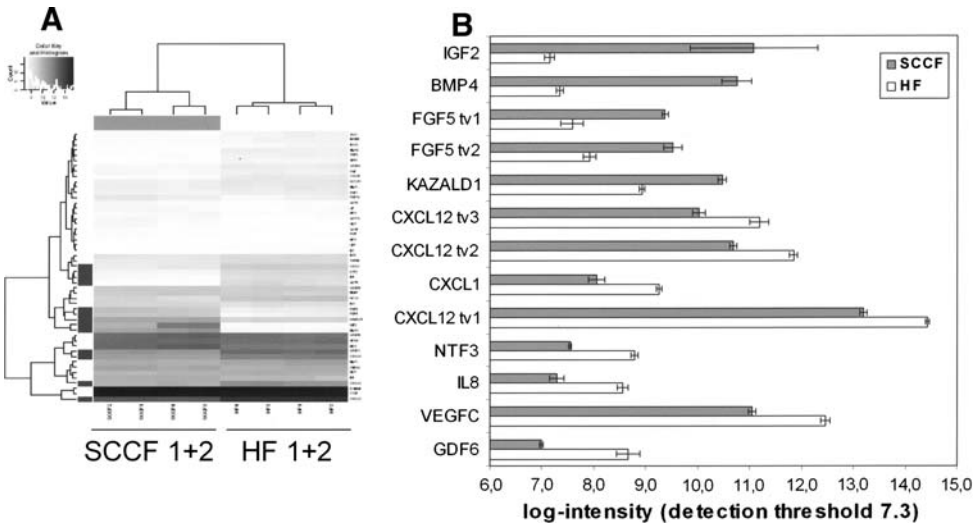

Fig. 3 Heatmap and dendrogram demonstrate significant differences between normal fibroblasts (HF) and SCCF (**a**). Thirteen significantly up- (down-) regulated transcripts for cytokines are shown in **b**

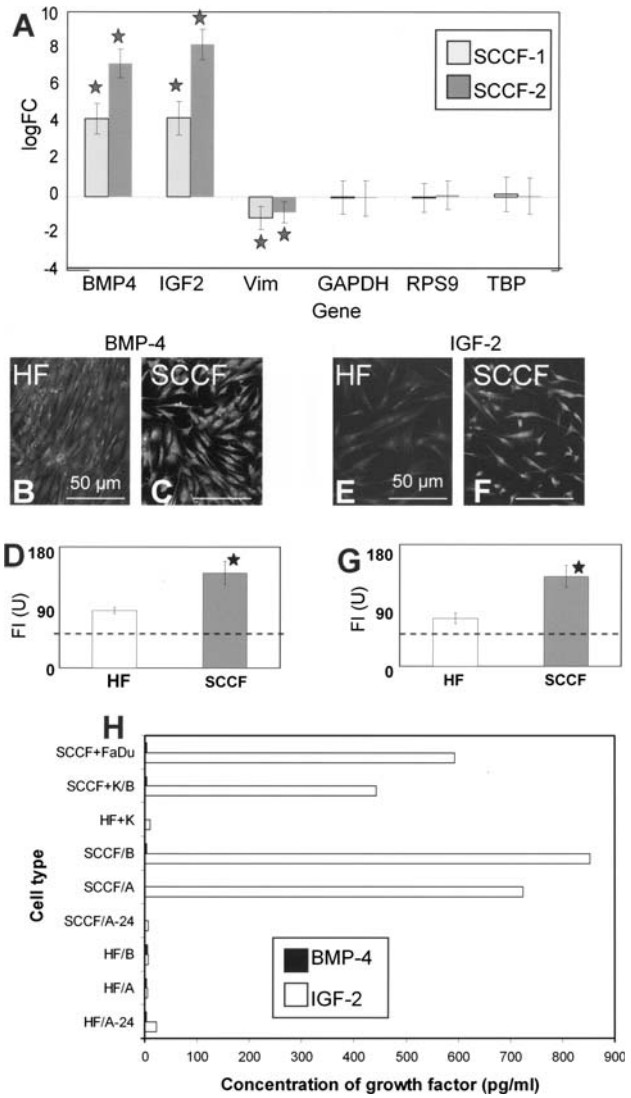

Fig. 4 Significant upregulation of mRNA for both IGF-2 and BMP-4 is detected by qRT-PCR in SCCF in comparison to normal fibroblasts (HF) (**a**). Both growth factors are also observed by immunocytochemistry in SCCF (**b**–**g**). The fluorescence intensity measured by quantitative immunocytochemistry is significantly higher (paired Student's *t* test; $P < 0.02$). The *dashed line* demonstrates level of autofluorescence. IGF-2 is secreted to the cultivation medium by SCCF cultured alone or accompanied by normal keratinocytes (SCCF + K) or FaDu cancer cell line (SCCF + FaDu). Normal fibroblasts (HF) secrete no IGF-2. No BMP-4 is secreted to the medium by HF or SCCF (**h**). In **h**, /A and /B stand for replicates of cocultivation experiment lasting 1 week and /A-24 stands for 24-h long cocultivation experiment

Discussion

This study presents new data about the nature of biological activity of stromal fibroblasts. The stromal fibroblasts significantly differ from non-tumor dermal fibroblasts in 560 differentially expressed genes. Although, the smooth muscle actin was not detected immunocytochemically, we detected upregulation of mRNA for this protein. This observation is important, because the so called cancer-associated fibroblasts frequently acquire properties of myofibroblasts (reviewed by De Wever and Mareel 2003). Paracrine secretion of growth factors, such as IGF-2 and BMP-4, can elucidate the biological activity of stromal fibroblasts to normal keratinocytes by markedly influencing their phenotype. The induced keratinocytes acquire appearance of squamous cell carcinoma keratinocytes or keratinocytes of wounded skin (Lacina et al. 2007a; Klíma et al. 2009). While IGF-2 was highly upregulated in SCCF at mRNA level, immunohistochemically detected in SCCF and produced to the medium by SCCF, we were not able to detect BMP-4 in the medium. However, it was significantly upregulated at mRNA level, and the protein product was clearly detected in SCCF. For that reason, we evaluated biological activity of both IGF-2 and BMP-4. We cannot exclude that BMP-4 is secreted to the medium under different experimental conditions or in tumors in situ. The two growth factors are capable to influence the differentiation status of cultured normal keratinocytes and their epithelial–mesenchymal transition. This phenomenon can explain our earlier observation of biological activity of stromal fibroblasts in vitro (Lacina et al. 2007a).

Our findings are in perfect concordance with detection of elevated levels of IGF-2 in serum of patients suffering from different types of carcinomas (for review see Ellis et al. 1998; Mathur et al. 2003; Trojan et al. 2006). High expression levels of IGF-2 were observed also immunohistochemically and at mRNA level in tumor and in stromal cells (Nosho et al. 2005; Sayer et al. 2005; Diehl et al. 2006; Kornprat et al. 2006; Pavelic et al. 2007; Weber et al. 2007). While IGF-2 is important for normal epithelial cells, it is also able to stimulate proliferation of tumor cells (Brady et al. 2007; Musselmann et al. 2008). We observed the positive effect of SCCF on epithelial–mesenchymal transition (Lacina et al. 2007a), and this phenomenon seems to be positively controlled by IGF-2 (Arciniegas et al. 2006). It is to be noted that IGF-2 plays a crucial role in the maintenance of stemness in embryonic stem cells (Bendall et al. 2007), which is reflected here by the observed expression pattern of keratins, typical for epidermal stem cells (keratin 19). Expression of IGF-2 and related genes is developmentally imprinted, and loss of this imprinting is typical for cancer, including squamous cell carcinoma of larynx (Grbesa et al. 2006). This process can be behind the upregulation of gene for IGF-2 in many types of cancer (for review see Jelinic and Shaw 2007), and harmonizes with observations of protumorigenic effect of this cytokine.

The second candidate molecule, which influences the normal/malignant keratinocytes, is BMP-4. During the embryonic development, this growth factor influences

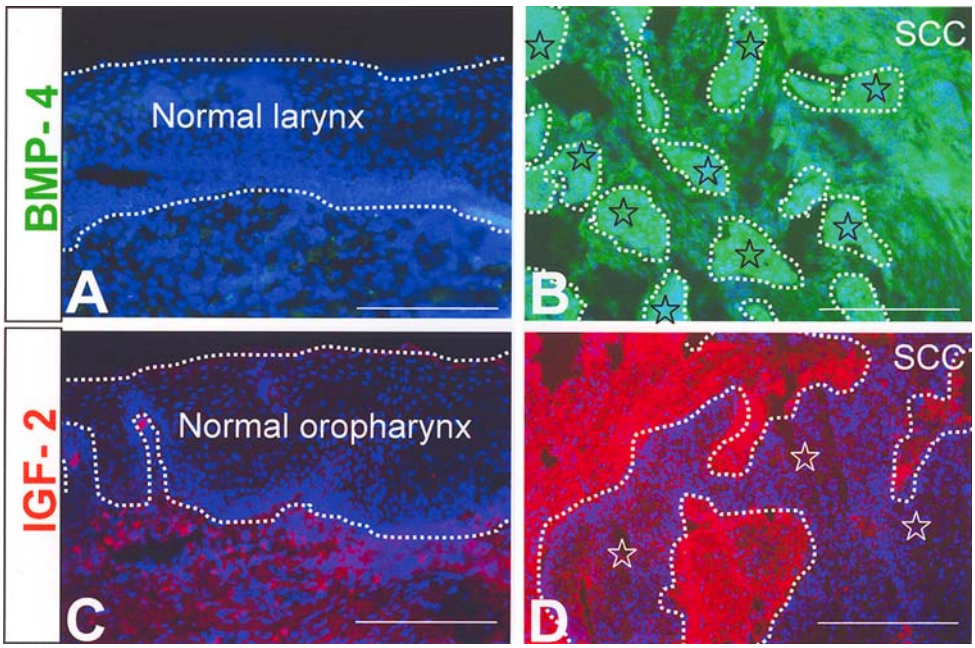

Fig. 5 Normal epithelium of larynx (**a**) and oropharynx (**c**) are negatives for both the IGF-2 and BMP-4 except the sporadic leukocytes positive for IGF-2 subepithelially. Squamous cell carcinoma of larynx (**b** SCC) exhibits positive signal for BMP-4 in tumor epithelium (surrounded by *dashed line* and marked by *asterisks*) and moderate specific signal in surrounding stroma. Squamous cell carcinoma of oropharynx (**d**) exhibits no activity in tumor epithelium but very strong signal in stromal tissue. Nuclei are counterstained by DAPI; *bar* 30 μm

differentiation of ectoderm to epidermis and inhibits the neurogenesis (Wilson and Hemmati-Brivanlou 1995; Aberdam 2004). This growth factor, moreover, induces expression of marker of embryonic stem cells Oct-4 in epidermal keratinocytes that are able to transdifferentiate to other cell types (Grinnell and Bickenbach 2007). This protein is also expressed in malignant tumors, including squamous cell carcinoma (Jin et al. 2001). It induces epithelial–mesenchymal transition in embryo and in tumors, and it also seems to be contributing to aggressive growth in tumors (Karafiat et al. 2005; Rothhammer et al. 2005; Hamada et al. 2007; Thériault et al. 2007), which is consistent with our observations.

The presented findings support many observations indicating the similarity between wound healing and cancer (Dvorak, 1986) and indicate the role of stromal fibroblasts and mesenchymal–epithelial interaction in the control of both processes. The tumor stromal fibroblasts are able to influence the phenotype of normal nonmalignant keratinocytes. This finding is not surprising, as this interaction crucially influences the development of squamous epithelia and their appendages (reviewed by Fuchs 2007).

In the light of our experiments, in which the activity of both mentioned growth factors was blocked by specific antibodies, the mutual epithelial–mesenchymal interaction in tumors can be controlled, and the growth factors and/or their receptors may be regarded as potential therapeutic targets (García-Echeverría et al. 2004).

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