The role of epithelial microenvironment in the progression of tumors

Doctoral theses

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I. INTRODUCTION

Malignant tumours are complex, heterogeneous systems. Besides the accumulation of evolutionary and genetic mutations, alterations in the microenvironment significantly contributes to their formation. The real nature of tumours can only be understood when tumour cells are examined together with their environment.

In the carcinogenesis of epithelial tumours, besides the physical and chemical carcinogenes, also certain viruses are significantly involved. For most cases of the two tumour types of epithelial origin in focus of our study – head- and neck and cervical carcinomas – infection of high-risk human papilloma viruses HPV-16 and HPV-18 is characteristic.

The benign and malignant transformation of the epithelium is histologically well characterised. Starting from the mild form of dysplasia, in its moderate and severe forms the stratified feature of the epithelium is compromised. Once tumour cells pass the basement membrane, they become in direct contact with the main components of the stroma, called fibroblast cells. Stromal fibroblasts are a heterogeneous cell population able to synthesize, maintain and organise the extracellular matrix. Due to the dynamic ralationship with tumour, fibroblasts become activated, so called tumour-associated fibroblasts, while tumour cells acquire an invasive nature. Invasive carcinomas are frequently accompanied by the accumulation of connective tissue that is produced by activated fibroblasts and carcinoma cells.

An important role is attributed to matrix metalloproteinases (MMPs) in the degradation of the extracellular matrix of both the basal membrane and the stroma, a process that may lead to metastasis formation of the malignant tumour. During wound healing and malignant transformation of the normal epithelium, the expression of MMP-7 and MMP-9 shows an excessive increase. Tetraspanin (CD151) binds the inactive pro-form of MMP-7 and thereby

promotes matrix degradation. In general, the enzymatic activity of MMPs is regulated by endogeneous inhibitors such as the family of tissue inhibitors of metalloproteinases (TIMPs).

In the process of metastasis formation, tumour cells pass through various types of tissues, while their cell-matrix adhesions and motility persistently change. They find their way through the matrix and the walls of blood vessels. In the series of these events cell junctions routinely alter, underlining the key importance of matrix receptors, for example the family of integrins. By means of dimerisation between various α and β integrin subunits, the extracellular portion of these receptors readily interact with collagens, laminins, fibronectin and thrombospondin. CD44 is further important receptor that contributes to the proliferation, migration and metastatic dissemination of several tumour types.

Growth factors and cytokines secreted by tumour cells stimulate or inhibit stroma cells in a paracrine way. Amongst those, TGF-β1 – a polypeptide with dual functions – inhibits proliferation of epithelial cells, induces their apoptosis, accordingly acts as a tumour suppressor. Its tumour promoter role manifests in its ability to stimulate proliferation of fibroblasts and their differentiation to myofibroblasts, boosting their matrix production.

II. AIMS

Our primary aim was to map stromal alterations influencing the invasiveness of tumours. For this, we established two *in vitro* epithelial tumour models with similar histological characteristics but different localisation. In the cervix model, we co-culturing cervical carcinoma cells (CSCC7) with fibroblasts isolated from normal (NF) and tumorous (TF) areas of the cervix. In the second model, tongue squamous cell carcinoma cells (SCC-25) were co-cultured with normal fibroblasts (PDL) isolated from the periodontal ligament, which is a soft connective tissue interposed between the roots of teeth and the inner wall of the alveolar socket.

As a second aim, we assessed the altered functions of normal and tumour-associated fibroblasts. The following questions were addressed:

- How is the connective tissue in normal and tumorous cervix different in terms of basal membrane and extracellular matrix protein localisations and expressions?

- What kind of expression patterns differentiate the normal fibroblasts from tumour-associated fibroblasts?

- How and to what extent fibroblasts influence viability and proliferation of tumour cells?

- What factors produced by fibroblasts help or constrain invasion of tumours?

- How is the ability of the two tumour types to invade similar or different?

III. METHODS

The cervix carcinoma model was investigated at the molecular diagnostics laboratory at the 1st Department of Pathology and Experimental Cancer Research at Semmelweis University, research on head- and neck carcinoma was done at the oncology-molecular biology laboratory at the University Clinic for Ear, Nose and Throat Medicine at Innsbruck Medical University.

Normal and tumorous cervical fibroblast cells were cultured from surgical specimens of radical Wertheim hysterectomy. The CSCC7 cell line was obtained from Leiden University, PDL fibroblasts from University of Goettingen and SCC-25 squamous cell carcinoma cells from a commercial source.

Our cervical cancer model consisted of direct co-cultures of fibroblasts and tumour cells, which allowed a direct physical interaction between the two cell types. NF, TF, CSCC7 as well as PDL and SCC-25 cells were also co-cultured indirectly, separated by a cell culture insert.

In the cervical cancer model, NF and TF cells grew in the bottom wells and CSCC7 cells in the inserts, while in the head- and neck cancer model SCC-25 cells were seeded in the bottom wells and PDL fibroblasts in the inserts.

In both model systems, the dynamic relationship between tumour cells and various types of fibroblasts was examined by the following methods:

- proliferation assay (SRB)
- invasion assay
- migration assay (Boyden chamber)
- gene expression array (qRT-PCR)
- protein expression methods (immunocytochemistry, immunohistochemistry/tumour microarray, caseinase- and gelatinase zymography, Western blot, dot blot, ELISA)

IV. RESULTS

IV.1. The cervical cancer model system:

IV.1.1. Alterations in the composition of the extracellular matrix

A tissue microarray (TMA) was generated from 27 normal and 29 cancerous, formalin fixed and paraffin embedded tissue blocks from the area of portio vaginalis uteri. Sections were stained for SMA, laminin-1, laminin-5 and fibronectin. After signal quantification and statistical analysis, the intensity of SMA immunostaining was 5.2-fold higher (Mann Whitney test: $p<0.0001$), laminin-1 showed a 3.8-fold increase (Mann Whitney test: $p<0.0001$) and fibronectin showed a 1.2-fold increase (Mann Whitney test: $p=0.0031$) in cervical cancer compared to normal tissues.

IV.1.2. Cell proliferation studies

The proliferation of tumour cells were increased in direct co-cultures with fibroblasts, when compared to the monoculture.

Our results further showed that the conditioned medium from CSCC7 tumour cells did not influence the proliferation rate of NF fibroblasts, whereas CSCC7 cells exhibited an increased cell proliferation rate (Student's *t*-test: $p<0.0001$) when exposed to conditioned medium from NF cells for 96 hours.

The conditioned medium of TFs did not stimulate the proliferation of CSCC7 tumor cells.

IV.1.3. Changes in mRNA expression in co-cultures

Expression results from monocultures of fibroblasts showed that in TF cells, expression of *FN1, ITGA3, MMP3*

and *MMP14* genes decreased, whereas that of *ITGB1*, *ITGB3* and *THBS1* significantly increased compared with NF cells.

Gene expression analysis of the two fibroblast co-cultures revealed significantly decreased *FN1*, *ITGA3*, *MMP14* and *TIMP1* expressions in TF/CSCC7 samples, while *THBS1* expression was increased compared to NF/CSCC7. In tumour cells, solely the expression of *MMP7* showed a significant drop.

IV.1.4. Changes in protein expression in co-cultures

Significantly more laminin-1 and less laminin-5, fibronectin, type III collagen, CD44 and TIMP-1 were detected in the conditioned medium of TF cells compared to those of NF cells, by the dot blot assays.

CSCC7 cells grown in direct co-culture with NFs strongly increased their MMP-7 production.

In TF+CSCC7 samples laminin-1 and perlecan expressions were significantly upregulated, whereas laminin-5, fibronectin, type III collagen, CD44 and TIMP-1 were significantly decreased, when compared to that of NF+CSCC7 group.

In fibroblasts from indirect co-cultures the expression levels of examined proteins did not change significantly compared to those in the control samples. The indirect interaction between cells did not influence protein production of the tumour cells.

Enzymatic activities of MMP-1, MMP-2 and MMP-7 were assayed in cell culture supernatants from direct and indirect co-cultures. Pro-MMP-1 was only produced by fibroblasts and in both co-culture systems the highest level of protein abundance was detected in TF fibroblasts and the presence of CSCC7 lowered the expression. Both the inactive and active forms of MMP-2 were assayed using gelatinase zymography. In direct co-cultures, pro-MMP-7 secreted by CSCC7 cells could only be found in NF+CSCC7 samples.

Integrins α4 and α5 were only expressed by fibroblasts, integrins α6 and β4 by tumour cells, whereas integrins αv, β1, β3 and β5 were produced by both cell types. Our Western blot results also confirmed that the physical contact between fibroblasts and tumour cells had a stronger influence on integrin expression than did indirect co-culturing. The presence of TF cells increased the production of fibronectin- and laminin-binding integrins in CSCC cells.

The pro-MMP-7 binding and integrin stabilizing CD151 was presented primarily on the cell surface of CSCC7 and to a less extent on fibroblast cells as well.

Both the fibroblasts and tumour cells secreted proteases that are able to hydrolyse the CD44 transmembrane protein. The resulting soluble form could be detected from the conditioned medium in all cases.

IV.1.5. Regulating mechanisms

In our model systems, the regulatory importance of SDF-1 in the differentiation of fibroblasts to myofibroblasts could not be confirmed.

Active TGF-β1 can regulate synthesis of matrix proteins and was therefore monitored from cell culture suparnatants using the ELISA method. Only CSCC7 samples contained immunoreactive TGF-β1 and its expression in the NF+CSCC7 direct co-culture samples increased 3.63-fold (Mann Whitney teszt: $p<0.0001$) compared to the control.

IV.1.6. Migration

Conditioned media from fibroblasts did not stimulate migration of tumour cells in a Boyden-chamber.

CSCC7 cells migrated at a 2.7-fold higher rate (Mann Whitney test: $p<0.0001$) towards laminin-1 used in 25 μg/ml concentration as a chemoattractant, as compared with the same concentration of fibronectin when assayed for 24 hours.

IV.2. <u>The head- and neck cancer model system:</u>

IV.2.1. Changes in mRNA expression in co-cultures

Expression of *FN1*, *ITGA4, THBS1, MMP2*, *MMP3, TIMP1* and *TIMP3* genes could only be confirmed in fibroblast samples, while *ITGA6*, *ITGB6*, *CD44*, *MMP9* and *MMP14* were expressed only be tumour cells.

Co-culturing PDL fibroblasts and SCC-25 tumour cells significantly increased gene expressions of *ITGA5, TGFB* and MMPs and TIMPs examined.

IV.2.2. Changes in protein expression in co-cultures

Co-localisation of CD44 and MMP-9 proteins was observed on the surfaces of tumour cells using fluorescent immunohistochemical analysis of paraffin embedded tissue sections.

Co-culturing significantly promoted MMP-2 protein expression (Mann Whitney test: $p<0.05$) in fibroblasts, confirming our mRNA expression data.

Gelatinase test assaying supernatants of cell lysates from either cell types proved that MMP-9 bears a tumour cell-specific function, since its active form could only be found in SCC-25 cells. Both cell types express the inactive form of MMP-2, however, we could detect active form only in the supernatant of tumour cells. In SCC-25 cells and their environment, co-cultures increased the levels of the active forms of both MMP-2 and MMP-9 compared to the case of monocultures.

Fibroblast cells produced elevated amounts of MMP-inhibiting TIMP-1 and TIMP-3 when co-cultured with SCC-25 cells.

In both cell types co-culturing increased the expression level of α5 integrin.

IV.2.3. Regulating mechanisms

In contrast with the cervical model system, in our head- and neck cancer model the regulatory role of SDF-1 proved to be important. A 2.1-fold increase in protein expression (Kruskal-Wallis test: $p<0.0001$) could be confirmed in cell culture supernatants by using the ELISA method.

TGF-β1 protein expression was comparable in both PDL and SCC-25 samples (8.5-37.8 pg/ml). Indirect interaction of the two cell types did not influence the level of the active protein.

Beyond the role of SDF-1 and TGF-β1 matrix modulators, based on previous observations from our research group, we expanded out examination to the potential regulatory role of IL-1β. IL-1β was secreted by SCC-25 cells, and its receptor was localised to fibroblast cells. IL-1β treatment of PDL cells at 1.5 ng/ml for 24 hours significantly increased the expression of *MMP1* and *MMP3* genes (Student's *t*-test with Welch correction: $^{**}p<0.01$ and $^{*}p<0.05$).

IV.2.3. Invasion and migration

The effect of co-culturing on the motility of SCC-25 cells was examined using invasion and migration tests. Tumour cells became significantly more invasive in the indirect presence of fibroblasts (Student's *t*-test with Welch correction: $p<0.05$) and also significantly more cells migrated through the insert covered with Matrigel (Student's *t*-test with Welch correction: $p<0.01$) compared to the control cultures.

V. CONCLUSIONS

1. In primary cultures tumour cells are only viable in the presence of fibroblasts.
2. The physical contact with stromal elements is essential for the spreading of invasive tumour cells that have passed through the basal membrane. Direct contact of normal fibroblasts and tumour cells results in the secretion of substantial amounts of active TGF-β1, MMP-7 and TIMP-1.
3. Proliferation of cervical cancer cells is increased by factors produced by normal fibroblasts, but not by tumour-associated fibroblasts.
4. Normal fibroblasts produce fibrillar elements of the stroma, as well as regulatory components of matrix degradation, which are necessary for the local invasion of tumour cells.
5. Tumour-associated fibroblasts promote progression and migration of invasive tumour cells by elevated secretion of laminin-1 and perlecan, and decreased production of type IV collagen. Meanwhile, their metalloproteinases loosen the structure of the basal membrane and cleave the fibrillar collagens of the interstitial stroma. These mechanisms result in stromal localisation of basal membrane proteins. Tumour cells expressing primarily laminin-binding integrins effectively migrate along laminin-1 that appears as a poorly organised network in the stroma. In cervical cancer laminin-5 produced by tumour cells decorates the leading edges of tumorous nests.
6. MMP-7 or MMP-9 activated by and membrane-anchored to tetraspanin cleaves the extracellular fragment of CD44, leading to decreased adhesion of tumour cells, which may facilitate tumour spreading.

7. In head- and neck carcinomas, cytokines produced by tumour cells – primarily IL-1β – regulate MMP-1 and MMP-3 expression, while TGF-β1 regulates MMP-2, TIMP-1 and TIMP-3 in fibroblasts.
8. Fibroblasts promote tumour cell invasion in part by the paracrine regulation of gene expression of various MMPs, and by secreting MMPs, which can be considered as a reaction to the presence of cytokines produced by tumour cells in head- and neck carcinomas.
9. Extracellular matrix components produced by stomal cells are fundamental in the progression and invasion of tumour cells of epithelial origin.

VI. CANDIDATE'S PUBLICATIONS

VI.1. Candidate's publications related to the thesis:

1. **Fullár A**, Dudás J, Oláh L, Hollósi P, Papp Z, Sobel G, Karászi K, Paku S, Baghy K, Kovalszky I. Remodeling of extracellular matrix by normal and tumor-associated fibroblasts promotes cervical cancer progression. BMC Cancer 15: Paper 256. (2015) **IF: 3,319**

2. Dudás J*, **Fullár A***, Romani A, Pritz C, Kovalszky I, Schartinger VH, Sprinzl GM, Riechelmann H. Curcumin targets fibroblast-tumor cell interactions in oral squamous cell carcinoma. Experimental Cell Research 319:(6) pp. 800-809. (2013) **IF: 3,372** *: Társ elsőszerzők

3. **Fullár A**, Kovalszky I, Bitsche M, Romani A, Schartinger VH, Sprinzl GM, Riechelmann H, Dudás J. Tumor cells and carcinoma-associated fibroblasts interaction regulates matrix metalloproteinases and their inhibitors in oral squamous cell carcinoma. Experimental Cell Research 318:(13) pp. 1517-1527. (2012) **IF: 3,557**

4. Dudás J, **Fullár A**, Bitsche M, Schartinger VH, Kovalszky I, Sprinzl GM, Riechelmann H. Tumor-produced, active Interleukin-1β regulates gene expression in carcinoma-associated fibrobroblasts. Experimental Cell Research 317:(15) pp. 2222-2229. (2011) **IF: 3,580**

VI.2. <u>Candidate's publications not related to the thesis:</u>

1. Dudás J, Bocsi J, **Fullár A**, Baghy K, Füle T, Kudaibergenova S, Kovalszky I. Heparin and liver sulfate can resouce hepatoma cells from topotecan action. Biomed Research International 2014: Paper 765794. 8 p. (2014) **IF: 2,706**

2. **Fullár A***, Baghy K*, Deák F, Péterfia B, Zsák Y, Tátrai P, Schaff Zs, Dudás J, Kiss I, Kovalszky I. Lack of matrilin-2 favors liver tumor development via Erk1/2 and GSK-3β pathways in vivo. PLOS One 9:(4) Paper ep3469. 11 p. (2014) **IF: 3,534** *: Co-first authors

3. Horváth Zs, Kovalszky I, **Fullár A**, Kiss K, Schaff Zs, Iozzo RV, Baghy K. Decorin deficiency promotes hepatic carcinogenesis. Matrix Biology 35: pp. 194-205. (2014) **IF: 3,648**

4. **Fullár Alexandra**, Baghy Kornélia, Deák Ferenc, Kiss Ibolya, Kovalszky Ilona: Hepatocarcinogenesis in matrilin-2 knock out mice. Orvosképzés 87:(1) pp. 9-20. (2012)

5. Péterfia B, Füle T, Baghy K, Szabadkai K, **Fullár A**, Dobos K, Zong F, Dobra K, Hollósi P, Jeney A, Paku S, Kovalszky I. Syndecan-1 enhances proliferation, migration and metastasis of HT-1080 cells in cooperation with syndecan-2. PLOS One 7:(6) Paper e39474. 13 p. (2012) **IF: 3,730**

6. Baghy K, Dezső K, László V, **Fullár A**, Péterfia B, Paku S, Nagy P, Schaff Zs, Iozzo R, Kovalszky I. Ablation of the decorin gene enhances experimental hepatic fibrosis and impairs hepatic healing in mice. Laboratory Investigation 91: pp. 439-451. (2011) **IF: 3,641**

VI.3. Oral and poster presentations related to the thesis:

1. **Fullár Alexandra**, Dudás József, Kovalszky Ilona. Tumor cells and cancer-associated fibroblasts co-operation of the head and neck tumors invasion. Young Pathologists Meeting (FiPAT), Zamárdi, 2012. Sep 21-22. Abstract booklet: page 20. E.02

2. **Alexandra Fullár**, József Dudás, Ilona Kovalszky. Tumor cells and carcinoma-associated fibroblasts interaction regulates matrix metalloproteinases and their inhibitors in oral squamous cell carcinoma. PhD Scientific Days, Budapest, 2012. Apr 12-13. Abstract booklet page 198. P/VI-4

3. **Fullár Alexandra**, Lakóné Vigh Renáta, Oláh Lászlóné, Dudás József, Kovalszky Ilona. Matrix transformation of importance in the progression of cervical cancer. 70th Congress of Pathology, Siófok, 2011. Sep 29- Oct 1. Abstract booklet page 10.

4. **Fullár Alexandra**, Lakóné Vigh Renáta. Matrix transformation of importance in the progression of cervical cancer. PhD Scientific Days, Budapest 2011. Apr 14-15. Abstract booklet page 86. E/VI-2