The role of syndecan-1 and -2 in the biological behavior of the HT-1080 fibrosarcoma cell line

Ph.D. thesis

Bálint Péterfia

Pathological Sciences Doctoral School
Semmelweis University

Supervisor: Prof. Dr. Ilona Kovalszky DSc.

Official reviewers: Dr. Attila Patócs MD, Ph.D.
Dr. Ferenc Deák DSc.

Examination Committee Chair: Dr. Janina Kulka Ph.D.
Examination Committee Members: Dr. Zoltán Bán Ph.D.
Dr. Gábor Réz Ph.D.

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INTRODUCTION

Syndecans are transmembrane proteoglycans bearing glycosaminoglycan (GAG) chains on their variable extracellular domain, by which they can bind several extracellular matrix components and other cell surface proteins. The biological activity of syndecans is further modulated by proteolytic shedding, whereby the ectodomain with its GAG chains is liberated and becomes a soluble effector. The truncated core protein remains embedded in the cell membrane, although its fate and cellular function mediated by the remnant core protein is unclear.

Cell-, and tissue-specific expression pattern of syndecan family members is established during the embryonal development. Although syndecan-1 is typical of epithelia, it has also been detected in the condensing mesenchyme during tooth, limb and lung morphogenesis.

Dysregulation of syndecan expression has been reported during tumour formation and progression. In a number of neoplasms, expression patterns of syndecan-1 and syndecan-2 characteristically correlate with the tumour stage and grade. While an extensive number of studies investigated the role of syndecans in carcinomas, little is known about their function in tumours of mesenchymal origin.

Syndecan-1 is detectable in a number of mesenchymal tumours with cell surface or intracytoplasmatic localisation. In malignant mesothelioma, a connective tissue tumour with partial epitheloid differentiation, syndecan-1 is rarely expressed, and its presence is associated with a more epitheloid phenotype and longer survival. Similarly, cultured malignant mesothelioma cells produce less syndecan-1 than benign mesothelial cells, regardless of their phenotypic differentiation.

In another type of mesenchymal tumour, osteosarcoma, syndecan-2 expression is reduced compared to that in osteoblasts and osteocytes in normal bone, and the expression of the proteoglycan sensitises tumour cells to basal and chemotherapy-induced apoptosis. Overexpression of syndecan-2 enhances migration and invasion of HT-1080 cells into Matrigel. The same effect is triggered by the overexpression of syndecan-1 as well.

Taken together, syndecans appear to be important players in oncogenesis therefore potential therapeutic targets in cancer. Thus, identification of clinically relevant agonists or antagonists and understanding the molecular mechanisms underlying their action is of paramount importance.

OBJECTIVES

The main purpose of the work was to reveal the role of syndecan-1 in the biological behaviour of the HT-1080 fibrosarcoma cell line. To adress this issue, the following investigations were performed:

1. Examination of the effect of syndecan-1 overexpression on the proliferation and migration of HT-1080 cells. For in vivo studies, the introduction of syndecan overexpressing stable transfectants into mice.
2. Investigation the role of syndecan-1 ectodomain shedding in the function of this proteoglycan.
3. Questing the key signaling molecules in the background of syndecan-1 effects.
4. Examination the effects of other syndecans on the proliferation and migration of HT-1080 cells.
5. Exploration and characterization possible cooperations between different syndecan forms by cell culture experiments.

MATERIALS AND METHODS

To better understand the role in the HT-1080 fibrosarcoma cell line, syndecan expression levels were modulated in cells and their growth rate and chemotactic ability were studied. For in vivo testing, syndecan-1 overexpressing cells were also inoculated into mice.

For overexpression experiments plasmids coding for truncated and full length syndecan-1 tagged with green fluorescent protein (EGFP) were transfected into cells. Moreover, untagged syndecan-1, -2 and -4 were also overexpressed. Control cells were the untransfected (wt), or the EGFP expressing ones. For syndecan-1 and -2 gene silencing experiments, vector based RNA interference (RNAi) was applied using the BLOCK-iT™ Pol II miR RNAi system with an RNAi vector targeting β-D-galactosidase as a negative control. To obtain stable transfectants, cells were selected by addition of Geneticin or Blasticidin in case of microRNA-coding plasmids. Distribution of syndecan-1/EGFP fusion proteins in living HT-1080 cells was examined by MRC-1024 confocal laser scanning microscope. In the course of proliferation measurements the sulforhodamine B (SRB) colorimetric assay was used to quantify cells. Doubling time of cells was calculated from the log phase of their growth curves measured by the SRB assay at 4, 24, 48 and 72 hours after

seeding. Cell migration was measured using a 48-well micro chemotaxis chamber equipped with a 8 μm pore-size polycarbonate membrane filter. Extracellular matrix (ECM) gel from Engelbreth-Holm-Swarm murine sarcoma was placed in the bottom chamber as chemoattractant, except for the blank sample where it was omitted. To generate spontaneous lung metastases *in vivo*, HT-1080 tumour cells were transplanted into the foot pads of mice. For quantitative experiments RNA was isolated and quantitative reverse transcription PCR (qRT-PCR) was performed using in house designed primers specific for syndecan-1 cytoplasmic and ectodomains, syndecan-2, EGFP and GAPDH. Protein levels and distribution was detected by western blot, ELISA, FACS and immunofluorescent staining. Oligonucleotide arrays and Phospho-receptor tyrosine kinase arrays were performed in order to characterise signalling activities transmitting the effects of syndecan-1 in HT-1080 cells.

RESULTS

I. Expression and localization of EGFP-tagged syndecan-1

Recombinant chimeric syndecan-1/EGFP proteins were detected in living cells by their green fluorescence. Control cells transfected with empty EGFP vector exhibited diffuse cytoplasmic and nuclear fluorescence, whereas full length and truncated proteins localised to the cell membrane and the endomembrane system. The green fluorescence of cells bearing the truncated construct was less intensive than that of the full length. Anti-GFP immunofluorescent staining displayed similarly strong membrane signals.

II. Effects of syndecan overexpression on the amount of cell surface-bound and soluble syndecan-1

The qRT-PCR results obtained by primers specific for EGFP and the cytoplasmic domain of syndecan-1 showed, that chimeric syndecan-1/EGFP proteins were expressed succesfully rising the amount of syndecan-1 in the cells. Moreover, the expression of truncated variant was more effective, than that of the full length. There was no changes in the mRNA nor in the protein level of syndecan-1 ectodomain detected except the cells transfected with the full length construct, where increasement were detected in the medium and in the lysate of cells as well. This indicated that the

truncated construct did not influence the expression or the shedding of endogenous syndecan-1.

III. The effect of syndecan-1 overexpression on the proliferation and migration of HT-1080 cells

Both syndecan-1 constructs stimulated proliferation of HT-1080 cells. Oligonucleotid arrays, western blots and immunofluorescent staining results showed, that those genes that regulate the G1/S transition of the cell cycle showed altered expression / activation, including elevated CDK2 and cyclin E1 levels and enhanced phosphorylation of retinoblastoma protein.

Directed migration towards ECM proteins of HT-1080 cells transfected with syndecan-1/EGFP constructs was significantly higher than that of the EGFP control used as baseline values. The truncated variant enhanced migration the most intensively, significantly more than the full length did. In the background of enhanced migration, an increase in syndecan-2 protein expression was found in cells overexpressing full length or truncated syndecan-1, detected by Western blotting immunofluorescent staining and also by FACS analysis.

IV. Syndecan-1 promotes malignancy of HT-1080 cells in vivo

To estimate in vivo malignancy of the stable transfectants, they were injected into foot pads of SCID mice. Twenty-four days after injection, the average volume of primary tumours of the syndecan-1/EGFP transfectants were significantly larger than those of EGFP control cells. Moreover, the full length construct promoted *in vivo* proliferation significantly more, than the truncated one.

In the control EGFP group only one animal developed metastasis in its lung with negligible size. This number was 4 and 3 for full length and truncated syndecan-1 bearing groups, respectively. The area of lung metastases bearing syndecan-1 constructs was, significantly more in animals than that of the control EGFP. These data confirmed the results found in cell cultures.

V. Effects of other syndecans on the proliferation and migration of HT-1080 cells

To expand the scope of our study, (and exclude possible interferences caused by the GFP-tag), HT-1080 cells stably expressing full length syndecan-1, -2, and -4 without a GFP-tag were established and their behaviour was tested in vitro. Syndecan-1 significantly reduced doubling time compared with EGFP control cells. This effect was also

significant when compared to untransfected wild type cells. Overexpression of syndecan-2 and -4 caused significant reduction in the doubling time only in comparison with EGFP cells, but not wt.

Overexpression of either syndecan paralogue uniformly increased the migratory ability of HT-1080 cells up to two fold. Migration of EGFP control cells did not differ significantly from that of wt cells.

VI. The effects of syndecan-1 and -2 silencing on the proliferation and migration

Syndecan-1 or -2 silencing resulted in a significant decrease in the expression of the corresponding syndecan, while no significant changes in the expression of other syndecans were detected. Syndecan-2 silencing significantly inhibited chemotaxis and slightly the proliferation of HT-1080 cells. Antisense silencing of syndecan-1 did not inhibit cell migration or proliferation.

VII. Effects of truncated syndecan-1 are supressed by syndecan-2 silencing

To test the importance of syndecan-2 in the highly malignant phenotype of syndecan-1 overexpressing fibrosarcomas, syndecan-2 silencer plasmid was co-transfected with truncated syndecan-1 or an EGFP empty vector control.

The truncated syndecan-1 construct increased neither the proliferation rate nor the chemotaxic ability, when syndecan-2 was silenced at the same time.

VIII. Possible mechanisms of syndecan-1 and syndecan-2 cooperation

To identify key signalling events underlying the observed induction of syndecan-2 by syndecan-1 overexpression in HT-1080, the control EGFP transfectants and the cells bearing syndecan-1/EGFP constructs or syndecan-2 were assayed on a phospho-receptor tyrosine kinase (pRTK) array and by Western blot. Two of the 42 RTKs investigated, IGF1R and Axl, showed considerable increase in their phosphorylation levels in syndecan-1 transfected cells. Additionaly, Axl showed a moderate but significant dephosphorylation in the syndecan-2 transfectants. MAP kinases p44-42 and p38 were hyperphosphorylated in syndecan-1 as well as in syndecan-2 overexpressing cells. The expression of Ets-1 transcription factor was increased only in syndecan-1 bearing cells.

NEW OBSERVATIONS

1. Syndecan-1 increases the proliferation and chemotactic migration of HT-1080 fibrosarcoma cells *in vitro* as well as the growth and the metastatic potential of primary tumors *in vivo.*
2. Overexpression of full length syndecan-1 increases the amount of cell surface-bound and soluble syndecan-1, whereas the truncated one, that is expressed more effectively, does not affect expression or shedding of endogenous syndecan-1. The tumor promoter role of syndecan-1 observed in HT-1080 cells is independent of its ectodomain *in vitro,* however, *in vivo* the presence of the ectodomain further increases tumour proliferation.
3. Syndecan-1 increases the expression of CDK2 and cyclin E1, the Ets-1 transcription factor as well as syndecan-2 in HT-1080 cells, while it promotes the phosphorylation of retinoblastoma protein and IGF1R.
4. The proliferation of HT-1080 cells is promoted only by syndecan-1 transfection, but not by syndecan-2 or -4.
5. Enhanced migratory ability induced by syndecan-1 overexpression is mediated by syndecan-2, however, elevated expression of both syndecan-1 and -2 are needed to promote the proliferation.

LIST OF PUBLICATIONS

Publications in the topic of the thesis:

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