The role of syndecan-1 and TFPI-2 in uterine physiological and pathological invasion

PhD theses

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Budapest
2020

1. Introduction

In multicellular organisms, cells communicate with each other and with the components of the extracellular matrix (ECM) around them. The ECM is a dynamic structure that is constantly changing by tightly controlled fashion. In the ECM, protein-polysaccharide complexes (proteoglycans) and multiadhesive proteins (fibronectins and laminins) bind to the network of structural proteins (collagen backbone) and appear in both forms of the ECM, the interstitial matrix and the basement membrane.

The fibroblast-secreted matrix metalloproteinases (MMPs) and their regulatory inhibitors (TIMPs) are responsible for tissue homeostasis. Fibroblasts are characteristic cells of connective tissue that are not only responsible for the production of the stroma but also continuously reorganize that by producing ECM-degrading proteolytic enzymes. A good example of this dynamic equilibrium is the process of wound healing, in which growth factors and cytokines also play an important role.

To compare the events of physiological invasion and tumor invasion, we examined placenta and cervical cancer in our experiments because the invasive ability of trophoblast cells is similar to that of malignant tumor cells. In healthy pregnancy, endometrial and myometrial trophoblast invasion is tightly regulated, which is most characteristic for the first trimester. Even the slightest disruption of this physiological process can lead to pathological pregnancies such as preeclampsia (PE) or a more severe form of it, HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count). PE is one of the most serious obstetric diseases, as it is one of the leading causes of maternal and fetal morbidity and mortality. The disease diagnosis is based on new-onset hypertension, proteinuria, and edema occurring after the 20^{th} week of pregnancy. In contrast, local invasion of tumors is an unregulated, pathological process that can form distant metastases.

Stromal cells and ECM elements play an important role in the establishment of the tumor characteristics. Tumor cells remodel intact tissues in their microenvironment to support their own growth and invasion by producing growth factors and cytokines. This results the formation of tumor stroma, which is composed of tumor-associated fibroblasts (TAF), endothelial and inflammatory cells embedded in the ECM. A constant, dynamic relationship develops between tumor cells and the surrounding stroma, mediated by matrix proteins. Therefore, to understand the real nature of a tumor it is important to study cell surface and extracellular matrix proteins, as well.

My dissertation focuses on two protein molecules, tissue factor pathway inhibitor-2 (TFPI-2) and syndecan-1, both involved in the regulation of the pericellular microenvironment. TFPI-2 is most abundant in the placenta and its role in the physiological processes of pregnancy is not yet fully understood. Beside the placenta, it is found in the liver, skeletal muscle, heart, kidneys, and pancreas, produced by fibroblasts, endothelial, smooth muscle and epithelial cells. TFPI-2 affects extracellular matrix rearrangement by inhibiting serine proteinases, such as plasmin that breaks down collagen and activates proMMPs. Thus, in addition to inhibiting fibrinolysis, it also regulates invasive processes. The syndecan-1 is able to link events in the pericellular space to intracellular processes, thereby also being involved in cell invasion. TFPI-2 is likely to bind to heparan sulfate chains, which enhances its activity.

2. Aims

Our aim is to compare invasive processes in two tissues that are histologically different but can be characterized by invasive properties. For a comparative study of the events of physiological and pathological invasion, the placenta was chosen and this was to be compared with invasion taking place in cervical cancer. Studying tissue microarrays and tissue culture models, we sought answers to the following questions:

- Is the expression of TFPI-2 altered in placenta from patients with preeclampsia and HELLP syndrome compared to pregnant controls?
- Do we find alteration in maternal serum TFPI-2 concentrations in patients with preeclampsia?
- If we find some changes, can it be in context of
  - placental pathogenesis of early preeclampsia and HELLP syndrome,
  - or changes in syndecan-1 expression in patients with preeclampsia?
- What mechanisms underlie the silencing of *TFPI2* in cervical cancer?
- Is there a correlation between the presence of syndecan-1 and the survival of patients with cervical cancer?
- Are the expression and localization of pathological syndecan-1 relevant in cervical cancer?
- To what extent are the invasive abilities of the two tissue types similar or different?

3. Methods

In our studies, we processed placenta and cervical cancer samples as well as maternal blood samples from the material of the 1st Department of Obstetrics and Gynecology, Semmelweis University, and the Maternity Private Clinic of Obstetrics and Gynecology.

Third-trimester pregnant women in the study were grouped according to age of gestation (before/after week 35) and then the appearance of clinical signs characteristic of preeclampsia (PE): 1) early control (n=5), 2) early PE (n=7), 3) early PE associated with HELLP syndrome (n=8), 4) late control (n=9), 5) late PE (n=8). Some samples were collected in the first trimester. Immediately after delivery, some of the examined placentas were snap-frozen in liquid nitrogen and stored at -80°C until use, and the rest were fixed in 10% formalin and embedded (FFPE samples). Maternal serum was stored at -80°C until use.

Some of the tissue samples obtained from cervical cancer from Wertheim's surgery were processed as tissue culture explants and the other as FFPE samples.

For morphological studies, based on H&E stained sections, villous-rich placenta areas, and normal and tumor areas of cervical cancer were selected for tissue microarray (TMA) block preparation and immunohistochemistry.

Tissues obtained from freshly removed tumors and normal areas from one patient, and in 1-1 cases lymph nodes, were minced into smaller slices from which primary cell cultures were formed: normal fibroblast (NF), metastasis-associated fibroblast (MF), tumor-associated fibroblast (TF), own tumor (T). For our experiments, cervical cancer cell lines (CSCC1 and CSCC7) were obtained from the University of Leiden. To investigate the interaction between the tumor and its microenvironment, direct and indirect co-culture models were established by co-culturing

cervical cancer cells (T, CSCC1, CSCC7) and fibroblast cells (NF, MF, TF) in a common culture dish.

For immunocytochemical studies, monocultures and direct co-cultures were grown on coverslips.

For Western blot analysis, placenta samples which were frozen then powdered in liquid nitrogen, as well as cervical cancer tissue culture samples (mono- and co-cultures, transfected cell cultures) were used.

TFPI-2 concentrations in maternal serum samples were determined with a human TFPI-2 sandwich ELISA Kit.

PCR-based methods were used for gene expression assays after DNA and RNA isolation, such as nested PCR (HPV typing), mRNA microarray, real-time PCR, DNA methylation assay (BS-PCR, MS-HRM, pyrosequencing), miRNA prediction regulating *TFPI2*.

Of the predicted and validated miRNAs, *miR-23a* mimics and inhibitors were transfected.

4. Results

Examination of placental samples

Demographic and clinical data

High systolic and diastolic blood pressure and proteinuria were observed in the PE patient groups. In our cases in the control groups, blood pressure values were normal and no protein appeared in the urine. The weight of the placenta was significantly lower in the patient groups compared to the corresponding control groups. Neonatal birth weights were lower in the patient groups compared to the corresponding control groups, and this difference was significant in the late groups.

Changes of TFPI-2 expression level as pregnancy progresses

Analyzing microarray expression data of placentas from healthy pregnancies obtained from the GEO database (ID number: GDS4037), a significant increase in *TFPI2* mRNA levels was observed with advancing age of pregnancy (first versus second trimester: p=0.07; first versus third trimester: p=0.005; second versus third trimester: p=0.006). This data was also supported by TFPI-2 immunostaining of our first and third trimester samples.

Expression of placental TFPI-2 and syndecan-1 in the third trimester of pregnancy

We were interested in quantitative changes in placental TFPI-2 in PE and HELLP syndrome. TFPI-2 appeared in the patient groups with the same molecular weight but increased expression compared to the control group.

Immunohistochemistry of TMAs allowed the simultaneous examination of 37 third trimester placentas. While cytoplasmic TFPI-2 expression of syncytiotrophoblast showed no significant

difference between early and late controls in physiological pregnancy progressed in the third trimester (p=0.75), increased TFPI-2 expression was found in early patient groups compared to the early control group (early PE: p=0.001; early PE+HELLP syndrome: p=0.003 vs. early control). There was no expression difference between the late patient and control groups (late PE: p=0.26 vs. late control).

TFPI-2 expression was compared to previously published syndecan-1 expression. The expression of syndecan-1 changed similarly to the expression of TFPI-2, except for the late groups, since syndecan-1 also had increased expression in late PE compared to the late control.

Evaluation of TFPI-2 staining with the Densitoquant program confirmed the result of the semiquantitative evaluation.

Correlation between TFPI-2 expression, placental histology and clinicopathological parameters

Histological abnormalities of the placenta were expressed as maternal vascular malperfusion (MVPM), the higher the value, the more severe the lesion. The patient groups resulted in a higher mean MVPM value compared to controls, and this difference was significant in the early groups.

In control cases, TFPI-2 expression was negatively correlated with placental MVMP (R=-0.18, p=0.54), birth weight (R=-0.27, p=0.36), and placental weight (R=-0.52, p=0.08), however, the differences were not significant. In PE cases, TFPI-2 expression was significantly positively correlated with placental MVMP (R=0.43, p=0.04) and negative with birth weight (R=-0.68, p=0.0003) and placental weight (R=-0.74, p=0.0001).

Maternal serum TFPI-2 concentrations in healthy and abnormal pregnancies

As physiological pregnancy progressed, maternal serum TFPI-2 concentrations increased slightly (early vs. late control: p=0.036). In the early PE groups, this increase was heightened compared to the early control group, and the difference was significant (early PE: p=0.048; early PE+HELLP syndrome: p=0.19 vs. early control). However, there was no difference between the late groups (late PE: p=0.60 vs. late control).

This fact raises the possibility that overexpression of TFPI-2 plays a role in inadequate placental development, thereby promoting PE formation.

Importance of TFPI-2 in cervical cancer

Characterization and morphology of tissue culture cells

For morphological studies, we generated T tumor and NF, TF, and MF fibroblast cells. The established T primary culture and the C control tumor cells were comparable. Fibroblasts were placed in direct contact with T and C tumor cells then visualized by H&E staining. Both tumor cells showed epithelial morphology and formed nests in the direct co-cultures. Each fibroblast type is spindle-shaped, with a slightly elongated, oval nucleus. Overall, T is also a true tumor cell culture and is similar in many ways to the CSCC7 cell line.

mRNA expression changes

Three NF-TF pairs were compared by total gene expression (mRNA) microarray assays. Of the nearly 41,000 genes possible, 67 showed significant differences (GEO identification number: GSE148747). This is how *TPFI2* came to our attention. The result

of the mRNA array was validated by real-time PCR on the primary cell cultures (NF, MF, TF, T) and the CSCC7 cell line (C). *TFPI2* mRNA levels of TF (0.01x, $p<0.001$) and MF (0.23x, $p<0.001$) were significantly decreased compared to NF. Neither T nor C expressed *TFPI2*.

Changes in TFPI-2 protein level of cell cultures and tissue samples

Protein-level analysis of monocultures by Western blot confirmed gene expression changes. In direct co-cultures (fibroblast+tumor cell), changes similar to monoculture models were observed, additionally TFPI-2 expression in fibroblasts was decreased in the presence of tumor cell.

In FFPE cervical cancer samples, strong nuclear staining was observed in the basal layer of the epithelium, which later appeared with varying intensity throughout the epithelial layer. Moderate amounts of TFPI-2 were detected in the cytoplasm. Variable amounts of TFPI-2 were also detected in the cytoplasm of squamous cell carcinoma near the surface of the cervix and in tumor stroma cells. Moving away from the surface, this amount gradually decreased. In the deeper layers of the tumor sample, the protein was no longer detectable in either the tumor nests or the connective tissue.

Epigenetic regulatory mechanisms

The methylation status of the promoter region of the *TFPI2* gene in the 5 cell cultures/cell lines (NF, TF, MF, T, C) was examined by MS-HRM and pyrosequencing with our designed assays 4, 5, and 6. MS-HRM showed methylation only in tumor cells, both in monoculture and indirect co-culture. Pirosequencing confirmed the methylation of the *TFPI2* gene in cervical cancer cells. Four CpG sites were examined with this method and the results indicate CpG_4 as a possible major silencing site for *TFPI2*

on cancer cells, whereas NF, TF, and MF do not show methylation there.

Other epigenetic silencing sought an explanation for the disappearance of TF protein expression. MiR databases were used to identify miRNAs targeting the *TFPI2* gene. Five miRNAs (*miR-616-3p, miR-646, miR-554, miR-3529-5p, miR-23a*) were considered. Only the expression profile of *miR-23a* corresponded to the protein expression pattern of fibroblasts observed on Western blot. Transfection of *miR-23a-3p* and *-5p* mimics and inhibitors and their negative controls into NF cells highlights a potential key role of *miR-23a-3p* in inhibition of *TFPI2* translation in TFs.

Relationship between HPV and miR-23a

Based on literature data, HPV is able to inhibit *miR-23a*, so we examined the presence of HPV E6 DNA in the tumor FFPE sample of the examined patient, in the cell cultures generated from the sample (NF, TF, T), and in CSCC7. HPV16 was clearly detectable in tumors, while all fibroblasts were negative.

Investigation of syndecan-1 in cervical cancer

Abnormal localization of syndecan-1

In addition to syndecan-1, the expression of vimentin was also examined in normal and tumor cervical histological samples. Vimentin was homogeneously stained in the connective tissue of the intact cervix and also in the tumor stroma. Stromal cells in the cancerous area contained more vimentin than cells from non-tumor regions (1.6x, $p<0.0001$). Vimentin also appeared in the cytoplasm of some tumor cells, indicating epithelial-mesenchymal transformation. Syndecan-1 was expressed on the surface of

squamous cells. The cell surface syndecan-1 intensity of tumor cells was decreased and its presence in the interstitial stroma was also detectable. There was 7.3 times more syndecan-1 in the cervical cancer stroma than in the surrounding tumor-free connective tissue ($p<0.0001$). Syndecan-1 can also appear in the cytoplasm of cancer cells, rarely in their nucleus and perinuclear membrane.

Survival

Analysis of 51/55 patients in the survival group showed no significant association between stromal syndecan-1 expression and survival (log rank test: $p=0.2765$).

The extent of the decrease in cell surface syndecan-1 expression follows the histological grade (Spearman correlation: $p=0.0055$, r=-0.3696). Loss of membrane syndecan-1 expression was associated with a higher mortality rate (51/55). The overall survival at 5, 10, and 15 years was 64.7% ($p=0.0374$), 60.8% ($p=0.1721$), and 49% ($p=0.3666$). The initial survival benefit was significant until the seventh year.

Effect of co-culturing on the behavior of participating cells

NF and CSCC1 were co-cultured separately and together plated with the same number of cells. Tumor cell proliferation was stimulated by fibroblasts.

Simultaneously, by culturing NF cells that did not originally produce syndecan-1 in direct co-culture with CSCC-1, syndecan-1 also appeared on NF cells.

5. Conclusions

1. TFPI-2 is localized in the placenta primarily in the cytoplasm of the syncytiotrophoblast.

2. During a healthy pregnancy, the amount of TFPI-2 expressed in the placenta increases from the first trimester to the third trimester, but does not change in the third trimester as the pregnancy progresses. Third-trimester syncytiotrophoblast TFPI-2 expression is increased in early preeclampsia, with or without complications by HELLP syndrome, compared with early controls; in late preeclampsia, however, it is not higher compared to late controls.

3. Compared with early controls, maternal serum TFPI-2 concentrations in healthy pregnant women increase moderately in the third trimester of pregnancy, while they increase significantly in cases of early preeclamptic disease.

4. In preeclampsia, placental TFPI-2 expression is positively correlated with placental MVMP, while a negative correlation is found with placental weight and birth weight.

5. In maternal serum in physiological pregnancy, TFPI-2 and syndecan-1 show a similar expression pattern. However, in cases of preeclampsia, their quantitative change is opposite. TFPI-2 appears to inhibit syndecan-1 shedding. Elevated TFPI-2 expression in the placenta in the third trimester of pregnancy may be associated with abnormal placentation in cases of early preeclampsia, regardless of the presence of HELLP syndrome.

6. Inactivation of *TFPI2* gene occurs through the interaction of cervical cancer cells and tumor-associated fibroblasts, using two well-known epigenetic regulatory mechanisms for this purpose. One is promoter methylation in tumor cells, the other

is the stimulation of *miR-23a* expression in fibroblasts by previously unknown factors produced by tumor cells.

7. Based on its strategic role in inhibiting tumor invasion, TFPI-2 can be considered a tumor suppressor.

8. Irrespective of the final clinical outcome, our immunohistochemical and immunocytochemical observations confirmed that tumor cell-induced stromal syndecan-1 expression, like in other tumors, is a common event in cervical cancer.

9. Although *in vitro* studies have shown that tumor cell proliferation is stimulated by stromal syndecan-1, its negative effects *in vivo* have not been demonstrated, probably due to the role of additional regulatory factors.

10. Cell surface syndecan-1 expression may be a good prognostic factor.

6. Publications

Publications in context of the thesis:

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