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Hsulf-1 regulates growth and invasion of pancreatic cancer cells

I Abiatari, J Kleeff, J Li, K Felix, M W Büchler, H Friess

Background: Hsulf-1 is a newly identified enzyme with arylsulphatase activity that can regulate the sulphation state of cell-surface heparan sulphate proteoglycans (HSPGs). In vitro overexpression of this enzyme in pancreatic cancer cells decreases responsiveness to fibroblastic growth factor-2, as Hsulf-1 is upregulated in primary pancreatic adenocarcinoma.

Aim: To further analyse the functions of the Hsulf-1 enzyme in vitro and in vivo with respect to growth, invasion and tumorigenicity.

Methods and results: Transfection of Panc-1 pancreatic cancer cells with a full-length Hsulf-1 expression vector resulted in increased invasiveness and adhesiveness. An in vivo xenograft nude mouse tumour model showed a markedly reduced growth potential of Hsulf-1-expressing Panc-1 cells, which correlated with a considerably lower proliferation rate. Hsulf-1-positive nude mouse tumours showed better development of interstitial matrix structures, with increased blood vessel density in these tumours. In an orthotopic model, Hsulf-1-positive tumours exhibited enhanced local invasiveness. In human primary pancreatic cancers there was strong staining for sulphated HSPGs, which was markedly reduced in metastatic tissue samples.

Conclusion: Hsulf-1-mediated desulphation of HSPGs reduces the growth ability of Panc-1 pancreatic cancer cells, but increases the basal invasiveness of these cells, suggesting an important role of this enzyme in pancreatic cancer progression.

Pancreatic adenocarcinoma shows one of the most malignant phenotypes of all gastrointestinal tumours. A high rate of invasiveness, metastasis, rapid growth and frequent resistance to chemotherapy are some of the characteristics of this disease, which has an extremely poor prognosis, with an overall 5-year survival rate <5%. Genetic alterations such as k-ras, p53, p16 and Smad4 mutations as well as numerous epigenetic changes underlie the development and progression of the disease. Although these findings have increased our understanding of the pathogenesis of pancreatic cancer, the reasons for its aggressive behaviour are still not completely understood.

Membrane-associated heparan sulphate proteoglycans (HSPGs), which are the main components of basement membranes and extracellular matrix, are thought to have important roles in many cellular processes, including cell–cell or cell–extracellular matrix interactions. Besides a structural and cell-to-matrix anchoring function, proteoglycans containing heparan sulphate chains are also associated with binding of several growth factors, thereby playing a distinct role in malignant transformation and growth. The importance of HSPGs in growth factor signal transduction has been assessed specifically with respect to heparin-binding growth factors. These growth factors require the presence of heparan sulphate chains for high-affinity binding to their receptors. Two members of the HSPG family, syndecan-1 and glypican-1, are overexpressed in pancreatic cancer and influence heparin-binding growth factor signalling in this disease.

Hsulf-1 was recently identified as an arylsulphatase activity enzyme, which can influence the sulphation state and the biological function of HSPGs. Previous studies have shown that down regulation of Hsulf-1 in different types of malignancies, such as hepatocellular carcinoma, ovarian cancer and head and neck cancers, is associated with increased signalling of heparin-binding growth factors.

We have shown previously that Hsulf-1 expression is increased in pancreatic cancer and that forced expression of Hsulf-1 in pancreatic cancer cells results in reduced growth and decreased responsiveness towards fibroblastic growth factor-2. In our study, we further analysed the influence of Hsulf-1 in pancreatic cancer cell growth and invasion, both in vitro and in vivo.

MATERIALS AND METHODS

In vitro invasion assay

Panc-1 pancreatic cancer cells were stably transfected with a full-length Hsulf-1 expression vector or an empty control vector, as described previously. Invasion assays were carried out in a BD Biocoat Matrigel Invasion Chamber with 8-μm pore size (BD Biosciences, Heidelberg, Germany) according to the manufacturer’s instructions. The Matrigel was rehydrated with 500 μL Dulbecco’s modified Eagle’s medium (DMEM) (serum-free) and incubated in a 37°C, 5% carbon dioxide atmosphere for 2 h. 5×10⁶ cells/ml were added to the top chamber and incubated for 24 h. The non-invasive cells were removed from the upper surface of the membrane with cotton-tipped swabs. Cells adhering to the lower surface were fixed with 75% methanol and stained with 1% toluidine blue (Sigma-Aldrich, Taufkirchen, Germany). The whole membrane was scanned and the invading cells were counted. The assays were carried out in duplicate and repeated four times. The invasion index was expressed as the ratio of the percentage invasion of the Hsulf-1-transfected cells over the percentage invasion of the control cells.

Abbreviations: DMEM, Dulbecco’s modified Eagle’s medium; HSPGs, heparan sulphate proteoglycans
In vivo tumorigenicity study

2 x 10^6 Hsulf-1-transfected and control Panc-1 cells in 200 µl DMEM were injected subcutaneously at sites behind the anterior forelimb of 4-week-old athymic mice through a 26-gauge needle. The sites were examined daily to determine whether tumours had appeared. Afterward, tumour size was measured weekly for 10 weeks. Animals were killed and tumours were collected. Immediately after removal, a 1-mm^3 portion of the tumour was implanted into the pancreas of 4-week-old athymic mice. The remaining tumour samples were either snap frozen in liquid nitrogen and then maintained at −80°C or fixed in 5% formalin and embedded in paraffin wax after 24 h. One month after orthotopic implantation, animals were killed; grown tumours were excised, fixed and embedded in paraffin wax as described. All experiments on animals were officially approved.

Patients and tissue collection

In all, 10 pancreatic cancer samples and 5 pancreatic cancer metastasis samples were obtained from patients (median age 62.5 years; range 41–78 years) who underwent pancreatic resections for pancreatic cancer at the University Hospital of Bern, Switzerland and the University Hospital of Heidelberg, Heidelberg, Germany. Five normal human pancreatic tissue samples were obtained from previously healthy people through an organ donor programme (median age 45 years, range 20–74 years). Immediately on surgical removal, tissue samples were snap-frozen in liquid nitrogen and then maintained at −80°C until used for immunohistochemistry. The ethics committees of the University of Heidelberg and the University of Bern approved all the studies. Written informed consent was obtained from all patients.

Immunohistochemistry

Frozen tissue sections (4–5 µm thick) were immunostained with the monoclonal anti-heparan sulphate antibody (10-E4 epitope), obtained from Seikagaku Corporation (Tokyo, Japan), and the purified anti-mouse CD31 antibody obtained from BD Biosciences (Heidelberg, Germany). Tissue sections were air dried and fixed in acetone for 5 min. Thereafter, slides were placed in washing buffer (10 nM Tris-hydrochloric acid, 0.85% sodium chloride and 0.1% bovine serum albumin at pH 7.4) and immunostained. Paraffin wax-embedded tissue sections (2–3 µm thick) were deparaffinised in xylene and rehydrated in progressively decreasing concentrations of ethanol. Thereafter, slides were placed in washing buffer. Antigen retrieval was carried out by microwaving the tissue sections in 10 mM citrate buffer for 10 min. Sections were incubated with normal goat serum (DAKO Corporation, Carpinteria, California, USA) for 45 min to block the non-specific binding sites and then incubated with a mouse monoclonal Ki-67 antibody (DAKO; goat anti-rat horse radish peroxidase diluted 1:500 from Amersham Biosciences, Freiburg, Germany), for 45 min at room temperature. Thereafter, tissue sections were incubated with 60 µl streptavidine-peroxidase (KPL, Gaithersburg, Maryland, USA) for 35 min at room temperature and subsequently with 100 µl of the diaminobenzidine and chromogen (DAKO Corporation).

Statistical analysis

Data are expressed as the means (standard error of the mean (SEM)) unless indicated otherwise. Student’s t test was used for statistical analysis of the data. A p value <0.05 was taken as the level of significance.

RESULTS

Previously, we had studied the invasion capacity of Hsulf-1-transfected Panc-1 cells on growth factor treatment. Two clones (Sulf-26 and Sulf-38) were selected for further experiments as they express high levels of Hsulf-1 mRNA and show increased sulphatase activity after Hsulf-1 transfection. In this study, we examined the basal invasion potential of these Hsulf-1-expressing Panc-1 cells compared with control Panc-1 cells.

The Hsulf-1-expressing clone Sulf-38, which was previously characterised as the clone with the highest sulphatase activity, showed significantly increased invasiveness compared with control cells (338.7 (100.5%); p<0.05). Increased invasion was also observed in the other Hsulf-1 clone, Sulf-26, but to a lesser extent (113.8 (36.3%); not significant (NS); fig 1A).

As Hsulf-1 expression influenced the basal invasion ability of Panc-1 cells, we next sought to investigate the adhesion capacity of Hsulf-1-transfected cells compared with controls. Matrigel adhesion assays showed increased adhesion of Hsulf-1-expressing clones in the early phase of the experiment, which was significant for Sulf-38 after 6 h (120.0 (8.7) cells) compared with empty vector control cells (32.3 (9.10) cells, p<0.01), whereas increased adhesion of Sulf-26 clone was NS (77.0 (17.7) cells) at the same time point. At 24 h, there was no significant difference in adhesion of the Hsulf-1-transfected Panc-1 cells to the Matrigel surface in comparison to control cells (fig 1B).

After in vitro evaluation of the effects of Hsulf-1 expression in Panc-1 cells, the tumour growth potential in nude mice—that is, tumour incidence and tumour size—was compared between wild-type, empty vector and Hsulf-1-transfected Panc-1 cells. The potential of tumour cell growth was markedly reduced in Hsulf-1-expressing Panc-1 clones compared with wild-type and empty vector controls, which showed shorter time to tumour appearance and faster tumour growth (fig 2). After 10 weeks of growth, the Hsulf-1-positive tumours were significantly smaller than the empty vector-transfected (72.5 (15.1) mm^3) and wild-type tumours (81.39 (24.61) mm^3), measuring 14.69 (3.65) mm^3 (p<0.05) for Sulf-38 and 34.92 (9.37) mm^3 (NS) for Sulf-26.

Subsequently, histological and immunohistochemical analysis was carried out in nude mice tumours to determine the mechanisms of tumour growth in vivo of the transfected and control cells. Larger tumours in the groups of wild type and empty vector-transfected Panc-1 cells often showed signs of necrosis in central tumour areas, which was not observed in Hsulf-1-transfected cells. The gross morphological appearance was otherwise not different in transfected and control cells. Vessels in the tumour mass of the control tumours were often compressed in the cancer cells. In contrast, vessels were prominently developed in Hsulf-1-positive tumours. Moreover, analysis of the vessel density showed that tumours

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of Hsulf-1-transfected Panc-1 cells had a significant increase in vessel density, with 98.67 (28.62) vessels per unit area \( (p, 0.05) \) in Sulf-38 and 35.67 (12.25) vessels per unit area \( (NS) \) in Sulf-26 clones, compared with wild-type (19.67 (10.1) vessels) and empty vector-transfected Panc-1 cells (23.67 (4.50) vessels; fig 3A, 4A,B). Analysis of the proliferation rate, using an anti-Ki-67 (H-300) polyclonal antibody as a marker for cell proliferation, \( ^{20} \) showed a significantly lower number of proliferating cells per unit area in Hsulf-1-transfected Panc-1 cells, with 234.3 (26.5) proliferating cells per high-power field \( (p, 0.001) \) in Sulf-38 and 450.7 (73.9) proliferating cells per high-power field \( (NS) \) in Sulf-26 cells, compared with wild-type (583.0 (88.68) cells) and empty vector-transfected cells (621.3 (32.52) cells; fig 3B, 4C,D). Immunohistochemical analysis of tumours, using a monoclonal antibody against sulphated HSPGs, showed somewhat more pronounced expression in Hsulf-1-positive tumours compared with Hsulf-1-negative ones. However, the expression was mainly in the extracellular matrix in both Hsulf-1-positive and Hsulf-1-negative tumours (fig 4E,F).

To evaluate the in vivo invasion capacity of Hsulf-1-expressing Panc-1 cells, an orthotopic tumour model was used. One Hsulf-1-positive clone (Sulf-38) and one control clone were analysed. One month after implantation, tumours were found in two of three mice for both groups. One Sulf-38

Figure 1 In vitro evaluation of the invasion and adhesion capacity of Hsulf-1-transfected Panc-1 cells. (A) An in vitro cell invasion assay was carried out using 8-μM filters coated with Matrigel, as described in Materials and methods. Panc-1 cells (1.25×10⁵ cells/well) were seeded to the filters in 1% serum overnight and invaded cells were counted. The values shown are the mean (SD) obtained from four independent experiments. (B) An in vitro cell adhesion assay was carried out as described in Materials and methods. Panc-1 cells (5×10⁴ cells/well) were seeded on the Matrigel-coated surface and incubated in a humid chamber. Adherent cells were manually counted at the indicated time points. Values shown are the mean (SD) obtained from three independent experiments. EV, empty vector; WT, wild-type.

Figure 2 In vivo subcutaneous tumour growth of wild Hsulf-1-transfected Panc-1 pancreatic cancer cells in nude mice. In vivo tumorigenicity was carried out as described in Materials and methods. The mean of the tumour volume was calculated from measurable tumours at each time point. Data are derived from three independent experiments and are presented as the mean tumour volume (SD). EV, empty vector; WT, wild-type.

Figure 3 Effects of Hsulf-1 expression on vessel density and cell proliferation rate in subcutaneous nude mouse tumours. (A) Vessel density of tumours grown in vivo was immunohistochemically analysed as described in Materials and methods. Data are expressed as the mean number of proliferating cells per high-power field (SD). Values shown were obtained from three independent experiments. EV, empty vector; WT, wild-type.
tumour appeared necrotic, with local abscess formation. However, there was one liver metastasis in this mouse. In the other Sulf-38 mouse, there were no metastases, and the size of the primary tumour was 6.6 mm$^3$. In the control mice, there was no metastatic disease, and the size of the tumours was 9.4 mm$^3$ and 8.7 mm$^3$, respectively. Next, immunohistochemical analysis was carried out using a monoclonal anti-human epithelial membrane antigen antibody as a marker for human epithelial (cancer) cells. This analysis showed a well-developed fibrotic capsule surrounding Hsulf-1-negative tumours, thus preventing direct contact between cancer cells and mouse pancreatic tissue (fig 5A). Although Hsulf-1-positive tumours also developed this fibrotic capsule, there were areas of local invasion into the surrounding pancreatic tissue (fig 5B). Analysis of the liver metastasis of the Hsulf-1-positive tumour showed a rim of viable tumour cells surrounding the necrotic area in the centre of the metastasis (fig 5C).

We further analysed sulphated HSPG expression in pancreatic cancer, pancreatic cancer metastasis and normal pancreatic tissues using the same monoclonal anti-heparan sulphate antibody. Immunohistochemical staining showed high expression of sulphated HSPGs in the basal membranes and the extracellular matrix of pancreatic tissues. In the normal pancreas, sulphated HSPGs were mainly seen around the acinar complexes and pancreatic islets, as well as in the walls of the vessels (fig 6A,C). Pancreatic cancer tissues showed staining mainly in the basal layer of the ductal structures and in desmoplastic areas (fig 6D–F). Vessels of pancreatic cancer metastasis samples also showed positive staining; however, the metastatic cells themselves were devoid of specific staining (fig 6G).

**DISCUSSION**

HSPGs are cell surface and extracellular matrix macromolecules that consist of a core protein to which heparan sulphate glycosaminoglycan chains are attached. Cell surface HSPGs are linked to the plasma membrane by a glycosylphosphatidylinositol linkage or a transmembrane domain and regulate interactions between cells and their surrounding environment. These interactions have an important role in several biological processes, such as cell recognition, adhesion, migration and growth. Therefore, HSPGs have a major effect on the maintenance of the cell phenotype and neoplastic development.

Hsulf-1 is a newly identified member of the sulphatase family, which exhibits arylsulphatase activity and removes sulphate from the C6 position of glucosamine in the specific subregions of intact heparin. Hsulf-1 can influence the sulphation state and the biological function of HSPGs. Sulphation of HSPGs has been found to have a pivotal role in heparin-binding growth factor signalling. Several studies
have shown that desulphation of HSPGs by Hsulf-1 negatively influences this signal transduction pathway. Recently, we showed a significant up regulation of Hsulf-1 in primary pancreatic cancer and analysed the role of Hsulf-1 in modulating heparin-binding growth factor signalling in Panc-1 pancreatic cancer cells stably transfected with Hsulf. Hsulf-1-expressing Panc-1 cells showed decreased fibroblastic growth factor-2-induced growth and invasion.

In this study, we investigated the basal invasion and adhesion capabilities of Hsulf-1-expressing Panc-1 cells in comparison to control cells. Interestingly, the invasion capacity of Hsulf-1-positive Panc-1 cells was considerably higher than that of Hsulf-1-negative cells, indicating that desulphation of HSPGs results in increased invasiveness. This suggested an important role of the sulphation state of HSPGs in cell–extracellular matrix interaction. These findings were confirmed by using an orthotopic nude mice tumour model in which increased local invasion and liver metastasis were observed in Hsulf-1-positive tumours. Further studies using different cell lines and different tumour models are required, however, to firmly establish the role of Hsulf-1 in cancer cell invasion.

Hsulf-1 expression and the desulphation of HSPGs were shown to result in a growth disadvantage in different types of tumour cells. These findings were supported by our previous results. However, the exact biological mechanisms and the effect of Hsulf-1 on cancer cells in vivo are poorly understood. In addition, the in vitro observation of a growth disadvantage was in striking contrast with the in vivo findings of a significant up regulation of Hsulf-1 in pancreatic cancer tissues. Therefore, in our study, we investigated the growth of Hsulf-1-transfected Panc-1 pancreatic cancer cells in vivo in nude mice. Hsulf-1-expressing cells exhibited a decreased ability to form tumours compared with Hsulf-1-negative cells. Moreover, there was an inverse correlation between the sulphatase activity of the Hsulf-1-positive Panc-1 cells and their potential to grow in nude mice. Tumours of transfected cells showed a better developed and better organised extracellular matrix, with considerably increased vessel density, probably due to slower growth of the tumour. In line with this, the proliferation rate of the tumours of the transfected cells was considerably lower than that of Hsulf-1-negative tumours. Interestingly, this observation is in agreement with a recent study in myeloma tumours showing that Hsulf-1 and Hsulf-2 promote an increase in extracellular matrix deposition in tumours and a reduction in tumour growth. It can be hypothesised that a major factor of reduced growth ability of Hsulf-1-positive tumours is a reduction of heparin-binding growth factor signalling, as suggested previously. Pancreatic cancer is characterised by enhanced expression of a variety of growth factors that positively influence the proliferation of malignant cells. The sulphation status of cell-surface HSPGs seems to be an important mediator in this interaction.

In a recent study, we showed decreased expression of sulphated cell-surface HSPGs in Hsulf-1-overexpressing pancreatic cancer cells. In this study, we sought to analyse the sulphated HSPG expression status in Hsulf-1-positive and Hsulf-1-negative tumours in nude mice. Interestingly, we found prominent expression of sulphated HSPGs in Hsulf-1-positive tumours compared with controls. This was mainly owing to the pronounced presence of extracellular matrix component structures in these tumours. The epitope against which the monoclonal anti-heparan sulphate antibody was raised includes N-sulphated glucosamine residues, which are present in many types of heparan sulphate. Therefore, expression of heparan sulphate chains, which are abundantly present in the basal lamina and its surrounding interstitial matrix, was easily detectable. Thus it was difficult to distinguish cellular expression of sulphated HSPGs between Hsulf-1-positive and control cells in vivo. Of course, partial desulphation of cell-surface HSPGs by Hsulf-1 may also be responsible for this discrepancy. Analysis of pancreatic ductal adenocarcinoma tissues, which show high levels of Hsulf-1,
gave similar results. Thus, there was high expression of sulphated HSPG, with dominant localisation in extracellular matrix component elements, which are widely present in these neoplastic tissues.

Pancreatic cancer cells overexpress Hsulf-1 and also frequently show enhanced levels of two major members of the membrane-bound proteoglycan family, syndecan-1 and glypican-1,12 13 which are constant carriers of heparan sulphate. In addition, other proteoglycans, which can bear heparan sulphate chains in some proportion and under some conditions, such as CD-44,21 may also have some role in the localisation of heparan sulphate in pancreatic cancer. It is known that cell-surface HSPGs can be shed from the cell surface as soluble molecules, particularly in ligand non-responsive cells.21 As Hsulf-1-expressing cancer cells show changes in ligand–receptor interactions, it can be hypothesised that this mechanism is responsible for the high extracellular expression of sulphated HSPGs in pancreatic cancer tissues. It can therefore be speculated that the increased invasion ability of Hsulf-1-expressing cells is not directly promoted by desulphation of heparan sulphate residues, but rather is promoted by the consequent loss of HSPGs. The near absence of sulphated HSPGs in metastatic pancreatic cancer cells supports this hypothesis, whereas the

Figure 6 Localisation of sulphated heparan sulphate proteoglycans in pancreatic tissues. Immunohistochemical analysis was carried out as described in Materials and methods using a monoclonal anti-heparan sulphate antibody. (A–C) normal pancreas, (D–F) pancreatic cancer, (G) liver metastasis of pancreatic cancer. Note the absence of staining in the control section (B) compared with the positive section (A).
surrounding liver tissue shows high-sulphated HSPG expression. As it is evident that the sulphation state of HSPGs modulates the invasiveness of cancer cells, and as the expression of sulphated HSPGs in metastasis is markedly downregulated compared with primary cancer tissues, it can be speculated that the complete desulphation of HSPGs in cancer cells appears only in the process of invasion. With the use of increased numbers of samples and more specific analysis of these cell-surface proteins and their oligosaccharide residues, we may be able to show the intricacies of this process better.

In conclusion, enhanced endogenous levels of Hsulf-1 control the tumorigenicity of pancreatic cancer cells. Hsulf-1-mediated desulphation of cell-surface HSPGs negatively influences the proliferation capacity of Panc-1 pancreatic cancer cells, thereby reducing their ability to grow. However, overexpression of this gene in cancer cells contributes to increased invasiveness of these cells, which is obviously not mediated by heparin-binding growth factors. Considering the specific characteristics and differences of cultured pancreatic cancer cell lines and primary cancer cells, the exact function of Hsulf-1 in vivo is not known. None the less, our results further support the assumption that Hsulf-1 has a specific role in pancreatic cancer, with the ability to manipulate pancreatic cancer cell growth and invasion.

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