Heparanase expression is a prognostic indicator for postoperative survival in pancreatic adenocarcinoma

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Pancreatic ductal adenocarcinoma has a poor prognosis due to its aggressive biological behaviour. In the majority of cases, diagnosis is only established late in the course of the disease, when local invasive growth and distant metastasis have already occurred (Baumel et al, 1994; Murr et al, 1994; Robert Koch Institut, 2002). At that point, the opportunity for curative therapy, which is surgery at an early stage, has already passed. Even though complete resection may still be achieved, the rate of relapses is high (Connolly et al, 1987; Trede et al, 1990; Cameron et al, 1991). Adjuvant combination therapy may offer a small survival advantage but is associated with treatment related morbidity. It is therefore not unequivocally accepted (Kalser and Ahlgren, 1985; Ahlgren, 1985; Jekel, 1997; Klinkenbijl et al, 1999). The cytotoxic agent gemcitabine is the only drug currently known to be effective for palliation. Nevertheless, the absolute gain in survival is marginal (Burriss et al, 1997). A number of markers have been associated with therapy-resistant tumours or shorter survival. Their validity as prognostic criterion remains to be confirmed, though (Compton et al, 1997).

On the molecular level, several events including proto-oncogene mutations such as K-ras (Almoguera et al, 1988), tumour suppressor gene mutations in the p16INK4a, DPC4, and p53 genes (Berrozpe et al, 1994; Caldas et al, 1994; Redston et al, 1994; Schutte et al, 1997; Goggins et al, 1998), and overexpression of a variety of growth factors and their receptors (Korc, 1998) have been described in the carcinogenesis of pancreatic cancer. The underlying causes for the aggressiveness of this cancer are still not fully understood.

Invasion of tumour cells into the surrounding tissue requires loosening of cell–cell adhesion, invasion of the basement membrane and disassembly of the extracellular matrix. Chief components of basement membrane (BM) and extracellular matrix (ECM) such as collagens, fibronectin and laminin are substrates of metalloproteinases and cysteine and serine proteases, which are known to be upregulated in metastatic cancers (Chambers and Matrisian, 1997).

Recently, another ECM degrading enzyme, mammalian heparanase, has been cloned from human placenta tissue and platelets and a putative 65 kDa precursor and a 50 kDa recombinant active enzyme have been expressed. Preliminary results suggest that heparanase may be a cell membrane protein (Hulett et al, 1999; Vlodavsky et al, 1999). The enzyme cleaves another key component of BM and ECM, namely heparan sulphate proteoglycans (HSPG) which belong to the glycosaminoglycans. Heparanase expression and function may be regulated at the transcriptional and posttranscriptional level (Gilat et al, 1995; Andel et al, 2000), though this topic awaits further clarification.

In addition to the enzymatic degradation of the ECM heparanase acts by releasing growth factors such as bFGF, heparan sulphate (HS) fragments and other enzymes such as lipoprotein lipase from the ECM. Consistent with its molecular capabilities heparanase has been shown to stimulate wound healing and tumour angiogenesis in vivo (Elkin et al, 2001). Its expression is upregulated in a number of metastatic tumour cell lines and transfection of tumour cells with heparanase cDNA conferred a highly metastatic phenotype (Hulett et al, 1999; Vlodavsky et al, 1999).
Heparanase expression has been studied in human primary tumours and tumour cell lines. Its expression correlates with tumour progression and invasiveness in colorectal and hepatocellular carcinoma (Friedmann et al., 2000; El-Assal et al., 2001). In this study, we examined the expression of heparanase protein and mRNA in human PDA cell lines and tumours. In addition, we studied the correlation of heparanase expression and stage of disease with postoperative survival in the intent to identify putative prognostic markers.

MATERIALS AND METHODS

Cell Culture and DNA transfection

Eight pancreatic cancer cell lines (AsPC-1, BxPC-3, Capan-1, Hs 766T, PANC-1, HPAF-II, CFPAC, MIA PaCa-2), the colorectal cancer cell line HT29 and the cervical cancer cell line HeLa were obtained from American Type Culture Collection (Manassas, USA) and maintained in the recommended growth conditions. Transfection for overexpression of Heparanase in HeLa cells was performed on cells growing at ~70% confluence in T75 plates, using full-length Heparanase cDNA in pcDNA3 (Vlodavsky et al., 1999) and FuGENE 6 transfection reagent (Roche, Germany) according to the manufacturer’s instruction.

RNA-isolation and semiquantitative RT – PCR

RNA was isolated from cell lines with the RNeasy Kit (Qiagen, Germany) according to the manufacturer’s instructions. After reverse transcription of 5 μg total RNA by oligo(dT) priming (Superscript, Invitrogen, USA), the resulting cDNA was amplified by PCR using Taq DNA polymerase (Qiagen, Germany) and the heparanase primers 5’-GTGATGCAAGCAGCAACTTTG and 5’-TC AGATGCAAGCAGCAACTTTG. The PCR conditions were an initial denaturation of 5 min at 94°C and subsequent denaturation for 30 s at 94°C, annealing for 30 s at 58°C and extension for 1 min at 72°C (26 cycles). Standard β-actin primers and identical PCR conditions were used as internal input controls. PCR products were separated by 1.5% agarose gel electrophoresis and visualised by SYBR Green staining (Biozym Diagnostik, Germany).

Western blot analysis

Cells were dissolved in lysis buffer containing 5 m NaCl, 10% NP-40 (Igepal CA-630, Sigma-Aldrich, Germany), 1 m TRIS pH 8 and protease inhibitors (Complete Mini, EDTA free, Roche, Germany) at 4°C for 45 min and centrifuged at 13 000 r.p.m. for 20 min. The protein concentration of the supernatant was measured by the Bradford assay (BioRad, USA). 50 μg of each protein was separated by electrophoresis on 10% SDS/polyacrylamide gels and transferred to Hybond-P-Membrane (Amersham Pharmacia Biotech, UK) by semidyed electroblotting (Transblot, BioRad, USA). Western blot analysis was carried out with a mouse monoclonal anti-heparanase antibody as previously described (Vlodavsky et al, 1999). A monoclonal antibody against β-actin (Sigma-Aldrich, USA) was used to verify equal loading of western blots. The secondary antibody used was a horseradish-peroxidase-conjugated goat-anti-mouse antibody (Pierce, USA). Immunoreactive bands were detected by enhanced chemiluminescence (SuperSignal, Pierce, USA) and exposure in a luminous image analyser (LAS-1000plus, FujiFilm, USA).

Patients and tissue samples

Tissue specimens were chosen from 25 patients with chronic pancreatitis, from 50 patients with ductal adenocarcinoma of the pancreas undergoing surgery of curative intent between 1995 and 1999 and from five cases with normal pancreatic tissue. The PDA patients included 26 patients, in whom the tumours were R0 resected. Each tumour was re-evaluated with regard to typing, staging and grading. Tumour typing and staging were performed using WHO (Kloppel et al, 2000) and UICC (Sobin and Wittekind, 1997) criteria.

Immunohistochemistry

2 – 4 μm sections of formaline-fixed and paraffin-embedded specimens were deparaffinised and rehydrated. Endogenous peroxidase was blocked by 3% H2O2. Tissue was then demethylated in a microwave oven, incubated with citrate buffer, and blocked with goat serum. Sections were incubated with biotinylated goat anti-mouse IgG+IgM antibodies (BioGenex, USA), rinsed with washes with PBS sections were incubated with biotinylated goat anti-mouse antibody as previously described (Vlodavsky et al, 1999) or with non-immune mouse serum for negative control, respectively. Following washes with PBS sections were incubated with biotinylated goat anti-mouse IgG+IgM antibodies (BioGenex, USA), rinsed with PBS and incubated with peroxidase-labelled streptavidin. Colour was developed using Sigma Fast 3,3′-Diaminobenzidine (Sigma-Aldrich, USA). Counterstaining was performed with Mayer’s haematoxylin. Samples were evaluated by microscopy and semiquantitatively analysed for heparanase expression (intense, moderate or absent staining).

Riboprobe preparation and in situ hybridisation

A 482 bp fragment of the human heparanase cDNA (primers identical to RT – PCR primers) was subcloned into the pGEMT vector multiple cloning site (Promega, USA). The linearized vector was used as a template for in vitro transcription and digoxigenin labelling of antisense or sense riboprobe using SP6 and T7-RNA polymerase (Roche, Germany). In situ hybridisation was performed as described before (Vlodavsky et al, 1999; Friedmann et al, 2000). Briefly, 5 μm sections were dewaxed and rehydrated, denatured with 0.2 N HCl for 10 min and then digested with protease K (200 μg ml−1) at 37°C for 30 min. Digestion was stopped with two changes of H2O. Slides were prehybridised and hybridised as described (Vlodavsky et al, 1999; Friedmann et al, 2000). Probe concentration was 2 μg ml−1. Washes after hybridisation, incubation with anti-digoxigenin antibodies, and calorimetric detection were performed as described before (Vlodavsky et al, 1999; Fried-

Table 1 Clinical and histological characteristics of 50 patients with pancreatic cancers

| Heparanase expression | Yes (%) | No (%) | Chi square |
|----------------------|--------|-------|------------|
| UICC stage           |        |       |            |
| I                    | 4 (80) | 1 (20) |             |
| II                   | 5 (59) | 5 (50) | P=0.17     |
| III                  | 23 (85)| 4 (15) | n.s.       |
| IV                   | 6 (75) | 2 (25) |            |
| Lymph node metastasis|        |       |            |
| No                   | 11 (61)| 7 (39) | P=0.06     |
| Yes                  | 27 (84)| 5 (16) | n.s.       |
| Distant metastasis   |        |       |            |
| No                   | 32 (76)| 10 (24)| n.s.       |
| Yes                  | 6 (75) | 2 (25) |            |
| Grading              |        |       |            |
| 1                    | 2 (100)| 0 (0) | P=0.61     |
| 2                    | 27 (77)| 8 (23)| n.s.       |
| 3                    | 9 (69) | 4 (31) |            |

Correlation analysis of heparanase expression (total positive=38, total negative=12) with staging (UICC), lymph node and distant metastases and histological grading (1 well, 2 moderately, 3 poorly differentiated carcinoma) by Chi square test.
Heparanase expression in human pancreatic cancer cell lines

We examined eight human pancreatic cancer cell lines from different tissues of origin for the expression of heparanase mRNA and protein. Four of the cell lines (CFPAC, AsPC-1, HPAF-II, and Hs 766T) had been established from distant metastases (liver, ascites) and lymph node metastases, respectively. Heparanase was expressed in all cell lines, but could hardly be detected in two cell lines (BxPC-3, Hs 766T) as determined by Western blot (Figure 1A). The level of expression varied widely. Apparently, heparanase expression did not correlate with whether or not the cell line was derived from a metastatic site. We also looked for levels of mRNA expression by quantitative RT–PCR. Levels of mRNA correlated closely with protein expression in the tumour cell lines examined (Figure 1B).

Heparanase expression in human pancreatic cancer tissue

We analysed sections of tumour tissue from 50 patients (mean age 63 years, range 41–77 years, 27 male/23 female) that were operated for pancreatic cancer and from 25 patients with chronic pancreatitis. Of the 50 tumour patients, 26 underwent a potentially curative resection (R0 resection). The colorectal cancer cell line HT-29 and the cervical cancer cell line HeLa transfected with heparanase cDNA were used as positive controls. Untransfected HeLa cells were used as negative control [3]. β-Actin Western blot to confirm equal protein loading. (B) Semiquantitative RT–PCR of heparanase mRNA in human pancreatic cancer cell lines. DNA ladder [1], water control [2], AsPC-1 [3], BxPC-3 [4], Capan-1 [5], CFPAC [6], HPAF-II [7], Hs 766T [8], MIA PaCa-2 [9] and PAN-ε-1 [10].

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angiogenesis and cancer metastasis (Vlodavsky and Friedmann, 2001). Heparanase is expressed by a variety of cell types and helps the cells to cross physiological boundaries, as is the case with trophoblasts in the placenta, leukocyte extravasation, or tumour cell metastasis (Parish et al, 2001). There are as yet few identified molecular mechanisms of heparanase regulation. These comprise transcriptional and posttranscriptional mechanisms such as tissue-pH and regulation by NF-κB and proto-oncogenes (Schwarz et al, 1990; Gilat et al, 1995; Andela et al, 2000). In our study expression of heparanase mRNA and protein in vivo showed significant overlap. Transcriptional upregulation therefore appears to be the main way of PDA cells to benefit from the prometastatic and angiogenic effects of heparanase.

The key role of heparanase in tumorigenesis and the existing evidence for only one endogenous mammalian heparan sulphate degrading endoglycosidase (Hulett et al, 1999; Vlodavsky et al, 1999) has spurred efforts to develop therapeutic applications. So far, non-anticoagulant heparins and sulphated oligosaccharides show promising results in animal studies of tumour spreading (Miao et al, 1999; Parish et al, 1999). A drug of the latter group, Phosphomannopentaose sulfate (PI-88), slowed tumour growth and metastasis and reduced the vascularity of tumours both in vitro

**Figure 2** Heparanase expression and localisation in primary human pancreatic cancers (A,D,G), chronic pancreatitis (B,E) and histologically normal pancreas (C,F). HE staining (A,B,C) for histological evaluation. Immunohistochemical staining with a monoclonal α-heparanase Ab and in situ hybridisation with an antisense (G) and sense probe (H, negative control). Normal pancreas with absent (F) and chronic pancreatitis with moderate (E) heparanase expression. Carcinoma tissue with strong heparanase expression (D,G), or absent heparanase expression (M). Heparanase positive tumour cells invading lymph sheets (I). Lymph node metastasis with strong (K) or absent heparanase expression (I).
and in vivo in animals (Parish et al., 1999). Hopefully, this and other studies will emerge into clinical trials of the most promising of these drugs.

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Figure 3 Heparanase expression in pancreatic cancers depending on lymph node metastasis (n=32) vs absent metastasis (n=18).

Figure 4 Survival curves of 26 pancreatic cancer patients with R0 resection. Kaplan–Meier plots of postoperative survival curves of patients with or without heparanase expression in the primary carcinoma. Log-rank analysis proves a shorter postoperative survival period for patients with heparanase expression in their tumours (P<0.01).
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