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ORIGINAL ARTICLE

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Expression of thrombospondin-1 in pancreatic carcinoma: correlation with microvessel density

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Abstract Thrombospondin-1 (TSP-1) is a multifunctional platelet and extracellular matrix protein that is involved in angiogenesis. Under certain pathological conditions, e.g., malignant tumors, high concentrations of TSP-1 work as an angiogenic agonist. Here we examined 98 pancreatic carcinomas with respect to TSP-1 immunoreactivity and its correlation to intratumoral microvessel density (MVD), a representation of the overall degree of angiogenesis in carcinomas. Northern blot analysis for TSP-1 mRNA was performed in seven additional cases. Eighty-seven tumors showed strong TSP-1 immunoreactivity, nine carcinomas were only weakly positive, and two lesions were negative for TSP-1. TSP-1 immunoreactivity was detected in the extracellular matrix, mostly at the invasion front of the tumor. Using Northern blot analysis, we observed high levels of TSP-1 mRNA in three out of seven pancreatic carcinomas. The mean MVD in pancreatic carcinoma was 38.8 vessels per mm^2. Tumors with a high expression of TSP-1 showed a higher MVD and the correlation between TSP-1 immunoreactivity and microvessel density was highly significant (P=0.003). As a modulator of angiogenesis, TSP-1 is strongly expressed in most pancreatic adenocarcinomas and is likely to contribute to the extensive neovascularization and spread of this highly aggressive tumor.

Keywords Pancreas · Carcinoma · Thrombospondin-1 · Angiogenesis · Microvessel density · Pathology

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Introduction

Adenocarcinomas of the pancreas are aggressive tumors with a poor prognosis. In Germany, more than 11,000 patients die of this disease per year [25]. At present, the molecular biology of pancreatic carcinomas is not yet fully understood. Activation of oncogenes, such as the K-ras-oncogene, and growth factor expression [8], inactivation of tumor suppressor genes like p53, p16, DPC4, and DPC1/2, as well as DNA mismatch repair defect and microsatellite instability play an important role in the carcinogenesis and progression of pancreatic carcinomas [23].

Folkman et al. [9] suggested that a switch from the prevascular to a vascular phase is the prerequisite for the growth and spread of many solid tumors. Thus, angiogenesis contributes significantly to the progression of carcinoma. Intratumoral microvessel density (MVD) is believed to reflect the overall degree of angiogenesis in carcinomas. It is an independent prognostic indicator in several types of carcinomas, such as breast cancer [24], ovarian cancer [30], endometrial cancer [36], colon cancer [11], and lung adenocarcinoma [18]. For pancreatic cancer, such a correlation has yet to be established [26].

Thrombospondin-1 (TSP-1), a 450,000 dalton glycoprotein, is a multifunctional platelet and extracellular matrix protein that is thought to contribute to angiogenesis [29]. It is made up of three identical disulfide-linked chains [28]. Each polypeptide chain is composed of several domains interacting with different surface receptors as well as with other macromolecules such as collagens. TSP-1 is involved in diverse processes such as the regulation of cell growth, cell motility, inflammation, and wound healing. It modulates endothelial cell adhesion, motility and growth [31]. Under normal circumstances it is an inhibitor of neovascularization [13]. However, the effect of thrombospondin is concentration-dependent. At low concentrations, it inhibits angiogenesis, whereas at high concentrations it stimulates neovascularization [28]. Under certain pathological conditions, e.g. malignant tumors, the concentration of TSP-1 increases [27,34];

thus, the matrix-bound protein also works as an angiogenic agonist [28].

The purpose of the present study was to analyze the role of the TSP-1 expression in pancreatic carcinoma and to correlate these findings with MVD.

Materials and methods

Ninety-eight patients who underwent surgical resection of the pancreas for pancreatic carcinoma were examined retrospectively. There were 45 women and 53 men. The mean age was 62.1 years (range 42–81 years).

Staging was performed according to the TNM classification system as follows: There were ten patients with pT1, 78 patients with pT2, eight patients with pT3, and two patients with pT4 tumors. Sixty-one patients showed metastases in one (N_{1a}=27) ore more than one (N_{1b}=34) lymph nodes; in 11 patients, distant metastases occurred. Most of the carcinomas were ductal adenocarcinomas (91 cases). Two cases were classified as serous cystadenocarcinomas, and five cases as undifferentiated carcinoma. Three tumors were well differentiated, 38 moderately differentiated, 52 poorly differentiated, and five undifferentiated. Undifferentiated carcinomas are thought to have the same origin and similar pathways of pathogenesis as adenocarcinomas. Therefore, it seems reasonable to examine these tumors as a group. Seventy-seven tumors were localized in the head of the pancreas and 21 in the tail or body.

Immunohistochemical evaluation

Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded tissue. For TSP-1, the monoclonal mouse antibody thrombospondin (Ab-1, Cat BA18, Dianova, Hamburg, Germany) was used. Tissue sections (4 µm thick) were mounted on slides (SuperFrost plus, Mentzel, Germany), deparaffinized, and rehydrated in 100% and 95% ethanol. Endogenous peroxidase was blocked in 3% hydrogen peroxide-methanol for 30 min. Antigen retrieval was performed as described by Grossfield et al. [16]. The slides were placed in Tris-HCl buffer solution at pH 1 and heated in a microwave oven four times for 5 min at 100°C. Slides were cooled down at room temperature for 15 min. Normal horse serum was used for the blocking of non-specific binding for 20 min. The primary antibody was applied at a dilution of 1:1000 for 60 min at 37°C. Tissue sections were then incubated with biotinylated rabbit anti-mouse antibody, and the reaction was visualized using the ABC-complex (Elite PK6100, Vector, Burlingame, Calif. USA) and DAB (Serva, Heidelberg, Germany). Sections of invasive urothelial carcinoma and skin were used as positive controls. The extracellular TSP-1 immunoreactivity was graded semiquantitatively as low, moderate, or high as described by Grossfield et al. [15].

Microvessels were visualized immunohistochemically using the monoclonal mouse IgG anti-CD34 antibody (Biogenex, San Ramon, Calif. USA), which has been shown to be a sensitive and reliable marker of MVD [24]. We followed the instructions of the manufacturer. Briefly, 4-µm-thick tissue sections were mounted on slides (SuperFrost plus, Mentzel, Germany), deparaffinized in xylol and rehydrated in 100% and 96% ethanol. Endogenous peroxidase was blocked with 3% hydrogen peroxide. The staining procedure was automated, including blocking with H_2O_2 and detection with DAB (Ventana, Strassbourg, France).

To measure intratumoral MVD, the stained sections were screened without any knowledge of the patients data at 50× magnification to identify the highest vascular area within the tumor. In this hot spots, the individual microvessel count was evaluated at 200× magnification using an square ocular grid. Any cluster of cells stained by CD 34 and clearly distinguishable from the background was counted as a vessel. Branching structures were counted as a single vessel. Vascular counts for each case were calculated as the mean and median of ten fields and are expressed as the number of microvessels per square millimeter. This procedure is based on a method described by Martin et. al. 1997 [24] and Vermeulen et al. 1996 [35].

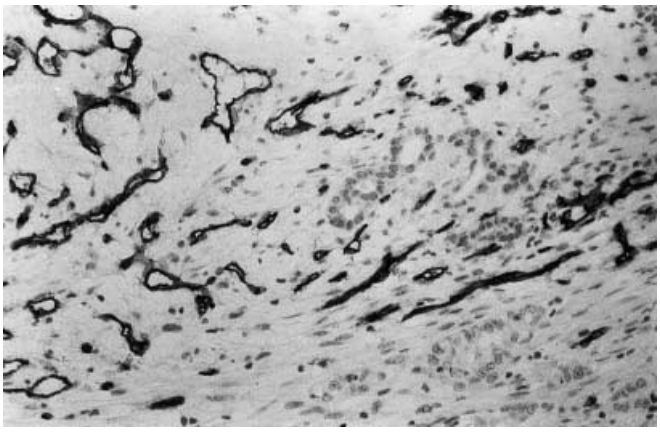

Fig. 1 Immunohistochemical staining of endothelial cells with CD34 (×200) in a case of moderately differentiated adenocarcinoma of the pancreas showing a high microvessel density

Northern blot analysis

Six specimens of normal pancreatic tissue and seven pancreatic carcinoma specimens were obtained immediately postoperatively, snap frozen in liquid nitrogen and stored at –80°C. These samples were taken from patients different from those examined in the immunohistochemical studies. Total RNA was extracted size-fractionated, and blotted onto nylon membranes as described previously [7]. The blot was prehybridized for 1 h at 42°C in a prehybridization buffer that contained 50% formamide, 0.1% SDS (Sigma, Taufkirchen), 5×SSC (Sigma), 2.5×Denhardt's (Sigma), 250 µl salmon sperm DNA, and 50 mM Na_2PO_4, pH 6.5. The blot was hybridized at 44°C for 16 h with labeled cDNA washed twice at 54°C in 1×SSC/2% SDS and twice at 56°C in 0.2×SSC/2% SDS. The blot was exposed at –80°C to a Kodak XAR-5 film with Kodak intensifying screens (Ultrascan XL; Pharmacia LKB Biotechnology, Uppsala, Sweden).

A 1.1-kb Eco/ RI fragment of human TSP-1/ cDNA was used for hybridization. Furthermore, a 190-base pair BamHI fragment of the mouse 7S cDNA [3] that cross hybridizes with human 7S RNA was used to verify equivalent RNA loading. All cDNA probes were randomly primer-labeled with dCTP [32].

Statistical analysis

The Mann-Whitney's U rank sum test was used to compare TSP-1 immunoreactivity and MVD. The Kruskal-Wallis analysis was used to examine the correlation between MVD and various clinicopathological data. A p-value $P \leq 0.05$ was considered statistically significant. All analyses were performed using SPSS for Windows software (SPSS, Chicago, Ill, USA).

Results

Microvessel density

The mean MVD in the highest vascular area of pancreatic carcinoma was 38.8 vessels per mm^2, with a range from 16.9 to 447.5 vessels per mm^2 (Fig. 1). The median MVC was 33.8 vessels per mm^2. In the surrounding non-neoplastic tissue, the MVD count was 16.9 vessels per

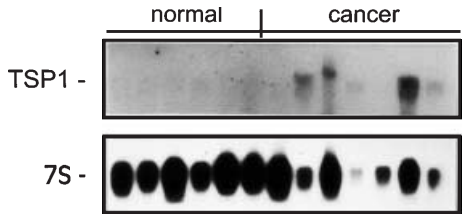

Fig. 2 Northern blotting of pancreatic mRNA. Three of seven pancreatic carcinoma samples show a band of approximately. 6.0 kb corresponding with high levels of TSP-1 mRNA. All normal control tissues are negative

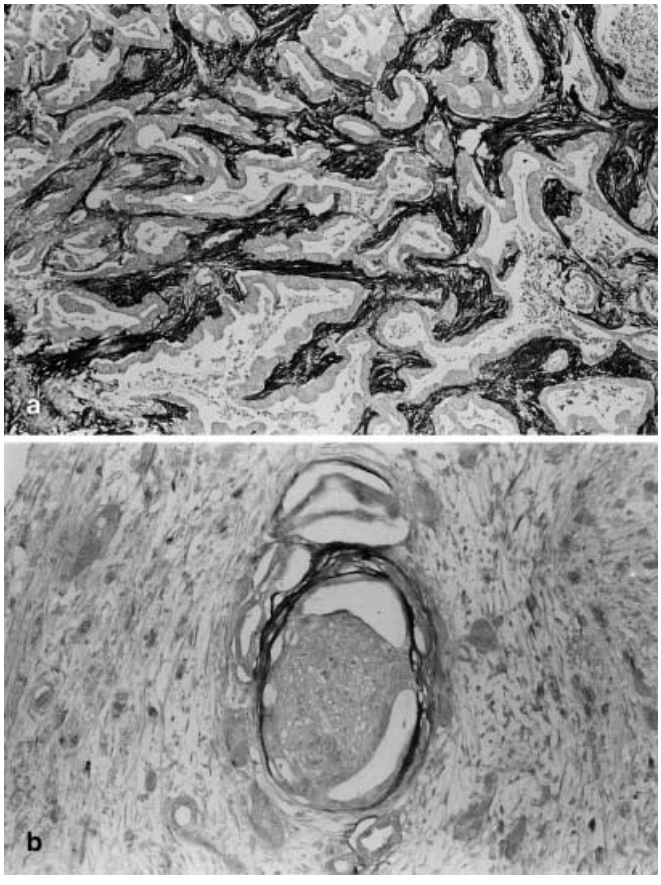

Fig. 3a, b Immunohistochemical staining for TSP-1 in pancreatic adenocarcinoma. **a** High TSP-1 expression is present in the desmoplastic tumor stroma. Epithelial cells are negative for TSP-1. **b** Tumor growth in a nerve sheet showing high TSP-1 expression in the peritumoral stroma tissue

mm^2. A distinction between preexisting capillaries and newly formed microvessels induced by the tumor was not possible. MVD did not correlate with tumor stage, histological grade, location within the pancreas, lymph node metastases, distance spread, or age or sex of the patient.

Thrombospondin-1

Using Northern blot analysis of total RNA, we observed a product of approximately 6kb in three of seven pancreatic carcinomas. This corresponds with high levels of TSP-1 mRNA. In normal pancreas, TSP-1 mRNA was always below the level of detection (Fig. 2).

Eighty-seven tumors showed strong TSP-1 immunoreactivity, nine carcinomas were only weakly positive, and two lesions were negative for TSP-1. Immunoreactivity for TSP-1 was detected in the extracellular matrix, mostly at the invasion front of the tumor (Fig. 3) and in blood vessels. The epithelium was negative.

TSP-1 immunoreactivity did not correlate with tumor stage, histological grade, location within the pancreas, lymph node metastases, distant spread, or age or sex of the patient.

Correlation between TSP-1 and microvessel density

The areas of greatest MVD were located throughout the carcinoma while the most intense TSP-1 immunoreactivity was located at the adjacent desmoplastic invasion front of the tumor. For statistical evaluation, MVD was classified as above or below the sample median. Tumors with a high expression of TSP-1 showed a higher MVD and the correlation between TSP-1 immunoreactivity and MVD was highly significant (P=0.003).

Discussion

Several lines of evidence suggest that tumor growth and metastatic spread depend on angiogenesis. Only angiogenic tumors seem to be capable of growing beyond a certain size [26]. Angiogenesis is a complex process regulated by soluble glycoproteins such as the fibroblast growth factor (FGF), the platelet-derived growth factor (PDGF), the vascular endothelial growth factor (VEGF), and the transforming growth factor beta (TGFβ)12.

TSP-1, which has been shown to play a role in angiogenesis, can function both as an inhibitor and as a promotor of neovascularization. At low concentrations, TSP-1 can inhibit the bFGF-induced endothelial cell migration [32]. Taraboletti et al [31] demonstrated a dose-dependent stimulating effect of TSP-1 on the motility of endothelial cells. Under pathological conditions, e.g., malignant tumors, the concentration of TSP-1 increases [27]. Thus, it is thought that TSP-1 is a pro-angiogenic modulator in malignant neoplasms [28].

TSP-1 is also a major activator of TGFβ_1 in vivo. Crawford et al. showed that similar lung and pancreas pathologies occur in TGFβ_1 null mice and in wild-type animals, with blocking of the TSP-1 dependent activation of TGFβ_1 [6]. Both TSP-1 and TGFβ_1 have been shown to mediate pancreatic tumor cell invasion through upregulation of the plasminogen/plasmin system [1]. Increased TSP-1 protein may act with selectively expressed TSP-1 receptors on tumor cells and endothelial cells, whereas CD36 is the main receptor [28].

We were able to demonstrate strong TSP-1 protein expression in the majority of pancreatic carcinomas. We

showed immunohistochemically that TSP-1 is expressed in the extracellular matrix of the desmoplastic tumor stroma. By in situ hybridization, Cramer et al. also observed a strong TSP-1 mRNA expression in mesenchymal cells surrounding tumor islands [5]. By Northern blotting we demonstrated high mRNA levels in three of seven additional pancreatic carcinomas, whereas in the normal pancreas TSP-1 mRNA was always below the level of detection. The fact that the fraction of carcinomas with high levels of TSP-1 mRNA was lower than that of tumors with TSP-1 immunoreactivity most likely reflects a lower level of sensitivity of Northern blotting or, perhaps, mRNA degradation in pancreatic tissue, which is rich in lytic enzymes. The different size of the TSP-1 mRNA in our Northern blot analysis may be due to altered splicing of TSP-1. Since the cases used for Northern blot analysis were different from the tissue used for immunohistochemistry, a comparison between the level of TSP-1 mRNA and protein expression was not possible.

Because pancreatic carcinoma is usually detected at an advanced stage, we were able to obtain only ten tumors at an early stage. All of these showed high levels of TSP-1 expression. However, we were not able to examine very early lesions before the induction of tumor vascularization or carcinoma in situ, which could provide further information about the role of TSP-1 in the vascularization of pancreatic carcinoma.

Recent studies suggest that TSP-1 may play an important role in many malignant tumors. Albo et al. demonstrated that TSP-1 promotes the invasion of pancreatic and breast carcinoma cells in vitro [1,2]. Patients with metastatic breast, lung or gastrointestinal cancer show higher plasma levels of this protein than do normal controls or patients with non-metastatic disease [34]. TSP also stimulates tumor metastasis in an animal model [33]. In our study, there was no correlation between TSP-1 expression and stage or grade of the carcinoma, a finding that is most likely due to the fact that early pancreatic cancer is often asymptomatic, and late tumor stages are usually not treated by surgery [21]. Therefore, there is a predominance of pT2 and pT3 tumors in our samples. The effect of thrombospondin could also be tissue-specific, because in urothelial cancer [16] and in melanoma [14], decreased levels of TSP-1 were associated with the metastatic phenotype.

The strongest expression of TSP-1 was seen at the invasion front of the tumor. Possibly, TSP-1 promotes tumor invasion. Yabkowitz et al. demonstrated TSP-1 mediated chemotaxis in metastatic squamous carcinoma cells, but not in non-metastatic tumor cell lines [38]. TSP-1 can upregulate the plasminogen-activator system [1], which is one of the proteolytic systems important for the invasion of malignant tumors.

There is growing evidence that the level of tumor angiogenesis is linked to the risk of metastases and thus to survival [37]. In histological sections, the degree of neovascularization can be determined morphometrically using antibodies to endothelial cell antigens such as CD31, CD34 and factor-VIII-related antigen. Anti-CD34 has been shown to be the most reliable and sensitive antibody for this purpose [24]. We found a mean MVD value of 38.8 vessels per mm^2 in tumor sections, as compared with 16.9 in normal pancreatic tissue. A differentiation between the original capillaries and neoangiogenetic microvessels can not be made. Survival data were not available. In our cohort, there was no correlation with parameters that have been shown to be linked to survival such as TNM stage and histological grade.

However, there was a strong correlation between MVD and TSP-1. Tumors with higher MVD showed an increased TSP-1 expression. In addition, there was a close proximity of MVD hot spots and TSP-1 immunoreactivity. This suggests that a high TSP-1 level in the extracellular matrix of the desmoplastic stroma increases tumor angiogenesis. Bertini et al. observed the same correlation at the mRNA level in invasive ductal breast carcinomas [4]. Our study is in concordance with the known pro-angiogenetic potential of higher concentrations of TSP-1. This interaction most likely constitutes one step in the complex process of angiogenesis in pancreatic adenocarcinoma. In contrast, cholangiocarcinoma of the liver is a relatively hypovascular tumor that also shows increased TSP-1 mRNA levels and increased protein expression [19]. This suggests that the function of TSP-1 may be tissue specific.

The interaction of thrombospondin and VEGF has not yet been fully understood. TSP expression is enhanced by the product of the p53 gene and is downregulated by p53 alteration [10]. A regulatory activity of p53 on VEGF is also documented [20]. In colorectal cancer Maeda et coworkers demonstrated an inverse expression of TSP-1 and VEGF with TSP-1 positive and VEGF negative cases showing a low MVD [22]. For pancreatic carcinoma correlation between VEGF expression and MVD is also described [12,17]. To understand this relationship additional investigations will be necessary.

In summary, TSP-1, a modulator of angiogenesis, is strongly expressed in the desmoplastic stroma of pancreatic carcinomas and may contribute to the extensive neovascularization and spread of this aggressive tumor.

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