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# Localisation of hyaluronate (HA) in primary tumors and nude mouse xenografts of human pancreatic carcinomas using a biotinylated HA-binding protein

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**Abstract** A biotinylated hyaluronate (HA)-binding protein isolated from bovine cartilage was used to analyze the distribution of HA in nude mouse xenografts derived from human pancreatic adenocarcinoma cell lines as well as in primary human pancreatic adenocarcinomas. The most reproducible results for the localisation of HA were obtained using cryostat sections. When the biotinylated HA-binding protein was applied to histological sections of nude mouse xenografts, the specific staining found could be inhibited by preincubating the HA-binding protein with an excess of HA or by hyaluronidase treatment of the tissue before staining. The highest HA concentration was found at the tumor boundaries, while in the central part of the tumor staining was slight or absent. In cryostat sections of primary tumors HA was found predominantly in the connective tissue immediately around tumor cells or at the border between the tumor and normal pancreatic tissue.

**Key words** Hyaluronate · HA-binding protein
Pancreatic adenocarcinoma · Metastasis · Invasion

## Introduction

Human pancreatic adenocarcinomas of ductular origin, which account for 80–90% of all malignant non-endocrine pancreatic tumors, are amongst the ten commonest malignant neoplasm in the Western industrial nations. The bad prognosis in pancreatic adenocarcinoma is due partly to early invasion of peripancreatic tissues and adjacent organs (Klöppel 1984). An important step in the process of invasion is cell migration, which depends on the composition of the tumor matrix and the ability of tumor cells to interact with various molecules of the extracellular matrix. One important molecule involved in this process is hyaluronate (HA), a high molecular weight glycosaminoglycan composed of repetitive disaccharide units each consisting of N-acetyl-D-glucosamine and D-glucuronic acid. A functional role for HA has been demonstrated for cell migration during embryonic development where migrating cells are associated with HA-rich matrices which are degraded by hyaluronidase when cells cease migrating and start differentiating (Toole 1981). Investigation of various tumors, such as human nephroblastoma (Allerton et al. 1970), liver carcinoma (Kojima et al. 1975), malignant mesothelioma (Roboz et al. 1985), several types of human lung tumors (Horai et al. 1981), mammary carcinoma (Takeuchi et al. 1976), parotid carcinoma (Takeuchi et al. 1985) and human pancreatic carcinoma (Cudkovicz 1956) has demonstrated a high concentration of HA in tumor tissues. Furthermore, in the experimental V2 carcinoma of rabbits with subclones of different metastatic potential, the HA content was three to four times higher in metastasizing tumors than in the same tumors grown in nude mice, where no metastatic potential was observed (Toole et al. 1979). Interestingly, the highest HA concentration in invasive tumors was located at the tissue interface between the tumor mass and the neighboring host tissue (Toole et al. 1979).

We have recently shown that human pancreatic adenocarcinoma cell lines synthesize and secrete HA (Mahlbacher et al. 1992). This might suggest that these tumor cells can synthesize their own extracellular matrix, and thereby facilitate the migration and invasion of the tumor cells. In the present study a biotinylated specific HA binding protein was used to investigate the distribution of HA in nude mouse xenografts derived from human pancreatic tumor cell lines as well as in primary tumors of the human pancreas.

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## Materials and methods

### Preparation of hyaluronate-Sepharose (Tengblad 1979)

HA (300 mg) derived from human umbilical cord, with an intrinsic viscosity of 100 (Sigma, Taufkirchen, Germany) was mixed with 40 ml EAH-Sepharose (Pharmacia, Freiburg, Germany) in 100 ml millipore-water. To this, 0.7 g N-ethyl-N'-(3-dimethyl-aminopropyl)-carbodiimid-hydrochloride (Sigma, Taufkirchen, Germany) was added and the reaction mixture was stirred for 24 h at room temperature. The pH was held at 4.5–5.8 by the addition of 0.1 M hydrochloric acid. The remaining unsubstituted aminogroups in the gel were blocked with 5 ml acetic acid for 6 h at room temperature. The gel was subsequently washed with 500 ml 1 M sodium chloride; 0.1 M Tris/HCl, 1 M sodium chloride, pH 8.1; 0.05 M formate and water, transferred to 0.5 M sodium acetate, pH 5.7 and was stored at 4° C.

### Preparation of cartilage extracts (Knudson and Toole 1985)

Bovine nasal cartilage, freshly obtained from the local slaughter house, was cut into small pieces and extracted with 9 vol 4 M guanidinium-chloride, 0.5 M sodium acetate, pH 5.8, containing the following protease inhibitors: 50 mg/ml phenylmethansulfonylfluoride and 10 mM ethylendiaminetetraacetic acid. The cartilage extract was homogenized 4 × 15 s with an Ultraturrax Polytron homogenizer (type PT 10–35, Kinematica GmbH, Luzern, Switzerland) and 2 × 30 s with an Ultraturrax homogenizer type TP 18/2 (Janka & Künkel KG, Hohenstaufen, Germany) at high speed. The extract was stirred for 24 h at 4° C, again homogenized (3 × 15 s) and stirred for 66 h at 4° C. The extract was centrifuged at 13000 × g, 4° C for 45 min and the supernatant was dialysed against distilled water and lyophilized.

### Trypsin treatment (Tengblad 1979)

Lyophilized cartilage extract (2.13 g) was dissolved in 66.5 ml protease buffer containing 0.1 M sodium acetate, 0.1 M Tris/HCl, pH 7.3 and incubated at 37° C with 1.6 mg of trypsin (from bovine pancreas, 30 U/mg, Serva, Heidelberg, Germany). After 2 h the reaction was stopped by adding 2 mg soyabean trypsin inhibitor (57 U/mg, Serva, Heidelberg, Germany).

### Biotinylation (Updike and Nicolson 1986)

Trypsinized cartilage extract was dialysed against distilled water, lyophilized and dissolved in 30 ml 0.1 M Hepes buffer, pH 8.0. The extract was centrifuged 15 min at 5000 × g at 4° C. The protein concentration of the supernatant was determined by the method of Bradford (1976) using a color reagent from Biorad. The protein concentration was usually 4 mg/ml. The volume was adjusted to 127 ml with Hepes buffer and 14.1 ml 2 mM sulfo-NHS-biotin (Pierce Chemical, Rockford, IL) in 0.1 M Hepes buffer pH 8.0. The reaction was performed at 25° C in polypropylene tubes for 30 min.

### Affinity chromatography

The trypsinized and biotinylated cartilage extract was dialysed against 0.4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.7 and run over a HA-Sepharose column with a flow rate of 10 ml/h. The gel was washed with 40 ml 1 M sodium chloride followed by a 150 ml linear gradient from 1 to 3 M sodium chloride to remove non-specifically adsorbed material. The proteins bound to hyaluronate were subsequently eluted with 80 ml 4 M guanidium-chloride.

### SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed under reduced conditions according to Lämmli (1970) and the proteins were stained with silver nitrate according to Hempelmann (1984). Western blot analysis was performed according to Towbin et al. (1979) and Burnette (1981). Biotinylated proteins were detected using streptavidin/biotinylated peroxidase (DAKO, Hamburg, Germany). The color reagent contained 0.3% 4-chloro-1-naphtol (Serva Feinbiochemica, Heidelberg, Germany) and 0.21% perhydrol (Merck, Darmstadt, Germany).

### Cell lines

Three cell lines previously established from human primary pancreatic adenocarcinomas in our laboratory (Patu 8988s, 8988c and 8902) were used in the experiments (Elsässer et al. 1992, 1993). Cells were grown on 55 $cm^2$ plastic dishes (Becton and Dickinson, Heidelberg, Germany) in Dulbecco's modified Eagles Medium (DMEM; Flow Laboratories, Meckenheim, Germany) containing 5% fetal calf serum (Gibco, Karlsruhe, Germany), 5% horse serum (Gibco, Karlsruhe, Germany), 2g/l Hepes (Serva, Heidelberg, Germany) and 50 mg/ml gentamycin (Gibco, Karlsruhe, Germany) and were cultivated at 37° C in a humidified chamber equilibrated with 2% $CO_2$.

### Nude mouse experiments

NMRI nu/nu nude mice obtained from Zentralinstitut für Versuchstierzucht, Hannover, Germany were kept under aseptic conditions at 26–28° C, 40–60% humidity and a light-dark cycle of 12 h. They received a special sterilized diet (Altromin 1414, Fa. Altromin, Lage, Germany). The body weights of the animals were measured once a week. For transplantation cultured cells were trypsinized, washed once in culture medium and resuspended in medium at a concentration of about $1 \times 10^6$ cells/ml. Subcutaneous injections of this suspension (200 µl) were made in the midline of the neck of nude mice. When the tumors reached a diameter of 1.5–2 cm or when the body weight decreased continously, the mice were killed by cervical dislocation and dissected.

### Morphological methods

Nude mice tumors were either fixed in Carnoy's solution or were snap-frozen for cryostat sectioning in isopentane cooled by liquid nitrogen. Carnoy-fixed tissue was embedded in paraffin wax according to standard procedures. Cryostat sections were cut on a 1720 Digital cryostat (Leitz, Wetzlar, Germany) and fixed in acetone at 4° C for 10 min, in acetone/methanol (1:2) at −20° C for 10 min and in 3.7% formaldehyde at 4° C for 10 min. Tissue from four human primary pancreatic tumors obtained during surgery was prepared for cryostat sections according to the same methods.

Paraffin sections were rehydrated and incubated for 30 min with 0.3% $H_2O_2$ in methanol; frozen sections were incubated for 10 min in the same solution. This was followed by three rinses with phosphate-buffered saline (PBS) and incubation with 10% goat serum (Gibco, Eggenstein, Germany). Paraffin sections were incubated for 45 min and frozen tissue was incubated for 15 min at room temperature. The sections were then covered overnight at 4° C with the eluted HA binding protein in PBS. After three rinses in PBS and incubation with the streptavidin/biotin-peroxidase-complex (Dako, Hamburg, Germany) for 30 min, sections were covered with a solution containing 1.5 mg/ml diaminobenzidine (Sigma, Munich, Germany) and 0.01% $H_2O_2$ for 6 min. Finally they were rinsed in running tap water for 5 min, counterstained with Hemalum, dehydrated and embedded in Entellan$^R$ (Merck, Darmstadt, Germany). Control sections were incubated only with normal goat serum instead of the HA-binding protein solution. The specificity of HA binding was demonstrated by: (1) control

sections were covered with a mixture of HA binding protein and HA preincubated for 30 min at 4° C (Alho and Underhill 1989), and (2) control sections were incubated with Streptomyces hyaluronidase (Sigma, Taufkirchen, Germany) for 4 h in a humidified chamber at 37° C with 100 turbidity reducing units (TRU/ml) of enzyme in 100 mM sodium acetate buffer, pH 5.0, in the presence of following protease inhibitors: 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 μg/ml Pepstatin A.

## Results

Trypsinized and biotinylated proteins eluted from the HA-Sepharose column were analysed by SDS-PAGE. Silver staining and Western blot analysis (Fig. 1) exhibited a 60 kDa biotinylated protein as the major component of the preparation. This is in contrast with previous investigations, which showed a molecular weight of 90 kDa for the HA-binding globular portion of the proteoglycan monomer (Tengblad 1979). This difference may be due to different conditions during trypsin treatment. In addition to the 60 kDa protein a band at about 45 kDa was observed which might be the HA-link protein (Baker and Caterson 1979).

Differences were observed with various protocols of tissue fixation, in the staining quality with the hyaluronic acid binding protein (HABP) (data not shown). The most intense staining was obtained with cryostat sections fixed for 10 min with methanol/acetone at −20° C or acetone at 4° C, and with paraffin sections from tissue fixed with Carnoy's solution. The specificity of the binding was controlled by either preincubation of the HABP with HA (Fig. 2c, e; 3d, e, f) or by preincubation of the tissue with hyaluronidase (not shown). In both cases no staining was observed. The distribution of HA was compared in nude mouse xenografts from three different pancreatic carcinoma cell lines fixed in Carnoy and four primary tumors using frozen tissue. The staining pattern in nude mouse tumors was most intense at the boundaries between the tumor and the host tissue and was restricted to connective tissue septa in proximity to the tumor cells (Fig. 2a, b). In contrast, the central tumor parenchyma showed no staining (Fig. 2a). In regions where tumor cells had invaded muscle tissue, the staining reaction was very pronounced especially in the connective tissue cords (Fig. 2d).

When primary tumors were stained with HABP, pronounced HA deposition was also seen at the border of the tumor, while the neighboring normal pancreatic tissue revealed only occasional small stained areas (Fig. 3a). The tumors investigated showed a strong desmoplastic reaction with tumor cell nests dispersed between the stromal tissue. Connective tissue at a distance from the tumor cell nests showed only a slight or no reaction with the HABP. In contrast, the loose connective tissue surrounding the tumor cells was strongly stained with HABP suggesting a close association of human pancreatic tumor cells with an HA-rich matrix (Fig. 3b).

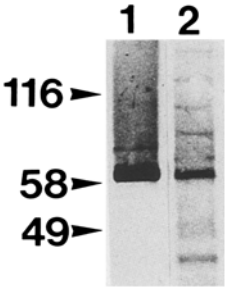



**Fig. 1** SDS-PAGE of trypsinized and biotinylated protein fraction 8/9. *Lane* 1: silver stain. *Lane* 2: Western blot analysis. Arrows indicate the two major biotinylated proteins with HA binding activity

## Discussion

A biotinylated HA-binding protein has been used to investigate the distribution of HA in nude mouse xenografts derived from human pancreatic adenocarcinoma cell lines and in primary human pancreatic tumors. In both cases, strong staining for HA was observed at the interface between tumor tissue and host or normal pancreatic tissue, respectively. This is in agreement with results in experimental tumor systems, especially in the V2 carcinoma in rabbits. This tumor invades the surrounding tissue when grown in the rabbit mesentery but when injected subcutaneously into nude mice no tumor invasion or metastasis occurred (Toole et al. 1979; Iozzo et al. 1985). Interestingly, in the rabbit mesentery, tumors able to invade the neighboring tissue had a three- to four-fold higher HA content than the non-invasive tumors in nude mice. Furthermore, the highest HA concentration was observed at the invasion front. These results support the hypothesis that the amount of HA as well as its distribution correlates with the invasive behavior of tumor cells. However, the staining does not necessarily reflect HA concentration, since the binding sites for the HA molecule can be masked by endogenous HA binding proteins or proteoglycans, staining represents rather areas where HA has free binding sites which could at least also be used by cell surface HA binding proteins of tumor cells.

In human primary pancreatic adenocarcinomas most HA is located around the tumor cells, which are dispersed as cell nests in an abundant connective tissue stroma, that is only slightly, or even not stained with HABP (Fig. 3b). Similar observations have been reported in human breast tumors (Takeuchi et al. 1976). In contrast, experiments with human lung tumors, which also have a high stromal content revealed the highest HA staining in connective tissue septa rather than in the vicinity of tumor cells (Knudson et al. 1989).

The extracellular matrix molecule HA is normally synthesized and secreted by fibroblasts, chondrocytes, oligodendrocytes, epithelial and embryonic cells (Prehm 1989; Mason et al. 1989; Philipson and Schwartz 1984; Alho and Underhill 1989; Toole 1981). However, we have recently shown that pancreatic tumor cells can also synthesize and secrete HA (Mahlbacher et al. 1992). This raises the possibility that the HA around pancreatic tumor cells is a secretory product by which tumor

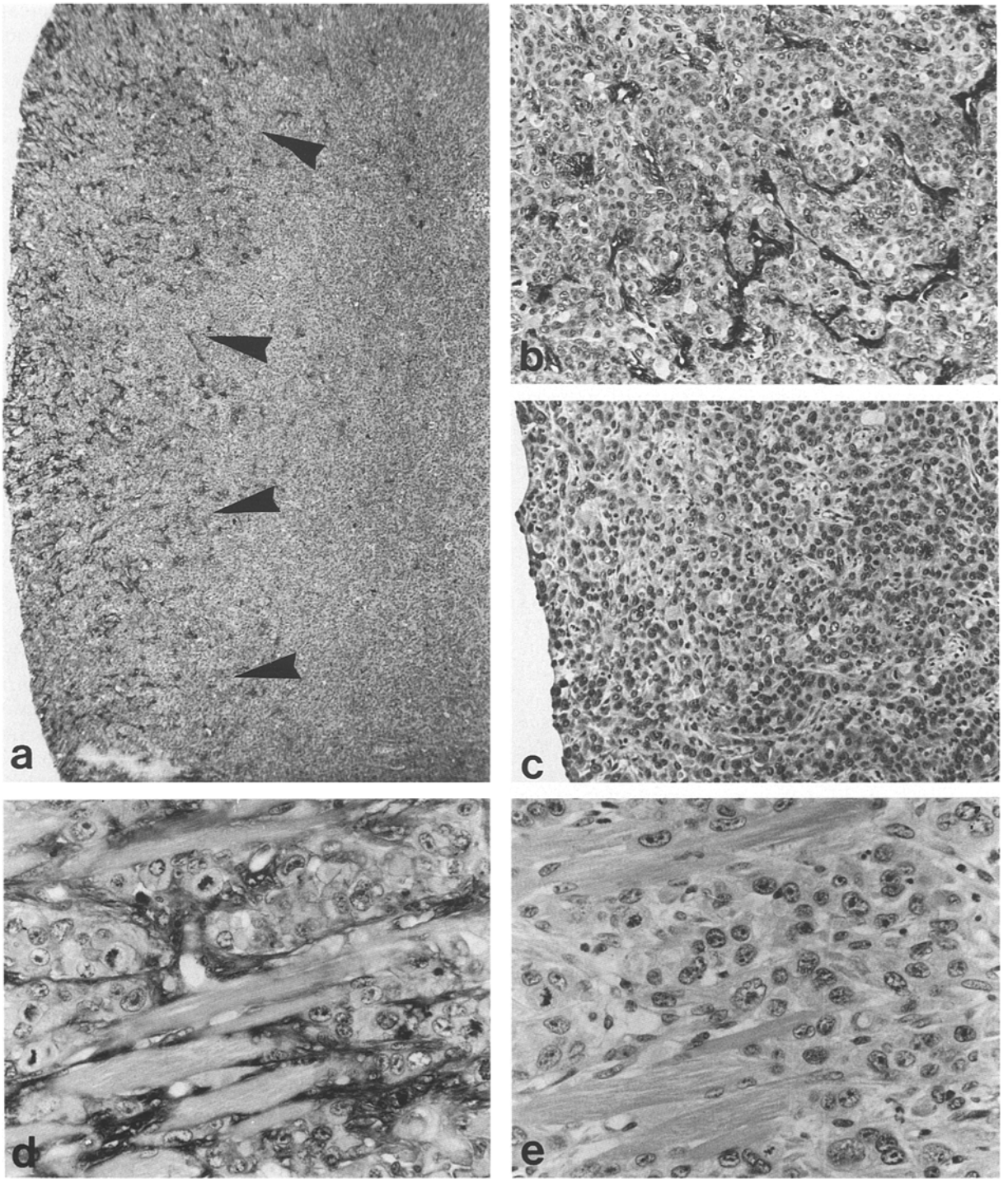
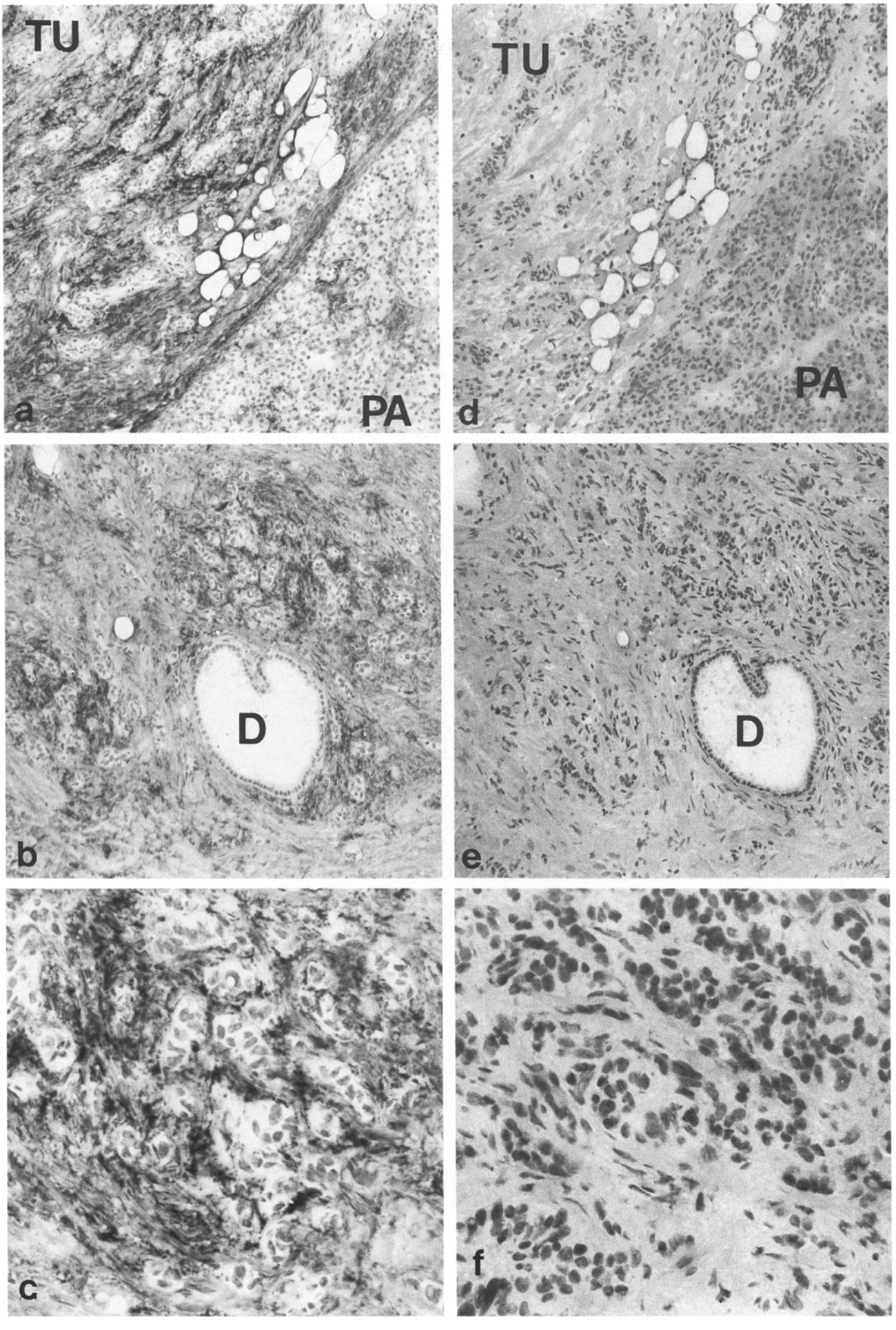



**Fig. 2** Distribution of HA in xenografts from PATU 8902 fixed with Carnoy's solution, stained with HABP and counterstained with hemalum. (**a**) The highest intensity occurred in the periphery of the tumor (arrowheads), while in the central part staining was slight or absent. (**b**) Higher magnification of (**a**). (**c**) As in (**b**) but HABP was preincubated with HA. (**d**) Infiltrated muscle tissue was rich in HA. (**e**) As in (**d**) but HABP was preincubated with HA. Magnification: (**a**): ×40; (**b**, **c**, **d**, **e**): ×130

**Fig. 3** Distribution of HA in frozen sections of primary tumors. ▶ (**a**) Tumor tissue (TU), adjacent to normal pancreatic tissue (PA), showed a strong positive reaction with HABP. (**b**) Tumor cell nests surrounded by connective tissue showing high HA concentration. (D) = pancreatic duct. (**c**) Higher magnification of (**b**). (**d**, **e**, **f**) As in (**a**, **b**, **c**) but after preincubation of HABP with HA. Magnification: (**a**, **b**, **d**, **e**): ×130; (**c**, **f**): ×320


TU
PA
a
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D
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cells modulate the composition of the surrounding matrix, facilitating their migration and invasion. An additional mechanism explaining the high HA concentration immediately surrounding tumor cells is the stimulation of HA synthesis in fibroblasts by secretory products of the tumor cells. This has been reported in various epithelial tumor cell systems including the pancreatic adenocarcinoma cell line DAN (Knudson et al. 1984). Although our results cannot differentiate between these two alternative processes as a source for HA, the accumulation immediately around the tumor cells implies a significant role for HA for the biology of human pancreatic tumors.

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