Differential Expression of the Hyaluronan Receptors CD44 and RHAMM in Human Pancreatic Cancer Cells

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ABSTRACT

To explore the putative role of hyaluronan (HA) in tumor invasion in pancreatic cancer, we investigated the expression of the HA receptors CD44s and RHAMM in a panel of human pancreatic cancer cell lines. Expression of CD44s has been found in only 1 of 10 cell lines included in this study. This cell line exhibits a highly differentiated phenotype without any metastatic potential when injected into nude mice. Since it has previously been shown that normal pancreatic duct cells express a high level of CD44s, our results indicate that pancreatic cancer may be accompanied by an almost complete loss of CD44s expression. As demonstrated by PCR amplification, this loss of CD44s expression is due to alternative splicing of CD44 pre-RNA. Although most of the pancreatic cancer cell lines express a complex but identical pattern of variant CD44 gene transcripts, only one higher molecular weight CD44 isoform can be detected in a subset of pancreatic cancer cell lines in Western blot analysis. This variant CD44 molecule represents the epithelial CD44 isoform (CD44v8–v10). When cells are cultured on Matrigel, the expression of additional CD44 variants is induced, suggesting that the extracellular matrix can influence the expression of CD44 isoforms and thereby may facilitate tumor invasion. This induction could be due to a regulatory process in the translation of the CD44 variant mRNAs expressed in pancreatic tumor cells. Molecular cloning of a cDNA encoding human RHAMM reveals that both HA receptors are structurally unrelated. In addition, they share an inverse expression pattern. RHAMM mRNA is overexpressed in pancreatic cancer cell lines exhibiting a poorly differentiated phenotype and a high metastatic potential when injected into nude mice. These results indicate that CD44 and RHAMM differentially contribute to invasion of pancreatic adenocarcinoma; however, these functions still remain to be determined.

INTRODUCTION

HA is a widely distributed high molecular weight glucosaminoglycan composed of repetitive disaccharide units, each consisting of N-acetylgalactosamine and D-glucuronic acid. HA has been shown to participate in various physiological processes including tissue morphogenesis, wound repair, inflammation, angiogenesis, cell motility, and tumor invasion (1). HA has been proposed to exert many of these effects on cells by either interacting with receptors on the cell surface or with HA-binding proteins in the ECM. Although several HA-binding proteins have been identified, only two cell membrane receptors, CD44 and RHAMM (receptor for HA-mediated motility), have thus far been characterized and cloned (reviewed in Ref. 2).

CD44 is an almost ubiquitously expressed transmembrane glycoprotein that has been shown to have a high affinity for ECM proteins including types I and VI collagen and fibronectin. CD44 has been shown to act as a cell adhesion molecule both in cell-cell and cell-matrix interactions. Larger variant isoforms (CD44v) are generated by alternative splicing of up to 10 exons in the membrane proximal region of the extracellular domain. Different combinations of variant exons can be used, giving rise to a broad range of CD44 molecules with altered biological functions. Whether variant CD44 proteins are capable of binding to HA is controversial. Furthermore, several lines of evidence suggest that the particular cellular background determines whether an individual splice variant binds to HA or not. Other ligands of variant CD44 proteins still remain to be identified. Expression of individual variants seems to be much more restricted and little is known about their putative physiological functions. Most interestingly, expression of variant CD44 proteins has been linked to tumor metastasis formation. For example, two CD44 isoforms containing sequences encoded by variant exon v6 confer full metastatic potential to nonmetastasizing pancreatic tumor cells of the rat (4, 14). In addition, coinjection of variant-specific mAb with the metastasizing cells inhibits metastatic spread in vivo (15, 16). Aberrant expression of splice

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The abbreviations used are: HA, hyaluronan; ECM, extracellular matrix; RT, reverse transcription; mAb, monoclonal antibody; HARC, HA receptor complex.
variants containing exon v6 has also been found on carcinoma cell lines from lung, breast, and colon (5). Subsequently, several tumors including human colorectal carcinoma (17–20), non-Hodgkin’s lymphomas (21, 22), gastric carcinomas (23), and melanomas (24) have been screened for expression of CD44 isoforms. These studies revealed that certain splice variants of CD44 may play a crucial role in the progression of various tumors, particularly in the process of tumor dissemination.

The HA receptor RHAMM has originally been identified as part of a multimERIC complex (HARC) that mediates HA-induced motility of H-ras-transformed fibroblasts (25). This complex occurs on the cell surface or is segregated into the media as soluble proteins. In the released form, HARC consists of four proteins with apparent Mr 72,000, 68,000, 58,000, and 52,000, whereas the membrane-bound form lacks the smallest 52,000 component (26). The capability of HARC to bind to HA has exclusively been attributed to the 58,000 and 52,000 proteins, and antibodies that specifically recognize these proteins have been shown to block HA-induced motility (25). cDNA clones which code for the HA-binding proteins were isolated from a phage expression library by immunoscreening (27). The encoded protein was either 52,000 or 48,000, depending on which of two possible initiation codons were used. Both proteins have collectively been called RHAMM. Interestingly, the cDNA encoding RHAMM does not include a putative transmembrane hydrophobic region, and is thus supposed to be a peripheral membrane protein bound to a transmembrane-docking protein designated as "connectin" (28). It has also been shown that the interaction of RHAMM with HA leads to protein tyrosine phosphorylation of several proteins, including the focal adhesion kinase p125FAK (29). These results indicate that upon binding HA, RHAMM may trigger a signal transduction cascade that initiates cell motility. Recently, it has been demonstrated that overexpression of the RHAMM gene by transfection into fibroblasts is transforming and causes spontaneous metastases (30), indicating that the HA receptor RHAMM might play a crucial role in tumorigenesis.

Previously, we have shown that human pancreatic adenocarcinoma cell lines synthesize and secrete HA (31). In addition, we analyzed the distribution of HA in human primary pancreatic carcinomas. The highest HA concentration was found at the tissue interface between the tumor mass and the normal tissue, suggesting that HA might play an important role in the process of invasion in this particular tumor (32). To further explore the role of HA in human pancreatic cancer, we investigated whether any differences in the expression of HA receptors on the surface of pancreatic adenocarcinoma cell lines exhibiting different grades of differentiation and metastatic potential in nude mice.

MATERIALS AND METHODS

Cell Lines.4 The cell lines PaTu 8988t, PaTu 8988s, PaTu 8902, GR93, and B254 were established from human primary pancreatic adenocarcinoma in our laboratory. Culture
laboratory of Dr. Peter Herrlich (Karlsruhe, Germany). The mouse mAb VFF-11 (17, 21), specific for alternatively spliced isoforms of human CD44 containing the variable v3/v4 exons, was obtained from Bender and Co. GmbH (Vienna, Austria).

Western Blot Analysis. Cell monolayers were lysed in RIPA buffer (37) and boiled in SDS gel sample buffer (40). Proteins were separated on denaturing 6% polyacrylamide gels (40) and transferred to polyvinylidene difluoride membranes (Millipore GmbH, Eschborn, Germany) using electrophoresis. Nonspecific interactions were blocked with 5% skim milk powder in PBS. Subsequently, the membranes were incubated at room temperature with primary antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako Corp.), for 2 h each. After each individual antibody incubation, the membranes were washed with PBS containing 0.05% Tween 20 (Sigma). Signals were developed with the enhanced chemoluminescence system (Amersham).

Northern Blot Analysis. Total cellular RNA was isolated using the Ultraspec RNA method (Biotex Laboratories, Inc., Houston, TX). Twenty mg of total RNA were subjected to electrophoresis through 1% formaldehyde-agarose gels and transferred to nitrocellulose membranes by capillary blotting. Hybridization was performed according to standard procedures (41) using either a CD44s probe [sequences from positions 123-904 of human CD44 (36) generated by RT-PCR as described above] or a CD44v3-v10-specific probe [sequences from positions 13-1004 of the CD44 variant region (5) synthesized via RT-PCR from the cell line PaTu 8902]. The CD44v3-v7 and CD44v8-v10 probes were generated from the CD44v3-v10 cDNA fragment using appropriate restriction sites. RHAMM mRNA expression was detected using a 1.4-kb fragment of the human RHAMM cDNA that contained the open reading frame. All cDNA fragments were radioactively labeled with [32P]dCTP using a megaprime labeling kit (Amersham).

Isolation of a cDNA Encoding Human RHAMM. Oligonucleotides corresponding to positions 271-291 (5'-TCAACTCAGAACAAC-3') and 949-969 (5'-TTCCAGCTGCCTCTAAGTC-3') of the published murine RHAMM sequence (27) were used to synthesize a cDNA fragment by RT-PCR from NIH3T3 fibroblasts. The purified PCR product was labeled with [32P]dCTP and used to screen 5.5 x 105 recombinant clones from both an oligod(T)- and random-prime dg11 cDNA library derived from the endometrium carcinoma cell line Ishikawa (42). Hybridization was performed at 42°C in 35% formamide, 4 x SSC, 25 x Denhardt’s solution, 0.1% SDS, 20 mM sodium phosphate (pH 6.4), and 100 μg/ml denatured salmon sperm DNA. Filters were washed twice in 2 x SSC and 2 x SSC-0.1% SDS at room temperature, followed by two washes in 1 x SSC-0.1% SDS at 42°C, for 30 min each. After three screens, positive clones were identified and subcloned using the EcoRI site in Bluescript plasmid (Stratagen).

To obtain the complete open reading frame of human RHAMM, a RT-PCR amplification was used. Total RNA from Ishikawa cells were transcribed into cDNA by reverse transcriptase from a specific primer (5'-GTTGCTTTTAGTGGCAGCTTG-3') corresponding to nucleotides 623-644 of the human RHAMM cDNA shown in Fig. 7B. To increase the specificity, cDNA synthesis was carried out at a high temperature (53°C). Purified cDNA was amplified by Vent DNA polymerase (New England Biolabs, Inc., Beverly, MA) using a nested primer (5'-CTGATGCTGACATCTCTTCG-3') corresponding to positions 544-564 of the human RHAMM cDNA (Fig. 7B) and the oligonucleotide 5'-AGGGCTGAAACTCTCACGTGCTGAGATC-3' selected from murine RHAMM sequences upstream of the initiator methionine codon. A prerequisite for extension of an oligonucleotide by Taq DNA polymerase is a specific annealing of the 3' end of the oligonucleotide to the template. Therefore, the 3' end of the chosen 5' oligonucleotide sequence was ATGC-3', the ATG being the methionine used to initiate translation in virtually all eukaryotic proteins and the C being found at position +4 in the published murine RHAMM sequence (27). For easier cloning, the oligonucleotides were flanked by BamHI and EcoRI recognition sites at their 5' ends, respectively. After 30 rounds of amplification (95°C for 1 min, 54°C for 1 min, and 72°C for 1 min), the reaction mixture was electrophoresed on a 1.5% agarose gel, and the major fragment that migrated at 0.58 kb was excised. The purified PCR product was digested with BamHI and EcoRI and inserted into the BamHI/EcoRI sites of the Bluescript vector (Stratagen).

All cDNA clones were sequenced in both directions using T3, T7, and sequence-specific primers. The sequences were analyzed using the PC-Gene (IntelliGenetics, Mountain View, CA) sequence analysis program.

Immunohistochemistry. Nude mice tumors derived from the cell lines PaTu 8988t, PaTu 8988s, and PaTu 8902 were generated by s.c. injection of 10⁶ cells into NMR1-nu/nu mice as described previously (33, 34). Carnoy-fixed tissue was embedded in paraffin wax according to standard procedures. Paraffin sections were rehydrated and incubated with 10% goat serum (Life Technologies, Inc., Eggenstein, Germany) at room temperature for 45 min. Incubation with the primary antibody diluted in PBS was performed overnight at 4°C followed by three washes with PBS. Specific binding of the antibodies was visualized using either FITC-conjugated anti-mouse IgG (Dako Corp.) or tetramethylrdamine-conjugated goat anti-rabbit IgG (Dako Corp.). Cells were mounted in Mowiol (Hoechst, Frankfurt, Germany) and analyzed using a Leitz Diplan fluorescence microscope.

RESULTS

A panel of human pancreatic adenocarcinoma cell lines listed in Table 1 were investigated in this study. Only representative data obtained from the cell lines PaTu 8988t, PaTu 8988s, and PaTu 8902 are shown here. These cell lines do not only differ in their grades of differentiation when grown as nude mouse xenografts (Table 1), but also in their metastatic potential. When injected i.v. into nude mice, the cell line PaTu 8902 showed the highest metastatic potential (34). Although the two cell lines PaTu 8988t and PaTu 8988s produced either no metastases or metastases restricted to the lung, respectively, metastases from PaTu 8902 appeared in the lung, in the pleural cavity, in blood vessels, and in the wall of the esophagus (33, 34). These cell lines are supposed to be representative cell lines with regard to cellular differentiation and metastatic potential in nude mice.
Table 1  Expression of HA receptor genes in pancreatic cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Type of CD44 mRNA”</th>
<th>Protein expression”</th>
<th>mRNA expression”</th>
<th>Pattern of growth as xenografted tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaTu 8988t</td>
<td>Liver MTS</td>
<td>st.</td>
<td>++</td>
<td>+</td>
<td>Well differentiated, gland forming</td>
</tr>
<tr>
<td>PaTu 8988s</td>
<td>Liver MTS</td>
<td>var.</td>
<td>–</td>
<td>++</td>
<td>Moderately differentiated, occasional gland formation</td>
</tr>
<tr>
<td>PaTu 8902</td>
<td>Primary cancer</td>
<td>var.</td>
<td>+</td>
<td>++</td>
<td>Poorly differentiated, no gland formation</td>
</tr>
<tr>
<td>B254</td>
<td>Primary cancer</td>
<td>var.</td>
<td>++</td>
<td>+++</td>
<td>Not tested</td>
</tr>
<tr>
<td>GR93</td>
<td>Primary cancer</td>
<td>var.</td>
<td>++</td>
<td>+</td>
<td>Not tested</td>
</tr>
<tr>
<td>HPAF</td>
<td>Ascites</td>
<td>st.</td>
<td>–</td>
<td>+</td>
<td>Moderately differentiated, occasional gland formation</td>
</tr>
<tr>
<td>PaTu BI</td>
<td>Primary cancer</td>
<td>var.</td>
<td>–</td>
<td>++</td>
<td>Poorly differentiated, no gland formation</td>
</tr>
<tr>
<td>Capan-1</td>
<td>Liver MTS</td>
<td>var.</td>
<td>–</td>
<td>++</td>
<td>Not graded</td>
</tr>
<tr>
<td>Capan-2</td>
<td>Primary cancer</td>
<td>var.</td>
<td>+</td>
<td>+++</td>
<td>Well differentiated, gland forming</td>
</tr>
<tr>
<td>Dan-G</td>
<td>Primary cancer</td>
<td>var.</td>
<td>++</td>
<td>Not tested</td>
<td>Poorly differentiated, no gland formation</td>
</tr>
</tbody>
</table>

a  CD44 gene transcripts were classified as either standard (st.) or variant (var.) type according to results obtained by RT-PCR analysis using oligonucleotides flanking the membrane proximal splice site of CD44. Note that the pattern of variant CD44 transcripts detected in the cell line Dan-G differs from that observed in other variant-type-positive pancreatic cancer cell lines (see Fig. 1).

b  CD44 expression was evaluated at the protein level with Western blot analysis using pan-CD44 polyclonal antibody; the relative score was as follows: –, detectable expression only by overloading the gel; +, weak; ++, moderately; +++, strong.

c  RHAMM mRNA overexpression was evaluated at the RNA level with Northern blot analysis; the relative score was as follows: +, weak; ++, moderately; +++, strong.

Detection of Alternative Spliced CD44 Gene Transcripts in Pancreatic Cancer Cells. Oligonucleotides were designed to flank the membrane proximal extracellular alternative splicing site of CD44 to detect the presence or absence of alternatively spliced CD44 transcripts in a panel of human pancreatic adenocarcinoma cell lines listed in Table 1. RNAs were isolated from the cell lines, reverse transcribed, and amplified using PCR. A single PCR product of 330 bp was detected in the nonmetastatic cell line PaTu 8988t, whereas in PaTu 8988s and PaTu 8902 a complex but identical pattern of amplification products ranging from 330 to 1350 bp was visible (Fig. 1). Only the cell lines PaTu 8988t and HPAF expressed standard CD44 gene transcripts, whereas almost all other cell lines examined have been found to express the same pattern of CD44 variant RNAs as shown in Fig. 1 (see also Table 1). A striking difference in the pattern of RNAs was detected only in the cell line Dan-G (data not shown).

To identify the variable exons inserted in each individual CD44 splice variant, the six most predominant amplification products (A-F, Fig. 1) were excised from the gel and purified. The cDNA fragments were subcloned and sequenced. The results schematically represented in Fig. 2 demonstrate that the cell line PaTu 8988t expresses only the standard form of CD44 which lacks additional sequences encoded by variant exons (Figs. 1 and 2, variant A). CD44 splice variants homologous to pMeta-1 and pMeta-2, which contain the variable exons CD44v4–v7 and CD44v6–v7, respectively, are not expressed in human pancreatic adenocarcinoma cell lines (Fig. 2). These variants have been shown to be causally involved in metastasis formation of pancreatic tumor cells of the rat (4, 14). Nevertheless, three other splice variants comprising the variable exon v6 are expressed in human pancreatic cancer cells (Figs. 1 and 2, variants D-F). The CD44 splice variant B (Fig. 2) that includes the variable exons CD44v8–v10 is also known as the epithelial variant of CD44 (43). Two different isoforms of the epithelial variant are known, i.e., CD44E and CD44R1 (43, 44). Both CD44E and CD44R1 contain a 132-amino acid insertion, but the two molecules differ from one another by just three amino acid substitutions. Recently, it has been demonstrated by transfection experiments that CD44R1, but not CD44E, is capable of binding...
Fig. 2 Schematic representation of CD44 splice variants expressed in human pancreatic cancer cells. Top, organization of the human CD44 gene as deduced from genomic cloning studies (7). \( \square \) extracellular alternatively spliced exons; \( \square \) transmembrane domain. A-F, splice variants that correspond to those shown in Fig. 1. A is identical to CD44s; B is the epithelial CD44 variant (44). TM, transmembrane domain; CD, cytoplasmic domain.

Expression of CD44 Proteins. To investigate the expression of CD44 proteins, we raised a polyclonal antibody (pan-CD44) against human CD44. Since the immunization of rabbits was performed with an epitope which is common to all CD44 proteins, the resulting antibody is pan-CD44 specific and recognizes standard as well as variant CD44 proteins. A Western blot analysis of whole-cell extracts from pancreatic cell lines separated under reducing conditions showed that the nonmetastatic cell line PaTu 8988t is the only one that expresses a high level of the \( M_r 85,000 \) standard CD44 molecule (Fig. 4). The standard CD44 isoform is almost absent in all other cell lines to HA (45). We synthesized a cDNA fragment coding for the complete extracellular domain of the CD44 epithelial variant using RT-PCR. Sequencing of the corresponding positions revealed that pancreatic cancer cell lines express the CD44R1, but not the CD44E isoform.

Since the PCR reaction was performed under saturating conditions, the abundance of individual amplification products does not reflect the relative abundance of the corresponding mRNAs. We performed a Northern blot analysis of total RNA isolated from these cell lines. RNAs were hybridized to either a CD44s probe or to various CD44v-specific probes (see “Materials and Methods”). In accordance with the results obtained with RT-PCR, the cell line PaTu 8988t expressed three CD44 transcripts (Fig. 3). These RNA species are known to be generated by alternative usage of transcription termination signals and not by alternative splicing of the CD44 pre-RNA (36). CD44 variant mRNAs are exclusively detected in the metastatic cell lines PaTu 8988s and PaTu 8902 using different CD44v-specific probes (Fig. 3). There is a strong hybridization to RNAs using the domain-specific probe CD44v8-v10, whereas signals obtained with the CD44v3-v7 cDNA fragment are weak (Fig. 3). These results indicate that the major RNA species in metastatic pancreatic cancer cell lines is obviously a variant that includes the variable exons v8–v10, i.e., CD44R1 (Fig. 2, variant B). Amplification products representing the CD44R1 isoform were also the predominant products found in the nonquantitative PCR reaction (Fig. 1, variant B).

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To increase the sensitivity of the detection, chemoluminescence system (Amersham). Immunoglobulins. Bound antibodies were detected with the enhanced horseradish peroxidase coupled with goat anti-rabbit immunoglobulins. Bound antibodies were detected with the enhanced chemoluminescence system (Amersham). kDa, kilodaltons; molecular weight in thousands.

Examined. The highly metastatic cell line PaTu 8902 and some other cell lines listed in Table 1 carry a single epitope-positive protein with an apparent molecular weight of 150,000 (Fig. 4). Surprisingly, several pancreatic cancer cell lines, including PaTu 8988t, lack expression of CD44 proteins and are thus classified as CD44 nonexpressors (Table 1 and Fig. 4). The staining pattern of protein extracts from the cell line Dan-G was significantly different. Four proteins with molecular weights of 130,000, 160,000, 200,000, and 230,000 were readily detectable in this cell line (data not shown). To ensure that the rather low expression of variant CD44 proteins found in pancreatic cancer cells was not due to insufficient experimental conditions, we used the keratinocyte cell line HPK II which is known to express a complex pattern of CD44 proteins as control in this study (Fig. 4; Refs. 5 and 38). To increase the sensitivity of the detection, we also performed immunoprecipitations of radiolabeled proteins using either the pan-CD44 antibody or anti-CD44 mAb F10-44-2 (38). The results were identical to those obtained using Western blot analysis shown in Fig. 4.

To demonstrate that the $M_{r}$ 150,000 CD44 protein detected in a subset of human pancreatic cancer cells corresponds to the epithelial CD44 isoform, we carried out immunofluorescence stainings of fixed cells using the pan-CD44 antibody as well as various exon-specific mAbs. These cell lines exclusively stained positive when incubated with pan-CD44 antibody and mAb 11.24 which recognizes an epitope encoded by the CD44 variable exon v9 (data not shown). These observations strongly suggest that the major higher molecular weight isoform expressed on pancreatic cancer cells is the epithelial CD44 variant, which includes additional peptide sequences encoded by the variable exons v8–v10 (Fig. 2, variant B). The antibodies used in this study found no epitopes in the nonexpressing cell lines (data not shown).

We also examined the expression of CD44 proteins in xenografted tumors derived from the cell lines PaTu 8988t, PaTu 8988s, and PaTu 8902. Paraffin sections of nude mouse xenografts from the cell line PaTu 8988t exclusively stained with pan-CD44 antibody, indicating that variant CD44 epitopes were absent (Fig. 5). In contrast to the results obtained using immunofluorescence staining, we observed an immunohistochemical staining with pan-CD44 antibody and mAb 11.24 (recognizing exon sequence v9) in paraffin sections of nude mouse xenografts from the cell line PaTu 8988s which has been classified as a CD44 nonexpressor cell line in vitro (Fig. 5). The staining pattern was identical to that observed in xenografts derived from the cell line PaTu 8902, but the signals were much weaker (Fig. 5). All other mAbs (11.9, 11.10, and VFF-11) did not react. Although we cannot exclude the possibility that the differences in the expression of CD44 isoforms observed when cells were examined in vitro and in vivo were simply due to different accessibility of CD44 epitopes under the experimental conditions, these results indicate that the microenvironment of the cells might influence the expression of CD44 isoforms.

**ECM Can Influence CD44 Isoform Expression.** These observations prompted us to investigate whether the ECM can influence the expression of CD44 isoforms. To address this question, pancreatic cancer cell lines were cultured on plastic dishes precoated with Matrigel, which contains various components of the ECM including laminin, type IV collagen, entactin, heparin sulfate proteoglycan, and others. During the second passage, a change of morphology of some, but not all cell lines was observed. For example, the cell line PaTu 8902 showed a markedly increased capacity for self-aggregation, leading to enhanced cell clumping. Cell lysates were prepared at that time and proteins were subjected to Western blot analysis using pan-CD44 antibody. The results obtained from the cell line PaTu 8988s and PaTu 8902 are shown in Fig. 6. When cultured on Matrigel, the $M_{r}$ 85,000 standard CD44 molecule was readily detectable in PaTu 8988s cells. In addition, several high molecular weight species of CD44v ($M_{r}$ 200,000–230,000) were faintly visible (Fig. 6). In PaTu 8902 cells, an increased expression of the epithelial CD44 variant was observed (Fig. 6). The influence of Matrigel on PaTu 8988t cells, however, failed to reach significance (data not shown). These results indicate that the influence of Matrigel might be restricted to those cells expressing alternatively spliced CD44 gene transcripts. Since these transcripts could also be detected in cells cultivated on plastic only, components of the Matrigel might induce a trans-
lational control mechanism, leading to the expression of CD44 variant proteins.

**Isolation of a cDNA Clone Encoding Human RHAMM.**

In attempting to isolate a human RHAMM cDNA clone, we synthesized a 700-bp cDNA fragment coding for murine RHAMM using RT-PCR from NIH3T3-fibroblasts and used this as a probe to screen $5.5 \times 10^5$ recombinant clones from both an oligo(dT)- and random-primed λgt11 expression library prepared from a human endometrium carcinoma cell line (Ishikawa) (42). Two cDNA clones of 0.85 kb (hRH.1) and 1.2 kb...
Expression of HA Receptors in Pancreatic Cancer

was followed by blots to a polyvinylidene difluoride membrane (Milli-

Fig. 6 Matrigel induces CD44 expression. Protein extracts were prepared from the cell lines PaTu 8988s and PaTu 8902 cultured on either plastic dishes (P) or plastic dishes precoated with Matrigel (MG). One hundred-μg aliquots were applied to SDS-polyacrylamide gel. The proteins were blotted to a polyvinylidene difluoride membrane (Millipore). Incubation with the polyclonal pan-CD44 antibody was followed by a second incubation with horseradish peroxidase coupled with goat anti-rabbit immunoglobulins. The enhanced chemiluminescence system of Amersham was used for detection of bound antibody. *kDa*, kilodaltons; molecular weight in thousands.

(hRH.2) in length were obtained which possess homology to murine RHAMM (Fig. 7A). Clone hRH.1 contains nucleotide sequences at its 5′ end that are not homologous to murine RHAMM cDNA and are probably due to an artifact during cDNA synthesis (Fig. 7A). Since we failed to isolate cDNA clones encoding the N-terminal part of human RHAMM by screening of the phage expression library, an alternate RT-PCR-based strategy was pursued (see “Materials and Methods”). A PCR amplification product of the expected size, approximately 580 bp, based on homology to the murine RHAMM sequence, was the major fragment generated. This cDNA fragment codes for the N-terminal part of human RHAMM as confirmed by sequencing of the cloned PCR product (Fig. 7A, clone hRH.3).

Sequence analysis of the cDNA clones obtained revealed that they encode a protein which possesses a significant homology to the published murine RHAMM sequence (71.3% identity, 6.6% similarity; Ref. 27). The RHAMM cDNA consists of 2039 bp and contains an open reading frame of 1221 bp. It contains two putative initiation codons, coding for proteins of either 47,500 or 43,200. Comparison of the amino acid sequence with all protein sequences stored in the SWISS PROT data base revealed no significant homologies to any known protein except murine RHAMM. Computer-based sequence analysis revealed three potential N-glycosylation sites as indicated in Fig. 7B. The cDNA encoding RHAMM does not include a putative transmembrane hydrophobic region or a signal sequence. Interestingly, a stretch of 63 bp that is repeated five times in the murine RHAMM sequence is included only once in human RHAMM cDNA (Fig. 7B). The functional relevance of this 21-amino acid repeated motif still remains to be elucidated. The C-terminal part of the protein contains two sequences of seven amino acids flanked by basic amino acids (Fig. 7B). This charged (BX7B) motif is known to be present in all HA-binding proteins characterized to date (46, 47). Although the cDNA cloned has all of the structural characteristics of a full-length cDNA, it is inconclusive from our data whether this clone actually represents the human RHAMM protein. However, only recently it had been shown by others that the murine RHAMM gene consists of 14 exons and that these exons can be alternatively spliced (48). Therefore, the possibility exists that our cDNA represents one splice variant of the human RHAMM.

Expression of RHAMM mRNA in Pancreatic Cancer Cells. To investigate the expression of RHAMM gene transcripts, we performed a Northern blot analysis of total RNA isolated from pancreatic cancer cell lines listed in Table 1. RHAMM mRNA was found to be expressed in all of the cell lines examined (Table 1 and Fig. 8). The major RHAMM transcript is 3.7 kb in length, as estimated using rRNAs as internal standards. The size of the RHAMM mRNA differs from those detected in human fibroblasts and hairy cell leukemia spleen cells which have been shown to express a 5.2-kb gene transcript (49). This observation indicates that different RHAMM isoforms may exist. Indeed, it has recently been demonstrated that neuronal cell lines express various RHAMM isoforms with molecular weights ranging from 48,000 to 116,000 (50).

Comparing pancreatic tumor cell lines, markedly different levels of expression were detected (Table 1). Cell lines exhibiting a less-differentiated phenotype such as PaTu 8988s or PaTu 8902 were found to overexpress RHAMM mRNA, whereas in the highly differentiated cell line PaTu 8988 the level of expression of RHAMM transcripts was low (Table 1 and Fig. 8). A notable exception was the cell line Capan-2 that was described previously as a more differentiated cell line. Since Capan-2 was also the only highly differentiated cell line included in this study which expressed variant CD44 transcripts, it might be possible that it has already undergone dedifferentiation during cell culture. Thus, although RHAMM protein expression has not been investigated, our results indicate that RHAMM is up-regulated in the more aggressive human pancreatic cancer cell lines, as determined by the overexpression of its mRNA (Table 1). It should be noted that all cell lines expressing variant CD44 mRNA were also found to express an increased level of RHAMM mRNA (see Table 1).

DISCUSSION

Human pancreatic adenocarcinoma derived from the duct epithelium accounts for 80 to 90% of all malignant nonendocrine pancreatic tumors and is among the 10 most frequent malignant neoplasms in the Western industrial world. The extremely poor prognosis may in part be attributed to the high invasive potential of pancreatic carcinoma cells leading to early invasion in peripancreatic tissues and adjacent organs. The aim of the present study was to further explore the potential role of HA in tumor invasion in pan-
Fig. 7 Structure and nucleotide sequence of the cDNA clones encoding human RHAMM. A, schematic representation of the overlapping Ishikawa cell cDNA clones from the random primed λgt11 expression library (R), the oligodT(prim)ed library (T), and the RT-PCR product (P). Bold line, open reading frame. The first AUG codon and the stop codon of human RHAMM are indicated. Left, name of each clone. The clone hRH.1 contains nucleotide sequences at its 5' end that are not homologous to murine RHAMM and are probably due to an artifact during eDNA synthesis. B, nucleotide and deduced amino acid sequence (single-letter code) of human RHAMM are shown. Numbers on the left correspond to the DNA sequence. The two possible initiation codons and the stop codon are indicated with bold letters. Underlined bold letters, potential N-glycosylation sites (N-X-S/T). Sequences which are putatively involved in HA binding (BX-B motif) are boxed. A 63-bp motif that is repeated five times in the murine sequence is underlined twice. The oligonucleotide sequences used to isolate the 5' end of the RHAMM cDNA are underlined. Note that the nucleotides upstream of the first initiation codon (lowercase letters) are selected from the murine RHAMM sequence.
Expression of HA Receptors in Pancreatic Cancer

been shown that normal pancreatic duct cells express a high
exhibits a highly differentiated phenotype and possesses no
of CD44-2 used in these studies (54, 55) does not distinguish between standard and variant CD44 molecules. This
raises the possibility that the invasion-promoting effect of CD44 reported by Takada et al. (54) may be mediated by an increased expression of variant CD44 proteins and not by CD44s. Indeed, it has recently been shown that the cell lines Panc-1, MIA PaCa-2, BxPC-3, and AsPC-1 express variant CD44 proteins (56). Work is in progress to transfect CD44s cDNA in the highly metastatic cell line PaTu 8902 to test whether expression of CD44s diminishes the metastatic potential of these cells.

Most of the pancreatic cancer cell lines included in this study express a complex but identical pattern of variant CD44 gene transcripts. Our results clearly demonstrate that CD44 splice variants homologous to pMeta-1 and pMeta-2 are not expressed in human pancreatic adenocarcinoma cells. These variants have been shown to be causally involved in metastasis formation of pancreatic tumor cells of the rat (4, 14). Although expressing several variant CD44 RNA species, only one higher molecular weight CD44 isoform was detectable in a subset of pancreatic cell lines. This variant CD44 molecule represents the epithelial isoform CD44R1 which comprises additional sequences encoded by the variant exons CD44v8–v10. The role of the epithelial variant CD44R1 in pancreatic cancer still remains to be elucidated. Recently, it has been demonstrated by transfection experiments in COS cells that CD44R1 is capable of binding HA (45). However, several lines of evidence suggest that the particular cellular background determines whether an individual splice variant binds to HA or not. Therefore, only gene transfer experiments of CD44R1 cDNA in nonexpressing pancreatic cancer cells, such as PaTu 8988t or PaTu 8988s, will give more insights in the potential role of CD44R1 in pancreatic cancer. Taken together, our results indicate that the major CD44 variants expressed in pancreatic cancer cell lines are CD44s and the epithelial CD44R1 isoform. As demonstrated using RT-PCR analysis, these variants were also found to be the predominant CD44 splice variants expressed in primary and metastatic pancreatic adenocarcinoma (57).

Although expressing various CD44 gene transcripts, we observe a puzzling absence of CD44 proteins in a subset of pancreatic cancer cell lines. These results are in contrast to a previously published study (56), in which expression of CD44 epitopes was reported for pancreatic cancer cell lines. The discrepancy concerning CD44 expression may be based on differences in the cell lines or specificities of the antibodies used in both studies. Although we cannot exclude the possibility that CD44 epitopes are masked (e.g., by folding of the protein or by posttranslational modifications), our findings provide evidence

\[\text{CD44s} \quad \text{RHAMM} \quad \text{control}\]

\[\text{28S} \quad \text{18S} \quad \text{28S} \quad \text{18S}\]

Fig. 8 Northern blot analysis of RHAMM transcripts in pancreatic cancer cell lines. Total RNA (15 μg) from the cell lines PaTu 8988t, PaTu 8988s, and PaTu 8902 was subjected to electrophoresis on 1% agarose gels containing 2.2% formaldehyde and transferred to nitrocellulose membranes. The filters were hybridized with a radiolabeled RHAMM cDNA fragment. Left, migration positions of the 28S and 18S rRNAs. Equal loading of RNA per lane was demonstrated by ethidium bromide staining of the gel before blotting.

\[\text{PaTu 8988t} \quad \text{PaTu 8988s} \quad \text{PaTu 8902}\]

creatic cancer. Our data obtained thus far indicate that human pancreatic cancer cells express at least two HA receptors, i.e., CD44s and RHAMM. Expression of CD44s was found in only 1 of 10 cell lines included in this study. This cell line exhibits a highly differentiated phenotype and possesses no metastatic potential in nude mice. Since it has previously been shown that normal pancreatic duct cells express a high level of CD44s (17, 39, 51), our results indicate that pancreatic cancer may be accompanied with an almost complete loss of CD44s expression. Interestingly, a down-regulation of CD44s by yet unidentified factors has also been reported for neuroblastoma cell lines (52). Moreover, in neuroblastoma an inverse correlation between the expression of CD44s and tumor progression was observed (53). In pancreatic cancer cells, however, this loss of CD44s expression is due to alternative splicing of CD44 pre-RNA as demonstrated with RT-PCR analysis using oligonucleotides flanking the membrane proximal splice site. Preliminary data in our laboratory indicate that loss of CD44s is functionally correlated with a decreased adhesion of pancreatic cancer cells to HA-coated substrates.\[^5\] Thus, it is possible that CD44s might act as an invasion suppressor molecule. This hypothesis is at odds with data recently published by Takada et al. (54) indicating that expression of CD44 in four pancreatic cancer cell lines (Panc-1, MIA PaCa-2, BxPC-3, and AsPC-1) is up-regulated compared to normal pancreatic cells, as demonstrated by flow cytometry. Furthermore, anti-CD44 antibody was found to suppress in vitro cell invasion to a Matrigel basement membrane, suggesting that CD44 has an important role in tumor cell invasion (56). It should be noted that the monoclonal anti-CD44 antibody F10-44-2 used in these studies (54, 55) does not distinguish between standard and variant CD44 molecules.

\[\text{F-F-} \quad \text{O-O-} \quad \text{Co} \quad \text{CoO-}\]

\[\text{5}\]

\[\text{V. Altmann, unpublished observation.}\]
that expression of CD44 gene transcripts does not necessarily reflect the expression of CD44 proteins. Similar results have also been reported for other cell lines. For example, in the large cell lung carcinoma cell line LCLC97, several variant CD44 gene transcripts were readily detectable whereas anti-CD44 antibodies showed no reactivity (5). Interestingly, when cells were cultured on Matrigel, the M_{r} 85,000 standard CD44 molecule and other high molecular weight CD44 proteins were detectable in PaTu 8988s cells in Western blot analysis. In PaTu 8902 cells, expression of the epithelial CD44R1 isoform was dramatically increased. These results indicate that Matrigel can influence the expression of CD44 proteins. Further investigations will be necessary to demonstrate whether Matrigel indeed activates certain factors that have an influence on CD44 gene expression. Although we do not know whether the ECM component HA plays a crucial role in the expression of variant CD44 molecules, a prerequisite is the expression of a HA receptor, which upon binding HA may trigger a signal transduction cascade. A major candidate gene is the HA receptor RHAMM.

Molecular cloning of a cDNA encoding at least a part or a splice variant of the human HA receptor RHAMM revealed that both receptors are structurally unrelated. In addition, they share an inverse expression pattern. The RHAMM mRNA level is elevated in pancreatic cancer cell lines showing a poorly differentiated phenotype and a high metastatic potential when injected into nude mice. Since RHAMM has been demonstrated to mediate HA-induced locomotion of various cell types including ras-transformed fibroblasts (25, 27), smooth muscle cells (58, 59), neuronal cells (50), and leukocytes (48, 60), it is tempting to speculate whether RHAMM might promote invasion of pancreatic cancer cells into the surrounding tissue by increasing cell motility in response to HA. This hypothesis is in good agreement with the observation that in primary pancreatic tumors the highest HA concentration was found at the tissue interface between the tumor mass and the normal tissue (32). Since earlier studies in our laboratory have shown that pancreatic tumor cells synthesize and secrete HA (31), an autocrine motility system for HA via RHAMM contributing to the invasive potential of pancreatic cancer is likely. Interestingly, it has recently been demonstrated that overexpression of the RHAMM gene by transfection into fibroblasts is transforming and causes spontaneous metastases (30), suggesting that the HA receptor RHAMM might play a crucial role in pancreatic cancer, although the relevance of RHAMM in human pancreatic tumors has still to be shown by further investigations.

Taken together, our results indicate that the HA receptors CD44 and RHAMM might differentially contribute to invasion of pancreatic adenocarcinoma, at least in the cell lines and their nude mouse xenografts used in this study.

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1618 Expression of HA Receptors in Pancreatic Cancer


Differential expression of the hyaluronan receptors CD44 and RHAMM in human pancreatic cancer cells.

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