CD44 Isoform Expression in Primary and Metastatic Pancreatic Adenocarcinoma

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Abstract

CD44 is the transmembrane adhesion molecule which binds hyaluronate. The gene encoding CD44 is found on chromosome 11p and comprises 20 exons. Differential splicing of the 10 extracellular juxtamembranous exons (v1-10) generates the major isoforms of CD44. The major CD44 isoform found on hematopoietic cells (CD44s) contains none of the variably expressed exons, while the major isoform expressed on epithelial cells [CD44(v8-10)] contains exons v8-10. Metastasis-specific isoforms of CD44 were first documented in a model of rat pancreatic adenocarcinoma [CD44(v4-7), CD44(v6-7)] and subsequently in other cancers. This study is the first characterization of CD44 isoforms in primary and metastatic human pancreatic adenocarcinomas. CD44 isoforms were analyzed in specimens of 15 primary and 6 metastatic pancreatic adenocarcinomas as well as in 6 specimens of control pancreata by two different methods. Radiolabeled reverse transcriptase-PCR coupled with 8% PAGE allowed analysis of the major isoforms of CD44, while Southern blot hybridization with [alpha-32P]dCTP-labeled probes permitted analysis for metastasis-specific CD44 isoforms containing CD44(v6) or CD44(v8-10). No differences in the expression of CD44(v8-10) and CD44s were found among the primary and metastatic pancreatic adenocarcinomas, and control specimens of pancreata. However, a novel CD44(v6) isoform was found in metastatic lesions and may represent the human homologue of the rat pancreatic adenocarcinoma metastasis-associated CD44 isoform.

Introduction

CD44 is the cluster designation given to the polymorphic transmembrane hyaluronate receptor to replace the synonyms: phagocytic glycoprotein (Pgp-1), Lutheran inhibitor (In(Lu)-related p80), Hermes antigen, HUCH-1, and extracellular matrix receptor (ECM-III) (1). The gene encoding CD44 is found on chromosome 11p and comprises 20 exons which span 50 kilobases (2). Alternative splicing, differential N- and O-linked glycosylation, chondroitin sulfation, and phosphorylation contribute to the heterogeneity of the molecule with apparent molecular masses ranging from 85–260 kDa (3). The CD44 protein has four characteristic regions: the amino terminal constant domain (exons 1–5), the juxtamembranous extracellular variable domain (exons 6–14), the transmembrane domain (exon 17), and the cytoplasmic domain (exons 18–20; Refs. 2 and 4). In addition to a 30% homology with the cartilage link protein and core proteoglycan, the amino terminal constant domain has regions which mediate binding to hyaluronate and chondroitin sulfate (3). Alternative splicing of exons 6–14 [referred to as variant exons: exon 6 (v1) to exon 14 (v10)] in the juxtamembranous extracellular variable domain generates multiple isoforms (2, 4). Isoforms lacking variant exons v1-v10 are referred to as CD44s (for standard) which is the most common isoform in hematopoietic cells while all other isoforms containing any of the variant exons are designated CD44v, followed by the number of the variant exon. For instance, the most common isoform found in epithelial cells contains variable exons v8-v10 and is designated CD44(v8–10). The short hydrophobic transmembrane domain is composed of 21 amino acids while the acidic cytoplasmic domain with two variable exons (exons 18 and 20) is believed to interact with actin, ankyrin, and protein kinase C (3).

The polymorphic CD44 glycoprotein is multifunctional and is expressed in a broad range of cell types, including epithelial, hematopoietic, and mesenchymal cells (1, 5). As an integral cell membrane glycoprotein, CD44 plays both structural and functional roles in cell-cell and cell matrix interactions. CD44 is the major ligand for hyaluronate and is known to bind collagen, laminin, and fibronectin as well (3). In addition to functioning as an extracellular matrix adhesion molecule, CD44 plays roles in cellular aggregation and movement as well as in lymphocyte maturation and trafficking (3).

The differential expression of CD44s and variant isoforms (CD44v) has been studied in a range of normal human tissues (5–7) and in selected tumors after the discovery that transfection of CD44(v4–7) or CD44(v6–7) isolated from a rat pancreatic cancer model into a nonmetastatic rat pancreatic cancer cell line established metastases on injection into nude mice (8–10), while coinjection of mAbs against CD44(v6) inhibited metastasis (11, 12). These findings prompted further investigation into the role of CD44 in metastasis in a range of human cancers. Investigators studying a variety of tumors through the use of a range of techniques have obtained varying results. Tumors arising form neuroectoderm such as neuroblastoma (13), small cell carcinoma of the lung (14), and gastrinomas (15) do not exhibit overexpression of CD44 variant isoforms. Overexpression of the standard hematopoietic isoform CD44s was found in metastases of ovarian carcinoma (16). In contrast, overexpression of the epithelial variant CD44(v8–10) was found in metastases of colon carcinoma (17) and in invasive gastric carcinoma (18) by some authors. Other authors found overexpression of CD44(v6)-containing isoforms in gastric carcinoma (5, 19), colon carcinoma (20, 21), and lymphoma (22). These apparent divergent findings need not be mutually exclusive, because each CD44 isoform may play a different role in promoting invasion and metastasis.

To date, no one has studied the expression of CD44 in human pancreatic adenocarcinoma which is usually metastatic at the time of diagnosis and has the worst prognosis of all gastrointestinal cancers (23). We analyzed clinical specimens of primary and metastatic pancreatic cancer by radiolabeled RT3-PCR coupled with PAGE (RT-PCR/PAGE) and Southern blot hybridization for expression of CD44s-, CD44(v8–10)-, and CD44(v6)-containing isoforms.

Materials and Methods

Clinical Specimens. Specimens of pancreatic tissues were obtained from patients undergoing surgery. Specimens of primary pancreatic adenocarcinoma were derived from resections in 15 patients (mean age, 61 ± 12 years; range, 41–75 years; 10 males and 5 females). Metastases found at laparotomy were the source of tissue in six patients not undergoing resection (mean age, 58 ± 13 years; range, 42–74 years; 5 males and 1 female). Three metastases (specimens
3, 5, and 6) were to the liver, while single metastases were taken from the terminal ileum (specimen 1), omentum (specimen 2), and mesentery (specimen 4). Control specimens of pancreatic tissue were obtained at the time of donor organ harvest in six different subjects (mean age, 44 ± 27 years; range, 2–70 years; 5 males and 1 female). Control specimen 5 came from a 70-year-old white male whose pancreas harbored changes of chronic pancreatitis and foci of ductular hyperplasia. All tissues were snap frozen in liquid nitrogen and stored at −70°C until analyzed. Frozen sections were stained with hematoxylin and eosin to establish degree of differentiation and guide collection of pancreatic cancer specimens with the smallest percentage of nonmalignant cells. mRNA was selected from total RNA (Oligotex-dT; Qiagen, Chatsworth, CA) that was extracted from multiple (12–16) 20-μm cryostat sections (24).

**CD44 Isoform Analysis by Radiolabeled RT-PCR.** Generation of all variable CD44 isoforms was accomplished by preparing cDNA (First-Strand cDNA Synthesis Kit; Pharmacia, Biotech, Uppsala, Sweden) from 5.0 μl of the mRNA followed by PCR with primers flanking the major extracellular juxtapancreatic splice site. The sense primer 5’-TCCGAGAGCAAGACCTCCTGGAGA-3’ and antisense primer 5’-CAGCTGGGTGGGATGTGTCTTGGT-3’ were used under the following conditions: 95°C for 1 min, 62°C for 1 min, 72°C for 1 min, 30 cycles. One-tenth (5 μl) of the PCR products were amplified an additional five cycles using the same conditions in order to incorporate [α-32P]dCTP. The labeled PCR products were resolved by 8% PAGE along with labeled CD44(v8–10), CD44(v8–9), and CD44s probes (kindly provided by K. Tanabe). Gels were exposed for 12 h and developed (X-OMAT AR; Eastman Kodak Company, Rochester, NY) for visualization of the major CD44 isoforms.

**Evaluation of CD44(v8–10) Epithelial Isoform.** PCR Southern blot analysis was performed by separating one-tenth (5 μl) of the PCR products on 1.5% agarose gels and transferring to the Hybond-N membrane (Amerham, Buckinghamshire, England), followed by UV cross-linking (Stratalinker; Stratagene, La Jolla, CA) and hybridizing (Rapid-hyb; Amersham Life Science, Arlington Heights, IL) with a CD44(v8–10) specific fragment. The membranes were exposed at ambient temperature for 72 h and developed to permit visualization of isoforms containing variant exons CD44(v8–10).

**Evaluation of CD44(v6)-containing Isoforms.** A 129-base pair CD44(v6) probe was generated by PCR amplification of mRNA from three pancreatic cancer cell lines (AsPC-1, BxPC-3, and Capan-1; American Type Culture Collection, Rockville, MD) using primers within variant exon 6. The sense primer 5’-TCCGAGACAGACATCCTGGAGA-3’ and antisense primer 5’-CAGCTGGGTGGGATGTGTCTTGGT-3’ were used under the following conditions: 95°C for 1 min, 62°C for 1 min, 72°C for 1 min, 30 cycles. The filters were washed in 2X SSC/0.1% SDS at ambient temperature for 15 min, 1X SSC/0.1% SDS at 65°C for 30 min, and 0.1X SSC/0.1% SDS at 65°C for 30 min. The membranes were exposed at ambient temperature for 72 h and developed to permit visualization of isoforms containing variant exons CD44(v8–10).

**Results**

**Determination of the Major CD44 Isoforms in Normal Pancreas, Primary Pancreatic Cancer, and Metastases.** The autoradiograms of the 8% PAGE seen in Fig. 1 demonstrate ubiquitous expression of both the hematopoietic isoform CD44s amplification product (83 base pairs) and the epithelial variant CD44(v8–10) amplification product (479 base pairs) in all specimens of primary pancreatic cancer, metastatic pancreatic cancer, and control specimens. An approximately 275-base pair variant, which comigrates with the CD44(v8–9) fragment (kindly provided by K. Tanabe) can be detected faintly in 10 of 15 primary pancreatic cancers (primary specimens 4–6, 8, 9, and 11–15), 4 of 6 metastatic pancreatic cancers (metastatic specimens 1 and 4–6), and 1 of 6 control specimens of pancreata (control specimen 2). Although approximately 20 additional low intensity bands are observed by radiolabeled RT-PCR with equal frequency in both normal and malignant pancreatic specimens, there appears to be no significant differences in the expression of the major CD44 isoforms in these clinical specimens. A more precise determination of CD44(v8–10) isoforms and other putative metastasis-specific isoforms was obtained by Southern blot analysis.

**Determination of CD44(v8–10) Isoforms in Normal Pancreas, Primary Pancreatic Cancer, and Metastases.** Fig. 2 depicts Southern blots hybridized with [α-32P]dCTP random-labeled CD44(v8–10) probe. The CD44(v8–10) amplification product (479 base pairs, Fig. 2, arrow) is the major isoform present in all samples. Cross-hybridization of the CD44(v8–10) probe with the CD44(v8–9) isoform (275 base pairs) is evident in primary pancreatic carcinomas 4, 5, 8, 9, and 12–15; metastases 1, 4, and 6; and faintly in control specimens 2 and 6. In addition, isoforms larger than CD44(v8–10) and intermediate between CD44(v8–9) and CD44(v8–10) are detected with the random-primed CD44(v8–10) probe. Bands can be seen at approximately 740 base pairs, which may represent CD44(v6–10) in primary pancreatic cancer specimens 5, 8, and 9 and in metastases 1 and 6 while not in control pancreas specimens. Bands of intermediate size between CD44(v8–9) at 275 base pairs and CD44(v8–10) at 479 base pairs may represent CD44(v9–10) at 377 base pairs or CD44(v7–9) at 407 base pairs as seen in primary pancreatic carcinomas 3–5, 8, 9, and 11–13; metastases 1, 2, 4, and 6; and not in specimens of control pancreatic tissue. No significant differences in CD44(v8–10) expression were discerned among samples of primary pancreatic cancers, metastases, and specimens of control pancreata.

**Determination of CD44(v6) Isoforms in Normal Pancreas, Primary Pancreatic Cancer, and Metastases.** Rehybridization of the Southern blot depicted in Fig. 2 with a CD44(v6) specific fragment is seen in Fig. 3. The most prevalent band hybridizing with the CD44(v6) probe is found at approximately 212 base pairs in primary pancreatic cancers 4, 9, and 12–15; metastases 1–3 and 6; and control pancreas specimens 2 and 3. An amplification product of this size can only represent an isoform of CD44 which includes only variant exon v6. The CD44(v6–10) isoform (740 base pairs) detected on the Southern blot probed with the CD44(v8–10) fragment (Fig. 2) is confirmed by cross-hybridizing of the CD44(v6) probe in primary pancreatic carcinomas 4, 5, 8, and 9 and metastases 1 and 6 (Fig. 3). Control pancreas specimen 3 exhibited an intense band which may represent CD44(v5–6) at 329 base pairs or CD44(v6–7) at 344 base pairs. Residual cross-hybridization of the random-primed CD44(v6) probe is seen at 479 base pairs which corresponds to CD44(v8–10). The new bands (212 base pairs, 329/344 base pairs) detected with the directed primed CD44(v6) do not hybridize with the CD44(v8–10) radiolabeled probe.

**Discussion**

The present study presents the first detailed analysis of CD44 isoforms in human pancreatic adenocarcinoma. Using radiolabeled RT-PCR/PAGE to survey the major CD44 isoforms, we find no differences in expression of CD44s and the epithelial variant CD44(v8–10) among specimens of primary pancreatic adenocarcinoma, metastatic pancreatic adenocarcinoma, and control pancreas (Fig. 1).

More sensitive analyses were carried out with RT-PCR/Southern blot hybridizations to detect putative metastasis-specific isoforms. Although no differences in expression of CD44(v8–10) were seen, the
Fig. 1. Major isoforms of CD44 by radiolabeled RT-PCR. The [α-32P]dCTP-labeled PCR products were resolved by 8% PAGE along with a no-cDNA control, a X 174 HaeIII digest ladder, and labeled CD44s, CD44(v8–10), and CD44(v8–9) fragments. All specimens show the CD44(v8–10) (479 base pairs) and the CD44s isoforms (83 base pairs). A band at 275 base pairs is believed to be the CD44(v8–9) isoform.

Hybridization of the radiolabeled CD44(v6) probe revealed two interesting CD44 isoforms in the malignant tissues (Fig. 3). A higher molecular mass CD44 isoform believed to be CD44(v6–10) on the basis of its size and cross-hybridizing to both CD44(v6) and CD44(v8–10) was found in 4 (27%) of 15 of the primary pancreatic adenocarcinomas, 2 (33%) of 6 of the metastatic pancreatic adenocarcinomas, and 0 (0%) of 6 of the control specimens of pancreata. The CD44(v6) probe also hybridized to a lower molecular mass CD44 isoform which could only represent an isoform containing CD44(v6). This isoform was found in 6 (40%) of 15 of the primary pancreatic adenocarcinomas, 4 (67%) of 6 of the metastatic pancreatic adenocarcinomas, and 2 (33%) of 6 of the control specimens of pancreata. Although the differential expression of this CD44(v6) isoform among the small collection of metastatic pancreatic adenocarcinomas and control specimens of pancreata does not reach statistical significance, the presence of this small isoform warrants further investigation given in vivo experiments in a rat model of metastatic pancreatic adenocarcinoma (8–12).
The overexpression of only one isoform of the CD44 cell surface glycoprotein may convey metastatic potential to a malignant cell (8–10). These functional studies in a model of rat pancreatic adenocarcinoma suggest that CD44(v6) is the pivotal exon in the CD44(v4–7) and CD44(v6–7) isoforms studied (11, 12). No experiments published to date support the causal role of CD44(v9)-containing isoforms in promoting metastasis. The finding of a metastasis-associated novel CD44(v6) isoform by RT-PCR in our study of human pancreatic adenocarcinoma is very interesting, although substantiation of its biological significance requires stable transfection of the CD44(v6) isoform into primary pancreatic ductal cells and injection of such cells into nude mice.

What little is known about the regulation of CD44 expression comes from transfection experiments. Overexpression of the oncoproteins ras (14, 25, 26) and src (26) lead to overexpression of CD44 isoforms in transfected cells. This dysregulation of CD44 transcription may lead to surface expression of isoforms inappropriate for the microenvironment, thereby permitting the malignant cell to detach from the basement membrane or from adjacent cells caused by diminished binding to hyaluronate (27) or by secretion of soluble CD44.

Fig. 2. PCR Southern blot analysis of primary pancreatic adenocarcinomas, metastatic pancreatic adenocarcinomas, and control specimens of pancreata hybridized with random [α-32P]dCTP-labeled CD44(v8–10) probe. CD44(v8–10) (479 base pairs, arrow) is seen in all specimens while CD44(v8–9) (275 base pairs) is confirmed in primary pancreatic adenocarcinoma specimens 4, 5, 8, and 9 and metastases 1 and 6. CD44(v6) is seen at 212 base pairs in primary pancreatic cancers 4, 9, and 12–15, in metastases 1–3 and 6, and in control pancreas specimens 2 and 3.

Fig. 3. The PCR Southern blots used for analysis of CD44(v8–10) were rehybridized with [α-32P]dCTP-labeled CD44(v6) probe. CD44(v6–10) is seen at 740 base pairs in primary pancreatic carcinomas 4, 5, 8, and 9 and metastases 1 and 6. CD44(v6) is seen at 212 base pairs in primary pancreatic cancers 4, 9, and 12–15, in metastases 1–3 and 6, and in control pancreas specimens 2 and 3.
which would compete for binding with membrane-bound CD44 (28). Enhanced hematogenous and lymphatic metastasis mediated by overexpression of CD44(v6)-containing isforms reflects mimicy of activated lymphocytes (7, 22, 29), while overexpression of CD44(v9)-containing isforms may promote local invasion through altered hyaluronate binding and metabolism (27). Therefore, the apparent discordant results among authors investigating the same or related neoplasms are not mutually exclusive, but rather may reflect the multifunctional nature of CD44.

References


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