Different Splice Variants of CD44 Are Expressed in Gastrinomas but not in Other Subtypes of Endocrine Pancreatic Tumors

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ABSTRACT

Endocrine pancreatic tumors are neuroendocrine neoplasms with malignant potential and give rise to varied clinical syndromes due to excessive secretion of multiple hormones. In this study 22 endocrine pancreatic tumors and 11 carcinoid tumors were examined for the expression of CD44 using a monoclonal antibody. CD44 gene activity of 11 endocrine pancreatic tumor tissues and five carcinoid tumor tissues was also studied by amplifying messenger RNA with the polymerase chain reaction followed by electrophoresis and blot hybridization. Strong immunoreactivity was detected on all gastrinomas examined (P < 0.001), and in two nonfunctioning endocrine pancreatic tumors. Such immunoreactivity was not observed in other subtypes of endocrine pancreatic tumors. The normal human pancreas, the acinar portion and ductal epithelial cells stained strongly positive but pancreatic islet cells did not show any significant immunostaining. Furthermore, in endocrine pancreatic tumors with metastatic disease, CD44-positive tumors had a tendency to metastasize to lymph nodes (P = 0.005), as compared with CD44-negative tumors which were locally invasive or metastasized to the liver. Although, in this limited material and short follow-up, we were not able to show any statistical significance, patients with CD44-negative endocrine pancreatic tumors had prolonged survival time compared with patients with CD44-positive tumors (73% versus 59% at 5 years; P = 0.7). Of 10 carcinoid tumors examined, all three foregut carcinoids and one midgut carcinoid stained strongly positive, whereas all other midgut carcinoids were negative. Analysis of CD44 splice variants showed that in all five gastrinomas there was overproduction of alternatively spliced larger molecular variants as compared with other types of endocrine pancreatic tumors and carcinoid tumors. The band pattern from one case of carcinoid tumor with a fulminant clinical course was similar to that of gastrinomas, whereas other carcinoid tumors expressed the epithelial form of CD44. The earlier identified splice variants which confer metastatic behavior on a pancreatic tumor cell line were not expressed in neuroendocrine tumors. Our data indicate that CD44 expression in endocrine pancreatic tumors correlates with the ability to give rise to lymph node metastases and may play a vital role in determining the fate of metastasizing cells. Moreover, because gastrin is not detectable in the normal human pancreas, the pancreatic ductal cell positivity for CD44 gastrin. Other hormones produced are insulin, vasoactive intestinal polypeptide, glucagon, and somatostatin giving rise to respective clinical syndromes. Nonfunctioning endocrine pancreatic tumors are clinically silent but secrete peptides such as pancreatic polypeptide, chromogranin, and human chorionic gonadotrophin α/β subunits which do not result in hormone related clinical syndromes.

Immunohistochemically, most neuroendocrine tumors are chromogranin positive (1). Not all gastrin-producing tumors show positive gastrin immunoreactivity. In one report 69% of gastrinomas were gastrin immunoreactive (1). The gastrin immunoreactivity is found on scattered cells; unreactive cells may represent cells producing other hormones or poorly granulated cells that store gastrin in amounts below the detection limit of immunohistochemistry (2). Furthermore, antibodies used in histopathologic diagnosis may not recognize precursor forms of gastrin. Such a mixture of reactive and unreactive cells is commonly seen in many endocrine pancreatic tumors.

There is a high degree of heterogeneity in the growth pattern of endocrine pancreatic tumors. Clinically, the spectrum of endocrine pancreatic tumors includes benign insulin-producing tumors and highly malignant tumors with rapid growth and a fulminant course. Realizing the increasing importance of the role of growth factors and extracellular matrix in neuroendocrine tumors (3), we sought to investigate the involvement of adhesion molecules in these tumors. Some tumors are characterized by the production and accumulation of high levels of hyaluronate and neoplastic cells sometimes have increased capacity to bind to this glycosaminoglycan (4, 5). CD44 is the principal receptor for hyaluronate (6, 7), and has been implicated in diverse processes involving specific cell-cell and cell-extracellular matrix interactions as well as cell migration. In the present study we sought to investigate whether neuroendocrine tumors of the digestive system express CD44 and investigate its role in malignant conversion.

MATERIALS AND METHODS

Immunohistochemistry. Neuroendocrine tumors were obtained from 33 patients by abdominal operation or by ultrasonically guided needle biopsies (diameter, 1.2–2.0 mm) of liver metastases. The material comprised primary tumors and metastases. Twenty-one patients presented with endocrine pancreatic tumors including, eight gastrinomas, eight nonfunctioning, two insulinomas, three VIPomas (watery diarrhea, hypokalemia, achlorhydria syndrome), and one glucagonoma. Ten patients presented with carcinoid tumors, one each from the duodenum, stomach, and lung and seven midgut carcinoids. Four endocrine pancreatic tumors and one duodenal carcinoid were from patients with the multiple endocrine neoplasia type I syndrome. Both primary and metastatic tissue was examined for one patient with nonfunctioning islet cell tumor (Table 1, patient 11). The tumor tissue was kept frozen, cryosectioned, and fixed in cold acetone for 10 min for immunohistochemical staining. Cryostat sections of normal human pancreas were obtained from cadaveric kidney donors. Sections of pyloric antrum were obtained from tissue obtained from a patient undergoing gastric resection.

The ABC immunoperoxidase method was performed as described before (8). Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol and endogenous avidin-binding protein was blocked by incubating the sections sequentially with avidin and biotin in Blocking kit (Vector Laboratories, Burlingame, CA). Either normal horse or goat serum diluted 1:5 was used to block unspecific binding of the secondary antibody to tissue. Anti-CD44 mouse monoclonal antibody (British Biotechnology) was used as pri-
mary antibody. Goat anti-rabbit Ig or anti-mouse Ig was used as the secondary antibody. Islet cells in normal human pancreas were stained with methyl green. To determine the cell types in the tissue, several antibodies were applied in the sequential sections. Rabbit polyclonal antibody against von Willebrand factor (Dakopatts) was used as a marker for endothelial muscle actin HHF 35 (9), and monocytes were identified by Leu M5 monoclonal antibody (Becton-Dickinson). Islet cells in normal human pancreas were obtained from surgical resection specimens from 14 patients with neuroendocrine tumors (four carcinoid tumors, five gastrinomas, two insulinomas, one glucagonoma, and three nonfunctioning endocrine pancreatic tumors) and one crine tumor (four carcinoid tumors, five gastrinomas, two insulinomas, one glucagonoma, and three nonfunctioning endocrine pancreatic tumors) and one crine tumor (four carcinoid tumors, five gastrinomas, two insulinomas, one glucagonoma, and three nonfunctioning endocrine pancreatic tumors) and one crine tumor (four carcinoid tumors, five gastrinomas, two insulinomas, one glucagonoma, and three nonfunctioning endocrine pancreatic tumors) and one crine tumor (four carcinoid tumors, five gastrinomas, two insulinomas, one glucagonoma, and three nonfunctioning endocrine pancreatic tumors) and one crine tumor.
carcinoid tumor stained strongly positive whereas in one primary midgut carcinoid tissue, tumor cells at the interface of the tumor tissue with the stromal cells stained positive. All other midgut carcinoids did not show any detectable immunostaining on the tumor cells. The stromal component of all tumors stained strongly positive. Endocrine pancreatic tumors with metastatic disease and CD44-positive immunostaining were also lymph node positive ($P = 0.005$); whereas CD44-negative tumors were lymph node negative and metastasized to the liver or were locally invasive. Although not statistically significant, CD44-negative tumors had a prolonged survival time compared with patients with CD44-positive tumors (73% versus 59% at 5 years; $P = 0.7$).

**Normal Adult Human Pancreas.** The ductal epithelial cells and the acini of normal human pancreas stained strongly positive for CD44. The normal islets did not show any detectable immunostaining (Fig. 1C). The islet was identified by antisera against chromogranin on serial section (not shown).

**Pyloric Antrum.** Scattered cells present deep in the gastric crypts of normal human pyloric antrum stained strongly positive for CD44. Similar cells stained positive with antiserum against chromogranin in serial section (not shown). Some of these positive cells were also positive in serial section with antiserum against gastrin.

**PCR Amplification and Blot Hybridization**

PCR amplification was performed on polyadenylated RNA from tumor tissues, two cell lines, and normal human pancreas. The primers P1 and P2 amplify across the site in the mRNA at which extra segments may be inserted to produce different forms of CD44. P1 has its origin 324 base pairs upstream from the insertion site in the cDNA of standard CD44 molecule and P2 is 158 base pairs downstream (16). Thus the amplified fragment of 482 base pairs will be that of standard CD44 only. Products of this length are detected in most of the tumor tissues examined (Fig. 2) except primary midgut carcinoids and cell

Fig. 1. **A.** Immunostaining of an endocrine pancreatic tumor (gastrinoma) with antibody against CD44 (H-CAM) showing strong immunostaining of tumor cell membranes. Counterstain with methyl green. In **B,** an endocrine pancreatic tumor (nonfunctioning), showed only stromal cell staining with antibody against CD44, whereas tumor cells did not show any detectable immunostaining. Counterstain with methyl green. In **C,** normal human pancreatic acinar cells stained strongly with antibody against CD44 but the islet cells did not show significant immunostaining. $\times$ 410.
line LCC-18. The epithelial form of CD44, a fragment of length 878 base pairs is observed in metastatic but not primary carcinoid tissues whereas both metastases and primary endocrine pancreatic tumors express this form (Figs. 2 and 3). Normal pancreas expresses the epithelial form as a faint band (Figs. 2 and 3). All gastrinomas (Tracks J-N, Fig. 3) expressed variants with larger molecular size. No products were seen with probes D2 and D3. With probes D1 and D5 different products were also detected (not shown).

**DISCUSSION**

A number of functions have been ascribed to CD44, such as binding to high endothelial cells in postcapillary venules of lymphoid organs as part of the process of lymphoid circulation (18); the binding to collagen, fibronectin, and hyaluronate to confer cell-matrix interactions (7, 19–21); and signal transfer in lymphocytes and macrophages. These functions are thought to reside in the surface glycoprotein with an apparent molecular weight of 85,000. CD44 with larger molecular weights (Mr 110,000–200,000) have been detected in cutaneous lymphomas, colon and vulvar carcinomas as well as in neutrophils and keratinocytes (22–24). In the present study we have shown that all gastrinomas express CD44. All other types of endocrine pancreatic tumors except two nonfunctioning tumors did not express CD44. Tumors positive for CD44 had a preponderance to metastasize to lymph nodes whereas tumors not expressing CD44 were locally invasive or had metastasized to the liver. Gastrinomas are known to metastasize early to regional lymph nodes and later on to the liver. Such behavior could be supported by the previous observation that rat pancreatic tumor cells transfected with a metastasis-associated CD44 splice variant may mimic lymphocyte behavior (25). Furthermore, CD44-negative tumors had a prolonged survival time (although not statistically significant) as compared to tumors which did express CD44. A similar observation regarding the aggressiveness of human melanoma cell variants has been of melanoma cells expressing high levels of CD44 formed more numerous lung nodules when injected into nude mice (26). In contradiction was the finding that CD44 was repressed in neuroblastoma cells with high metastatic potential (27).

In the normal adult pancreas, immunoreactive gastrin is not detected in pancreatic islets. In an earlier study gastrin immunoreactive cells were found in fetal and neonatal rats (28), but were not observed in rats older than 20 days. Because ductal epithelium expressed high levels of CD44, it was suggested that pancreatic gastrin is stored in islet A1- (or D-) cells (32, 33). However claims that D-cells produce gastrin have not been confirmed as most gastrin antisera that are potent in demonstrating antral and duodenal gastrin tumor cells do not resemble 6 cells by electron microscopy as reported previously (34). There are also conspicuous histological and histochemical differences between pancreatic D-cells and antral gastrin cells, indicating that they store different peptide molecules. Claims that D-cells produce gastrin have not been confirmed as most gastrin antisera that are potent in demonstrating antral and duodenal gastrin cells fail to react with D-cells (35). Gastrin-producing tumors are often associated with islet cell hyperplasia and signs of neof ormation of islets from ducts (31). Such stem cells or nesidioblasts could be induced to proliferate and differentiate into various peptide hormone-producing cells.

There is controversy in literature regarding the cell origin of gastrin-producing pancreatic tumors. It has been suggested that pancreatic gastrin is stored in islet A1- (or D-) cells (32, 33). However gastrin tumor cells do not resemble δ cells by electron microscopy as reported previously (34). There are also conspicuous histological and histochemical differences between pancreatic D-cells and antral gastrin cells, indicating that they store different peptide molecules. Claims that D-cells produce gastrin have not been confirmed as most gastrin antisera that are potent in demonstrating antral and duodenal gastrin cells fail to react with D-cells (35). Gastrin-producing tumors are often associated with islet cell hyperplasia and signs of neof ormation of islets from ducts (35, 36). We as well as others (38, 39) could demonstrate strong expression of CD44 in pancreatic ducts. Because ductal epithelium expressed high levels of CD44 as compared to normal pancreatic islets, this would support the hypothesis that gastrinomas originate from multipotent stem cells in ductal epithelium whereas other subtypes of endocrine pancreatic tumors arise from transformation of normal islet cells which are already differen-
tiated to produce identifiable peptide products. Positive staining of pancreatic acini was noticed by us as well as Picker et al. (38) but not by Heider et al. (39).

The vast functional repertoire of CD44 could be explained by the various splice variants of CD44. One of these is identified as an metastasis-inducing splice variant of CD44 which was recently observed in metastatic rat tumor cells (Bsr 73ASM) (40). None of the neuroendocrine tumors we examined expressed this variant nor the other recently reported variant conferring metastatic activity (41). As most of the tumors had metastatic activity it is likely that some variants with other exons than the ones examined are responsible for conferring metastatic behavior. This is not surprising as neuroendocrine tumors are slow-growing tumors as compared with breast and colon tumors similarly examined by Matsumura et al. (16). This is also supported by the fact that breast and colon tumors expressed variants of larger molecular size as compared with neuroendocrine tumors. As epitopes of CD44 are conserved in various forms of CD44, antibodies raised against the standard form could react with different forms of CD44. Most neuroendocrine tumors other than gastrinomas expressed the epithelial variant of CD44 as seen by PCR amplification which is further confirmed by our immunohistochemical findings. Immunohistochemically, CD44 expression was also seen in the stromal component of all neuroendocrine tumor. It is possible that activated fibroblasts and other stromal cell components in these tumors also express the epithelial variant of CD44 which is seen by PCR amplification and blot hybridization. This is supported by the observation that carcinoid cell line LCC18 did not express CD44. Another possibility is that tumor cells express small variants which are not detectable by immunohistochemistry. One carcinoid tumor (Table 2, patient II-4) expressed larger size variants and was also immunohistochemically positive. This patient had a rapid clinical course and was more resistant to treatment than other carcinoid tumors. Although a larger number of carcinoid tumors needs to be examined, it is possible that larger size variants expressed by tumor cells contribute to malignant behavior.

In conclusion, our study demonstrates that gastrinomas express larger molecular variants of CD44 as compared with other endocrine pancreatic tumors. Preponderance of lymph node spread was also seen in CD44-positive tumors. Thus CD44 may play a role in tumor dissemination and malignant behavior of neuroendocrine tumors.

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