Differential Expression of CD44 Splice Variants in Intestinal- and Diffuse-Type Human Gastric Carcinomas and Normal Gastric Mucosa

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ABSTRACT

Immunohistochemical screening of gastric adenocarcinomas from 42 different patients revealed variant CD44 expression in all specimens tested. Adenocarcinomas of the intestinal type were strongly positive for epitopes encoded by variant exons v5 and v6, whereas diffuse-type adenocarcinomas predominantly expressed only exon v5. Normal stomach mucosa was stained by an exon v5-specific monoclonal antibody within the foveolar proliferation zone and on mucosal surface epithelium. Areas of intestinal metaplasia reacted positively with monoclonal antibodies specific for exons v5 and v6.

Analysis of RNA expression revealed dramatic differences between normal mucosa and adenocarcinomas. Whereas in normal epithelium only two CD44 variant RNAs containing exons v5 and/or v6 could be detected, intestinal-type tumors yielded a much more complex pattern of amplification products which hybridized to exons v5 and v6. A similar complex expression pattern of CD44 variants was observed in three cell lines established from intestinal-type tumors. In a sample of a diffuse-type tumor, expression of exon v5, but not v6, could be detected, confirming the data obtained with immunohistochemistry. These differences in variant exon v6 expression observed between diffuse-type and intestinal-type stomach adenocarcinomas establish variant CD44-specific antibodies as a tool in gastric cancer diagnosis and also support the theory of different origins for these tumor types.

INTRODUCTION

Gastric cancer is one of the most frequent cancers worldwide and accounts for one sixth of all cancer deaths in the United States (1, 2). The mortality rates in different countries and ethnic groups differ considerably. The population in Japan, Chile, and Costa Rica currently suffers from the highest gastric cancer mortality rates. In the United States the risk for the black population exceeds that for the white population (1–3).

Of all gastric tumors, 97% are adenocarcinomas, which can be divided into two major histological types, “intestinal-type” and “diffuse-type” carcinomas, as classified by Lauren (4). Intestinal-type tumors, but not diffuse-type tumors, are often accompanied by chronic gastritis B and especially by intestinal metaplasia, which are thought to be precursors of dysplastic changes and of intestinal-type adenocarcinoma (5–10). Pathogenetic differences between the two adenocarcinoma types are also reflected by the observation that patients with diffuse-type carcinomas often belong to blood group A, indicating a possible influence of genetic factors on cancer risk (11), while environmental factors, e.g., Helicobacter pylori infection, are possibly important for the development of intestinal-type tumors (12, 13).

We have recently demonstrated that the expression of variants of the surface glycoprotein CD44 is necessary and sufficient to confer so-called spontaneous metastatic behavior to a nonmetastatic rat pancreatic adenocarcinoma cell line, as well as to a nonmetastatic rat fibrosarcoma cell line (14, 15). Whereas the smallest CD44 isoform, CD44s, is ubiquitously expressed in a variety of different tissues including epithelial cells, individual splice variants of CD44 are expressed only on a subset of epithelial cells. The CD44 variants are generated by alternative splicing such that the sequences of 10 exons are completely excised in CD44s but can be used in various combinations to yield larger variants (16, 17). The variants differ from each other by the amino acids inserted at a distinct site of the extracellular portion of the protein. Such variants are also detected in a variety of human tumor cell lines and in human tumor tissue. In particular, we have recently examined the expression of CD44 variant proteins in the course of colorectal carcinogenesis (18). Normal human colonic epithelium lacks expression of CD44 variants, and only weak expression is detectable in proliferating cells of the crypts. At later stages of tumor progression, e.g., in adenocarcinomas and in metastases, all malignancies express variants of CD44, thus establishing CD44 splice variants as a new tumor marker. It is obviously important to examine to what extent this new marker is found on different cancer cells. Furthermore, the monoclonal antibodies now available permit fine analysis of the CD44 variants expressed in different types of tumors.

Here we describe the detailed analysis of CD44 splice variants in different types of human stomach adenocarcinomas, compared to normal gastric mucosa, lymph node metastases, and established human stomach carcinoma cell lines.

MATERIALS AND METHODS

Cell Lines. The culture conditions for the stomach carcinoma cell lines 2474, 2957, and 3051 have been described (19). The human melanoma cell line MeWo was obtained from the American Type Culture Collection and was grown as described (20).

Antibodies. The polyclonal serum and mAbs directed against the variant region of the CD44 molecule have been described previously (18, 21). The exon specificity of these antibodies is depicted in Fig. 1. mAb VFF8 recognizes an epitope in the amino acid sequence of exon v5, as does mAb VFF6 (21). mAb VFF14 recognizes amino acid sequences on bacterial fusion proteins encoded by variant exons 8–10. The exact exon specificity of mAbs VFF11 and VFF14 has not yet been accurately defined. All mAbs were kindly provided by Dr. G. Adolff, Bender and Co. GmbH (Vienna, Austria).

Tumors and Tissues. Tumor samples and normal tissues were selected from the files of the Department of Pathology, University of Würzburg (Germany). The samples had been snap-frozen in liquid nitrogen immediately after surgical excision and stored at –80°C until usage. Normal tissues were obtained from 12 different tumor-bearing patients, from both the corpus and antrum regions of the stomach. Pathological tissues were obtained from a total of 47 patients, with an average age of 63 years. Of the primary carcinomas, 29 belonged to the intestinal type and 18 to the diffuse type, as described by Lauren (4). The stage of the tumors ranged from localized (PT1) to extensive (PT4), and the histological grade ranged from well differentiated (G1) to poorly differentiated (G3) adenocarcinomas.

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3 The abbreviations used are: CD44s, standard CD44; mAb, monoclonal antibody; PCR, polymerase chain reaction; cDNA, complementary DNA; PBS, phosphate-buffered saline.
The membranes were then incubated at room temperature with the polyclonal serum against the bacterial fusion protein encoded by variant exons v3-v10 (bar CD44v3-v10); the other bars indicate the approximate location of the epitopes of the monoclonal antibodies VFF4, VFF8, VFF9, VFF11, and VFF14. All monoclonal antibodies are presumably exon specific. For VFF14 and VFF11 the specificity has not yet been accurately defined.

Immunohistochemistry. Frozen sections were fixed in ice-cold methanol for 10 min, washed in PBS, and preincubated with normal goat serum (10% in PBS). They were then washed with PBS 3 times, followed by incubation with the primary antibody (in PBS with 1% bovine serum albumin) for 1 h. Endogenous peroxidases were then blocked with 0.3% H2O2 in methanol, and the sections were incubated with the secondary biotinylated antibody for 30 min (either anti-mouse or anti-rabbit F(ab')2 (DAKO Corp., Santa Barbara, CA), depending on the primary antibody used). Visualization of the immunocomplex was performed with horseradish peroxidase that had been coupled to biotin as a streptavidin-biotin-peroxidase complex (DAKO Corp.). After incubation with the streptavidin-biotin-peroxidase complex for 30 min the sections were incubated with 3,3-aminoethylcarbazol (Sigma Chemical Co.) for 5–10 min and then the reaction was stopped in HiO. The cells were counterstained with hematoxylin, mounted with glycerol-gelatin, and then viewed by microscopy.

Western Blot Analysis. Cells were lysed by sonication and then boiled in sodium dodecyl sulfate-gel sample buffer for 3 min (22). Equal amounts of protein (as measured by staining of the gel with Coomassie Brilliant Blue) were electrophoretically resolved on denaturing 6% polyacrylamide gels (22). The proteins were transferred to polyvinylidene difluoride membranes (Millipore GmbH, Eschborn, Germany) using a Transblot apparatus (Bio-Rad Laboratories, Munich, Germany). Nonspecific interactions were blocked by preincubation of the membranes with a milk powder suspension (10% dry milk in PBS). The membranes were then incubated at room temperature with the polyclonal serum anti-CD44v3-v10 (see Fig. 1), followed by an additional incubation with the polyclonal serum and with some of the monoclonal antibodies. With the monoclonal antibodies, the functional activity of the mAbs was ascertained by immunohistochemical staining of human skin keratinocytes (data not shown), which previously were shown to express variant CD44 exons v3-v10 (18, 20). Almost all tumors that were positive with the polyclonal serum also reacted with a mAb directed against exons v5 (VFF8 in Table 1; an example is shown in Fig. 2f). In contrast, reaction with the v6-specific antibody VFF4 was more restricted, because only 26 of 42 tumors stained positively (Table 1), suggesting either that the splice variants did not contain these exon sequences or that the epitopes were concealed. Interestingly, 23 of the 26 VFF4-positive tumors were adenocarcinomas of the intestinal type, whereas 14 of the 16 v6-negative cases were signet ring carcinomas of the diffuse type (an example is shown in Fig. 2e).

From 10 patients both primary tumors and lymph node metastases were available. Five of these pairs belonged to the intestinal type and five belonged to the diffuse type. Epitopes recognized by the polyclonal serum were present on both primary tumors and metastases in all of these 10 tumor pairs (Table 2; Fig. 2, a and b, shows the primary tumor 9069/90 of Table 2 and the corresponding lymph node metastasis). All of the tumor samples (primary tumors and metastases) reacted with the exon v5-specific mAb VFF8. All samples belonging to the intestinal type were positive upon incubation with the exon v6-specific mAb VFF4, whereas of the signet ring carcinomas only one pair belonged to the VFF4-positive group (see Table 2). We observed no difference in staining intensity between primary tumors and metastases, nor did we find consistent differences in the percentage of variant CD44-positive cells between primary tumors and metastases (Table 2). This was particularly true for lymph node metastases of VFF4 (exon v6)-negative signet ring carcinomas of the diffuse type, which also did not react with this mAb (Table 2).

Immunohistochemical Detection of CD44 Variants in Normal Gastric Mucosa. To explore whether the expression of CD44 splice variants in gastric tumors is the result of the transformation process or whether these are already expressed in normal gastric tissue, cryostat sections of normal gastric mucosa derived from 12 different patients were tested for immunohistochemical staining with variant CD44-specific antibodies. In fact, all 12 samples stained positively with the polyclonal serum and with some of the monoclonal antibodies. With the polyclonal serum (exons v3-v10) and mAb VFF8 (recognizing exon sequence v5) we obtained positive reactions on the mucoid surface epithelium, in the foveolar proliferation zone, and in areas of intestinal metaplasia (an example is shown in Fig. 2d).

RESULTS

Immunohistochemical Detection of CD44 Variants in Gastric Tumors. CD44 variant proteins differ from the standard CD44 protein by insertion of extra amino acids, encoded by 10 “variant” exons, into a distinct position in the extracellular domain of the protein (scheme in Fig. 1) (14, 16, 17, 20). We have raised a polyclonal serum and monoclonal antibodies against the human variant region of CD44 to explore the expression of CD44 variants in human tissues and tumors (18, 21). The specificity of these antibodies with respect to the fusion proteins encoding the variant exons is indicated in Fig. 1. We have used these antibodies to examine expression of CD44 proteins in human gastric malignancies (Fig. 2). The polyclonal serum that is directed against the v3-v10 fusion protein (CD44v3-v10; Fig. 1) stained 42 of 42 cryostat sections of stomach tumors (Table 1; examples in Fig. 2, a and b). The staining was heterogeneous with respect to intensity and distribution, in that between 5% and 100% of the tumor cells were stained with variable intensity.

To identify the variant exon sequences exposed on the surface of the tumor cells, we screened tumor cryostat sections with exon-specific monoclonal antibodies. The functional activity of the mAbs was ascertained by immunohistochemical staining of human skin keratinocytes (data not shown), which previously were shown to express variant CD44 exons v3-v10 (18, 20). Almost all tumors that were positive with the polyclonal serum also reacted with a mAb directed against exon v5 (VFF8 in Table 1; an example is shown in Fig. 2f). In contrast, reaction with the v6-specific antibody VFF4 was more restricted, because only 26 of 42 tumors stained positively (Table 1), suggesting either that the splice variants did not contain these exon sequences or that the epitopes were concealed. Interestingly, 23 of the 26 VFF4-positive tumors were adenocarcinomas of the intestinal type, whereas 14 of the 16 v6-negative cases were signet ring carcinomas of the diffuse type (an example is shown in Fig. 2e).

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Fig. 2. Immunohistochemistry of normal gastric mucosa and gastric adenocarcinomas. A focal, accentuated, anti-CD44v3-v10-positive reaction is seen in tumor cells of a moderately differentiated adenocarcinoma (intestinal type, according to the system of Lauren) of the stomach (a), as well as in the regional lymph node metastasis (b). In normal gastric mucosa with chronic gastritis the foci of intestinal metaplasia react positively with mAb VFF4 (c, arrows) and also with mAb VFF8 (d, arrows), accompanied by an additional reaction of mucoid surface and foveolar epithelium (d, arrowheads). Nearly all goblet cell carcinomas of the stomach (diffuse type, according to the system of Lauren) show a negative reaction with mAb VFF4 (e) and, in contrast to adenocarcinomas of the intestinal type, the normal mucoid epithelium is negative (e, arrowheads). In most cases a positive reaction with mAb VFF8 is seen in these goblet cell carcinomas (f), and the residual normal mucoid epithelium shows immunoreactivity (f, arrowheads). Avidin-biotin-peroxidase complex method. a and b, anti-CD44v3-v10 polyclonal serum, × 140; c, VFF4, × 80; d, VFF8, × 80; e, VFF4, × 210; f, VFF8, × 210; counterstain, hematoxylin.

<table>
<thead>
<tr>
<th>No. of positive tumors/no. of tested tumors</th>
<th>Anti-CD44v3-v10 serum</th>
<th>mAb VFF11 (v3/4)</th>
<th>mAb VFF8 (v5)</th>
<th>mAb VFF4 (v6)</th>
<th>mAb VFF9 (v7)</th>
<th>mAb VFF14 (v8-v10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adenocarcinomas</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse type</td>
<td>17/17</td>
<td>0/17</td>
<td>14/17</td>
<td>3/17</td>
<td>0/17</td>
<td>0/17</td>
</tr>
<tr>
<td>Intestinal type</td>
<td>25/25</td>
<td>0/25</td>
<td>25/25</td>
<td>23/25</td>
<td>0/25</td>
<td>0/25</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>42/42</td>
<td>0/42</td>
<td>39/42</td>
<td>26/42</td>
<td>0/42</td>
<td>0/42</td>
</tr>
</tbody>
</table>

Interestingly, the latter regions also stained positively with mAb VFF4 (recognizing exon sequence v6), whereas other parts of normal gastric epithelium did not react with this mAb (Fig. 2c). All other mAbs (VFF11, VFF9, and VFF14) did not react. Thus, in restricted areas of gastric mucosa a splice variant of CD44 is expressed that carries exon sequence v5, resembling the expression on cells of the diffuse-type carcinomas. Areas of gastric mucosa affected by intestinal metaplasia carry epitopes of both exon v5 and v6, resembling the expression
EXPRESSION OF CD44 VARIANTS IN GASTRIC CANCER

Table 2: Expression of variant CD44 epitopes in primary gastric tumors and corresponding lymph node metastases

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Adenocarcinoma</th>
<th>Intensity b</th>
<th>Positive cells (%)</th>
<th>Intensity</th>
<th>Positive cells (%)</th>
<th>Intensity</th>
<th>Positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse</td>
<td>645/3-10</td>
<td>++</td>
<td>100/100 c</td>
<td>++</td>
<td>20/90</td>
<td>-</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>12589/3-10</td>
<td>++</td>
<td>80/50</td>
<td>++</td>
<td>40/30</td>
<td>-</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>12924/3-10</td>
<td>++</td>
<td>70/80</td>
<td>++</td>
<td>70/70</td>
<td>+</td>
<td>30/30</td>
</tr>
<tr>
<td></td>
<td>25501/3-10</td>
<td>++</td>
<td>90/70</td>
<td>++</td>
<td>40/20</td>
<td>-</td>
<td>0/0</td>
</tr>
<tr>
<td>Intestinal</td>
<td>33383/3-10</td>
<td>++</td>
<td>80/30</td>
<td>++</td>
<td>90/90</td>
<td>+</td>
<td>20/90</td>
</tr>
</tbody>
</table>

a The numbers refer to the tumors and the corresponding lymph node metastases (not separately indicated). The tumors are included in the collection presented in Table 1.
b Intensity (because there was no difference in intensity between primary tumor and lymph node metastasis, staining is indicated for both together): -, negative; +, weak; ++, moderate; + + +, strong.
c Percentage of positive tumor cells in the primary tumor.
d Percentage of positive tumor cells in a lymph node metastasis from the same patient.

observed on intestinal-type tumors. These findings would be compatible with the idea that the tumors originate from these normal cells and maintain the expression pattern of the cells from which they arise.

CD44 Variant-specific RNA Expression in Gastric Tumors and Normal Gastric Mucosa. To examine whether the patterns of splice variants expressed in normal tissue and tumor cells were identical or different, RNAs were isolated from normal tissue and tumors, reverse transcribed, amplified by PCR, and hybridized to exon-specific probes. Using primers located 5' and 3' of the site of insertion of the variant exons (see "Materials and Methods"), we obtained one predominant PCR product, as revealed by ethidium bromide staining of the agarose gel, both in normal mucosa and in the gastric tumor samples (data not shown). Its size is indicative of CD44s. PCR products of minor abundance were detected by blotting onto nylon membranes and hybridization with exon-specific probes (Fig. 3). Hybridization with a v5- or v6-specific probe revealed expression of exon v5- and v6-containing RNAs. Furthermore, there was a striking difference in the patterns of RNAs expressed in normal tissue and in tumors. Among different tumors the patterns of RNA were also different.

RNA derived from 10 of the 12 normal gastric mucosa samples that were also examined by immunohistochemistry (see preceding paragraph) gave rise to two predominant bands hybridizing to exon v6 and to two bands with similar sizes hybridizing to exon v5 (four representative examples are shown in Fig. 3). The relative abundance of the v5- and v6-containing transcripts differed markedly from one sample to another, indicating that molecules containing exons v5 and v6 are regulated by a different mechanism in normal mucosa.

Fig. 3. Southern blot analysis of PCR amplification products from individual samples of normal gastric mucosa, from primary stomach tumors, and from corresponding lymph node metastases. The PCR primers were specific for CD44 exons adjacent to the variant exon sequences. cDNAs were produced by reverse transcription and were tested by glyceraldehyde phosphate dehydrogenase PCR prior to CD44s amplification, to check for quality and abundance of the cDNAs synthesized (data not shown). The PCR products obtained with CD44 standard primers (see "Materials and Methods") were resolved on 1.2% agarose and transferred to Hybond N+ membranes (Amersham), and the same filters were hybridized consecutively to probes specific for exon v5 (positions 243-356) (A) or exon v6 (positions 360-482 of the published human variant CD44 sequence) (20) (B). Lanes 1-5, five different primary stomach adenocarcinomas (PT) with corresponding lymph node metastases (LN), which were not included in the collection screened by immunohistochemistry; lanes 1-4, intestinal type; lane 5, diffuse type. Lanes 6-9, normal gastric mucosa from four different patients, from the corpus (lanes 6-8) and antrum (lane 9) regions.
not part of the same transcript. RNA derived from tumor samples yielded a complex and variable pattern of splice products. The tumors contained larger splice variants at least in part, up to sizes that would be compatible with the presence of all variant exon sequences (Fig. 3). Note, however, that not all of the epitopes were accessible to immunohistochemistry. Comparison of the various tumor samples revealed that the expression patterns of CD44 variant RNAs containing exon v5 or v6 differed among the different tumors. Furthermore, even among primary tumors and lymph node metastases derived from the same tumors, differences in the PCR patterns were observed. Both diffuse-type and intestinal-type tumors showed strong expression of exon v5-containing transcripts (Fig. 3A, lanes 1-5). There was, however, a clear difference in hybridization to the exon v6-specific probe between intestinal-type and diffuse-type tumors. Whereas in all four samples of intestinal-type tumors amplification products containing exon v6 could be detected (see Fig. 3B, lanes 1-5), the sample of a diffuse-type tumor showed only very weak hybridization to the v6-specific probe (Fig. 3B, lane 5).

The data from PCR amplification and hybridization revealed that tumor cells express a much more complex pattern of variant CD44 transcripts than does normal gastric mucosa. It should be added, however, that the samples used for RNA preparations were heterogeneous with respect to the content of different types of cells and might contain macrophages and lymphocytes, which could contribute to the complex splice pattern. We therefore examined cell lines to explore whether the differences in the splice pattern of the various tumor samples are also observed in different clonal tumor cell lines.

Expression of CD44 Variants in Stomach Cancer Cell Lines. Only a few laboratories have succeeded in establishing cell lines derived from stomach tumors. Three such cell lines derived from intestinal-type tumors have been isolated and characterized recently (19). Two of these lines (2957 and 2474) were isolated from a lymph node metastasis, and the other one (3051) was established from a primary tumor. The cell lines 2957 and 3051 express variant CD44 proteins with apparent molecular weights between 90,000 and 180,000, as judged from staining with the variant-specific polyclonal serum (anti-CD44v3-v10) of proteins extracted from the cells (Fig. 4). The staining pattern of protein extracts from the third cell line (2474) was not significantly different from that of the melanoma cell line MeWo, which was previously shown to lack expression of CD44 variants (18, 20). Reverse transcription-PCR amplification revealed only weak variant CD44 expression in the cell line 2474, in contrast to the two other cell lines. PCR products indicating CD44 variant expression were observed by ethidium bromide staining of the agarose gels (Fig. 5). In an experiment performed with cDNA derived from primary tumors and from normal mucosa, as well as with cDNA from MeWo cells, ethidium bromide staining revealed no bands larger than 0.44 kilobase (data not shown). In the cell lines 2957 and 3051 the abundance of CD44 variants even exceeded the amount of the CD44s-specific band (0.44 kilobase). Southern blotting of the fragments on nylon membranes and hybridization with CD44 variant-specific probes revealed a complex pattern of variant CD44 transcripts. The two cell lines with a similar pattern in Western blotting also revealed a similar pattern in Southern blot analysis, whereas the cell line 2474 (negative in Western blotting) showed much weaker signals (Fig. 6). In the MeWo cell line no variant CD44 transcripts were visible (data not shown).

The various transcripts seen in the cell lines suggest that the complex splice pattern detected in human samples not only was due to contaminating cells but also was the result of a splice disorder in the tumor cells. Further, coexpression of CD44s and CD44 variants in the cell lines suggests that not all of the abundant CD44s transcripts seen in PCRs from tumor samples were derived from contaminating stromal and blood cells.

![Fig. 4. Western blot analysis of several human cell lines with the CD44v3-v10-specific polyclonal serum. Arrows, CD44 variant epitope-positive proteins. The same amount of cell lysate was loaded in each lane, as judged by Comassie Brilliant Blue staining (data not shown). Cell lysates were resolved on 6% sodium dodecyl sulfate-polyacrylamide. Numbers, gastric tumor cell lines, as described in "Materials and Methods." MeWo represents a CD44 variant-negative melanoma cell line used to identify nonspecific binding of the antibodies.](cancerres.aacrjournals.org)
exons v6 and v7 are necessary and sufficient to confer metastatic behavior to tumor cells in the spontaneous metastasis assay (14, 15). Here we show that similar splice variants are also expressed in human gastric tumors. The methods of reverse transcription-PCR amplification and hybridization with variant exon-specific probes, as well as immunohistochemistry of frozen sections using variant exon sequence-specific monoclonal antibodies, permit semiquantitative and qualitative conclusions. Upon immunohistological staining of cryostat sections of gastric tumors, we found expression of CD44 splice variants in 42 of 42 cases. The staining pattern resembles that observed for E-cadherin, a protein that mediates epithelial cell-cell interaction (25).

It is premature, however, to derive conclusions regarding function from this similarity in staining. Furthermore, cell lines established from gastric cancers were also positive for variant CD44 expression. On a restricted number of cell types in the normal gastric mucosa, expression of variants of CD44 was also detected. PCR analysis of RNA derived from different primary tumors, metastases, and normal gastric mucosa revealed that a complex pattern of different splice variants is expressed in tumors, in contrast to the expression of only one or two splice variants in normal gastric mucosa. The pattern of expression differs from one tumor to another, whereas expression in normal gastric mucosa cells is uniform.

Defined areas of normal gastric mucosa carry CD44 molecules with epitopes encoded by exons v5 and v6. The intensity of staining of these cells is loosely comparable to that of gastric carcinoma cells. Thus, cells of the gastric mucosa belong to the restricted group of normal tissues in which expression of CD44 variants occurs (18). Exon sequences v3 and v7-v10 do not seem to be expressed in normal gastric mucosa or are not accessible to the antibodies.

According to PCR data, normal gastric mucosa synthesizes two major splice variants hybridizing to exon v6. It is, however, not clear whether these variants are synthesized in the same type of cells. Hybridization with exon v5 reveals two RNA species, obviously not identical to those hybridizing to exon v6. Although mucoid surface epithelium or metaplasias stain uniformly with the v5-specific mAb in immunohistochemistry, these cells appear to be represented at different levels in the normal mucosa samples investigated by reverse PCR (Fig. 3A). These observations, therefore, signal caution in the evaluation of tumor samples by PCR. The positive cells may be absent in the sample chosen. Additionally, false-positive data can be generated by, for instance, contaminating resting and activated lymphocytes (21, 26). It is also difficult to draw quantitative conclusions from PCR data.

In contrast to normal tissue, tumor samples show a much more complex pattern of CD44 splice variant expression at the RNA level (Fig. 3). This indicates loss of stringent splice control in the tumor cells. Similar observations have been made in mammary tumors.4 In contrast, colorectal tumors seem to express a few well defined CD44 splice variants (18).5

All samples of lymph node metastases originating from gastric tumors expressed CD44 variants (see Table 2 and Fig. 3). This result resembles the findings in lymph node metastases of colorectal tumors and mammary carcinomas (18).6 When screened with an exon v6-specific probe, each pair of primary tumor (intestinal type) and metastasis showed rather similar expression patterns (Fig. 3B), whereas no obvious correlation between primary tumors and lymph node metastases with respect to v5-containing transcripts could be detected (Fig. 3A). This suggests that there are tumor cell populations in the primary tumors and lymph node metastases that are identical with respect to exon v6 expression, indicating a possible role of this exon sequence in the metastatic process. Involvement of this exon in the metastatic process was reported earlier (14). In contrast, exon v5-containing transcripts seem to appear at random in primary tumors and metastases. Thus, exon v5 does not seem to confer a selective advantage to the tumor cells, although they synthesize exon v5-containing transcripts, possibly due to a splice disorder in these cells. The possibility also remains that exon v5 sequences stem from contaminating cells.

It is noteworthy that most of the investigated diffuse-type adenocarcinomas did not express exon v6. This was confirmed at both the protein and RNA levels (Table 2; Figs. 2e and 3B, lane 3). More challenging is the observation that even lymph node metastases of these tumors are devoid of exon v6-containing proteins and transcripts (Table 2; Fig. 3B, lane 3). If these splice variants confer any advantage to the cells, the tumor pathways must be different. From the size of some PCR products, exon sequences v7 through v10 should be included in these molecules. The lack of reactivity with mAbs VFF9 and VFF14 suggests that the respective epitopes are concealed (e.g., by post-translational modification or folding of the protein).

The data from clonal tumor cell lines show that the complex and differing patterns of variant CD44 expression in tumors cannot be the result of contamination with infiltrating blood cells alone. The cell lines produce a multitude of large CD44 variants and only little CD44s. Thus, in the surgical samples CD44s could well be derived from nontumor cells.

Both immunohistochemical and PCR data indicate differences between diffuse-type and intestinal-type adenocarcinomas with respect to CD44 splice variant expression. Most signet ring carcinomas express a CD44 variant containing exon sequence v5, at both the RNA

**DISCUSSION**

In the rat, splice variants of CD44 carrying exons v4 through v7 or exons v6 and v7 are necessary and sufficient to confer metastatic behavior to tumor cells in the spontaneous metastasis assay (14, 15). Upon immunohistological staining of cryostat sections of gastric tumors, we found expression of CD44 splice variants in 42 of 42 cases. The staining pattern resembles that observed for E-cadherin, a protein that mediates epithelial cell-cell interaction (25). It is premature, however, to derive conclusions regarding function from this similarity in staining. Furthermore, cell lines established from gastric cancers were also positive for variant CD44 expression. On a restricted number of cell types in the normal gastric mucosa, expression of variants of CD44 was also detected. PCR analysis of RNA derived from different primary tumors, metastases, and normal gastric mucosa revealed that a complex pattern of different splice variants is expressed in tumors, in contrast to the expression of only one or two splice variants in normal gastric mucosa. The pattern of expression differs from one tumor to another, whereas expression in normal gastric mucosa cells is uniform.

Defined areas of normal gastric mucosa carry CD44 molecules with epitopes encoded by exons v5 and v6. The intensity of staining of these cells is loosely comparable to that of gastric carcinoma cells. Thus, cells of the gastric mucosa belong to the restricted group of normal tissues in which expression of CD44 variants occurs (18). Exon sequences v3 and v7-v10 do not seem to be expressed in normal gastric mucosa or are not accessible to the antibodies.

It is noteworthy that most of the investigated diffuse-type adenocarcinomas did not express exon v6. This was confirmed at both the protein and RNA levels (Table 2; Figs. 2e and 3B, lane 3). More challenging is the observation that even lymph node metastases of these tumors are devoid of exon v6-containing proteins and transcripts (Table 2; Fig. 3B, lane 3). If these splice variants confer any advantage to the cells, the tumor pathways must be different. From the size of some PCR products, exon sequences v7 through v10 should be included in these molecules. The lack of reactivity with mAbs VFF9 and VFF14 suggests that the respective epitopes are concealed (e.g., by post-translational modification or folding of the protein).

The data from clonal tumor cell lines show that the complex and differing patterns of variant CD44 expression in tumors cannot be the result of contamination with infiltrating blood cells alone. The cell lines produce a multitude of large CD44 variants and only little CD44s. Thus, in the surgical samples CD44s could well be derived from nontumor cells.

Both immunohistochemical and PCR data indicate differences between diffuse-type and intestinal-type adenocarcinomas with respect to CD44 splice variant expression. Most signet ring carcinomas express a CD44 variant containing exon sequence v5, at both the RNA
and protein levels, whereas intestinal-type carcinomas express both exons v5 and v6. Also a subpopulation of normal gastric epithelial cells expresses exon v5 alone. Intestinal metaplasia, a precancerous lesion that has been suggested to be an intermediate in the carcinogenesis of intestinal-type tumors, expresses exons v5 and v6, similarly to intestinal-type tumors. This difference between diffuse-type and intestinal-type tumors with respect to expression of exon v6 allows, for the first time, discrimination between these two types of gastric cancer by molecular markers. Furthermore, even presumptive precursor lesions are easily distinguishable from normal gastric mucosa due to specific expression of exon v6 within these lesions. In addition, the difference in v6 expression supports the theory that diffuse-type and intestinal-type carcinomas of the stomach arise from different precursor cells of the normal stomach mucosa and progress by different pathways.

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