CD44 glycoproteins in colorectal cancer; expression, function and prognostic value

Wielenga, V.J.M.

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Chapter 7

CO-EXPRESSION OF C-MET AND HEPARAN SULFATE PROTEOGLYCAN FORMS OF CD44 IN COLORECTAL CANCER

Vera J.M. Wielenga, Robbert van der Voort, Taher E.I. Taher, Lia Smit, Cees van Krimpen, and Steven T. Pals
ABSTRACT

In colorectal cancer patients, prognosis is not determined by the primary tumor but by the formation of distant metastases. Among the molecules that have been implicated in the metastatic process are the receptor tyrosine kinase c-Met and CD44 glycoproteins. Recently, we provided evidence for functional collaboration between these two molecules. CD44 isoforms decorated with heparan sulfate (HS) chains (CD44-HS) bind the c-Met ligand, the growth and motility factor hepatocyte growth factor/scatter factor (HGF/SF). This interaction strongly promotes c-Met signaling, a pathway mediating a broad range of biological activities including cell-growth and -motility, angiogenesis, and tumor invasion and metastasis. In the present study, we have assessed the expression of c-Met, HGF/SF, and CD44-HS in human colorectal cancer. We demonstrate that c-Met is strongly expressed on colorectal adenomas, carcinomas, and colorectal cell lines, while HGF/SF mRNA levels are increased levels within tumor tissue. Both heparitinase treatment and experiments with an HGF/SF mutant with strongly reduced affinity for heparan sulfate, indicate that interaction of HGF/SF with HS-moieties promotes activation of c-Met in colorectal carcinoma (HT-29) cells. Survival analysis shows that CD44-HS (CD44v3) expression predicts unfavorable prognosis in patients with invasive colorectal carcinomas. Our findings suggest that collaboration between CD44-HS and the c-Met signaling pathway plays an important role in colorectal tumorigenesis. In tumors overexpressing CD44-HS, constitutively enhanced c-Met signaling may promote tumor growth, invasiveness and metastasis, resulting in poor patient survival.

INTRODUCTION

The molecular genetics of colorectal cancer are among the best understood of any solid neoplasm (Kinzler et al., 1996; Korinek et al., 1997; Morin et al., 1997; Eppert et al., 1996). Several important molecules implicated in the tumorigenetic process act on the cell cycle, resulting in a disturbed homeostasis between cell proliferation and apoptosis (Kinzler et al., 1996). The main cause of tumor related death in colorectal cancer, however, is the
formation of distant metastases, rather than the growth of the primary tumor. Although relatively little is known concerning the molecular mechanisms underlying this complex process, recent studies have identified CD44 glycoproteins (reviewed in Wielenga et al., 1999a) and the c-Met receptor tyrosine kinase (reviewed in Jeffers et al., 1996a) as potentially important components of the metastatic cascade.

CD44 is a family of transmembrane receptors generated from a single gene by alternative splicing and differential glycosylation (Lesley et al., 1993; Stamenkovic et al., 1989; Günthert et al., 1991; Screaton et al., 1992; Jackson et al., 1995). Important biological processes involving CD44 glycoproteins include cell adhesion (Aruffo et al., 1990), lymphocyte homing (Jalkanen et al., 1987; Lesley et al., 1993; DeGrendele et al., 1997), hematopoiesis (Lesley et al., 1993, and tumor progression and metastasis (Lesley et al., 1993; Naor et al., 1997; Wielenga et al., 1999a; Günthert et al., 1991; Koopman et al., 1993). In colorectal cancer, CD44 glycoproteins, which are normally detected only in the lower crypt epithelium of the intestinal mucosa, are overexpressed (Heider et al., 1993; Mulder et al., 1995; Ropponen et al., 1998; Yamaguchi et al., 1996; Wielenga et al., 1998, 1999a,b). This overexpression is an early event in the colorectal adenoma-carcinoma sequence (Wielenga et al., 1993; Kim et al., 1994) suggesting a causal relation to loss of APC tumor suppressor gene function. Indeed, recent studies in Apc and Tcf-4 mutant mice indicate that CD44 expression in normal and neoplastic intestinal epithelium is regulated by the Wnt-signaling pathway (Wielenga et al., 1999b).

The precise mechanisms via which CD44 promotes tumorigenesis have not yet been elucidated. CD44 functions as a molecular linker between extracellular matrix (ECM) molecules, specifically hyaluronate, and the cell and cytoskeleton (Lesley et al., 1993; Aruffo et al., 1990; Kalomiris et al., 1988; Tsukita et al., 1994). Recently, CD44 isoforms decorated with heparan sulfate-side (HS) chains have been shown to bind and present growth factors (Bennett et al., 1995; Tanaka et al., 1993; van der Voort et al., 1999). We demonstrated that CD44-HS binds the growth and motility factor hepatocyte growth factor/scatter factor (HGF/SF). This interaction strongly promotes signaling through c-Met, the high-affinity receptor for HGF/SF (van der Voort et al., 1999). The HGF/SF - c-Met pathway is essential for normal murine
embryonal development (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995) and affects a wide range of biological activities including angiogenesis, cell motility, growth, and morphogenesis. In addition, there is ample evidence for a key role of HGF/SF - c-Met pathway in tumor growth, invasion and metastasis (reviewed in Birchmeier et al., 1996; Jeffers et al., 1996a; Maggiora et al., 1997). For example, c-Met was isolated originally as the product of a human oncogene, Tpr-met, which encodes a consitutively dimerized/activated chimeric c-Met protein possessing transforming activity (Cooper et al., 1984; Rodrigues et al., 1993). The generation of an autocrine loop as a result of coexpression of wild-type c-Met and HGF/SF molecules in the same cell is also oncogenic (Jeffers et al., 1996b). The tumorigenicity of both Tpr-Met and autocrine HGF/SF-Met signaling has been verified in transgenic mouse models, which develop tumors in many different tissues including mammary glands, skeletal muscles and melanocytes (Liang et al., 1996; Takayama et al., 1997). c-Met activation has also been shown to promote the metastatic spread of cancer, a finding that likely is due to its stimulatory effects on a variety of processes such as angiogenesis, cell motility, and protease secretion (Jeffers et al., 1996c). Recently, missense mutations in c-Met were found to be associated with human papillary renal carcinomas (Schmidt et al., 1997). These mutations deregulate the enzymatic activity of the receptor, thereby unleashing its oncogenic potential (Jeffers et al., 1997).

To explore whether collaboration between CD44-HS and the HGF/SF - c-Met pathway is an option in colorectal cancer, the present study investigates the expression of these molecules during colorectal tumorigenesis.

MATERIALS AND METHODS

Antibodies. Mouse monoclonal antibodies used were 3G5 (anti-CD44v3, IgG2b)(R&D Systems, Abingdon, UK); 3G10 (anti-desaturated uronate from heparitinase treated heparan sulfate; 'HS-stub', IgG2b) (David et al., 1992); PY-20 (anti-phosphotyrosine, IgG2b) (Affiniti, Nottingham, UK); anti-human c-Met (IgG2a)(R&D Systems, Abingdon, UK). Polyclonal antibodies used were C-12 (rabbit anti-c-Met, IgG)(Santa Cruz Biotechnology, Santa Cruz, CA); HRP-conjugated rabbit anti-mouse (DAKO, Glostrup, Denmark); HRP-
conjugated goat anti-rabbit (DAKO); HRP-conjugated swine anti-rabbit (DAKO); biotin-conjugated rabbit anti-mouse (DAKO). In addition we used RPE-conjugated streptavidin (DAKO).

Cell lines. The colon carcinoma cell lines SW480, SW620, colo 201, colo 205, colo 320 and HT-29, were purchased from the American Type Culture Collection (ATCC, Rockville, MD). HT-29 cells were cultured in modified McCoy's 5A medium (Gibco BRL/Life Technologies, Paisley, UK), while SW480 and SW620 were cultured in L-15 (Leibovitz) medium (Gibco BRL/Life Technologies). The other cell lines were cultured in RPMI 1640 (Gibco BRL/Life Technologies). All media were supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin (all Gibco BRL/Life Technologies).

Purification of wild type and mutant HGF/SF. The construction of pVL1393 vectors (Pharmingen, San Diego, CA) containing wild type or mutant HGF/SF (HP1) cDNA was described elsewhere (Hartmann et al., 1998). HGF/SF (wild type and HP1) was produced in a Baculovirus system as described previously (Hartmann et al., 1992). In brief, sf 9 insect cells were transduced with an amplified virus stock and after 3 days media were pooled and analyzed for scattering activity in the MDCK dissociation assay (Weidner et al., 1990). Then, HGF/SF was purified with Ni-NTA-resin from the QIA expressionist system (Qiagen, Hilden, Germany). HGF/SF concentrations were measured by ELISA as described previously (van der Voort et al., 1997), and in addition, HGF/SF (wild type and mutant) was analyzed by Western blotting using anti-goat-HGF/SF.

Enzyme treatments. For enzymatic cleavage of GAGs, cells were treated with either heparitinase (Flafobacterium heparinum, EC 4.2.2.8, ICN Biomedicals, Aurora, OH) or chondroitinase ABC (Proteus vulgaris, EC 4.2.2.4, Boehringer Mannheim, Almere, The Netherlands) in PBS at 37°C for the periods indicated. Enzyme treatments were followed by immunoprecipitation.

Immunoprecipitation and Western blot analysis. Immunoprecipitation was
performed as described (van der Voort et al., 1997). The only modification were that, for precipitation of CD44, cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP-40, 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 1 mM sodium orthovandate (Sigma), 2 mM EDTA, and 5 mM NaF. For precipitation of c-Met, cells were lysed in 10 mM Tris-HCl (pH 8), 150 mM NaCl, 10% glycerol, 1% NP-40, 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 2 mM sodium orthovandate (Sigma), 5 mM EDTA, and 5 mM NaF.

Western blotting of immunoprecipitates and total cell lysates was essentially performed as described previously (Taher et al., 1996), with the modification that, for analysis of phosphorylated proteins, membranes were blocked and stained in 2% BSA, 20 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 0.05% Tween-20 (Sigma). Films were scanned with an Eagle Eye II video system (Stratagene, La Jolla, CA) and band intensities were determined with ONE-Dscan software (Stratagene). c-Met phosphorylation was expressed as the ratio of phosphorylated c-Met to precipitated c-Met.

**Tissue samples.** The study set consisted of 54 primary colorectal carcinomas (Mulder et al., 1994), removed at operation between January 1, 1983 and January 1, 1986 at the Department of Surgery, Reinier de Graaf Hospital, Delft, The Netherlands, of which snap-frozen tissue and follow-up till June 1, 1992 (6.5-9.5 years) was available. The mean age of the patients at diagnosis was 69.7 (range 39-92) and the male to female ratio was 28/40. Colorectal tissue samples of 6 adenomas and 6 normal controls, removed at operation between January 1, 1992 and January 1, 1999 were obtained from the tissue bank of the Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

**Immunohistochemistry and statistical analysis.** Frozen tissue sections were tested for the expression of CD44v3 or c-Met by immunohistochemistry as described previously (Heider et al., 1993; Koopman et al., 1993). A single modification was that HRP-conjugated rabbit anti-mouse and HRP-conjugated swine anti-rabbit were used as secondary and tertiary antibodies, respectively. All slides were read by two independent observers, and discrepancies were
solved by consensus. The tumor samples were scored as described previously (Mulder et al., 1994; Wielenga et al., 1998): 0 (low/negative) = less than 10% of the cells positive; 1 (intermediate) = 10 to 50% of the cells positive; 2 (high) = more than 50% of the cells positive. Survival functions were estimated by the Kaplan-Meier method and comparison of survival functions between groups was performed by the Log-rank test.

RNA isolation and RT-PCR. RNA isolation and first strand cDNA synthesis were performed as described previously (van der Voort et al., 1997). PCR was performed with 1.5 U Taq DNA Polymerase (Gibco BRL/Life Technologies), 200 µM dNTPs (Pharmacia Biotech, Uppsala, Sweden) and 1.5 mM MgCl₂ (2 mM for GAPDH) in 1 x PCR Buffer (both Gibco BRL/Life Technologies). Primers used were HGF-1 (5'-CGACAGTGTTTCCCTTCTCG-3') in combination with HGF-3 (5'-GGTGGGTGCGACACACAC-3'), or GAPDH-D (5'-GGCAGAGATGATGACCCTTTTGGC-3') in combination with GAPDH-U (5'-AAGGTGAAGGTCGGAGTCAACG-3'). PCR was started with a 5 min denaturation step at 95°C, after which amplification was performed in 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C (55°C for GAPDH) for 1 min (30 s for GAPDH) and elongation at 72°C for 2 min (30 s for GAPDH). After a final elongation step for 10 min at 72°C, samples were cooled on ice and analyzed by electrophoresis in an 1.5% agarose TBE-gel containing ethidium bromide.

FACS analysis. For FACS analysis cells were blocked with 10% pooled human serum (CLB, Amsterdam, The Netherlands), 1% BSA (Fraction V) (Sigma) in PBS at 4°C for 15 min and washed with FACS buffer (1% BSA in PBS), respectively. Then, the cells were incubated with the primary antibodies for 1 h, washed, and incubated with the secondary antibody for 30 min. All incubations were performed in FACS buffer at 4°C. Stained cells were analyzed by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA).

HS-proteoglycan dependent phosphorylation of c-Met in HT29 cells. HT29 cells were cultured in 6-well plates until subconfluent (60-80%) and then
Figure 1. Expression of CD44v3 and c-Met by normal colon mucosa and colorectal carcinomas. (A and C) Normal colon mucosa and (B and D) colorectal carcinoma serial frozen tissue sections were stained for (A and B) CD44v3 or for (C and D) c-Met by immunohistochemistry. (A) Normal colon mucosa showing weak focal expression of CD44v3 in the lower part of the crypts. (B) Invasive colorectal carcinoma with strong CD44v3 expression. (C) Normal colon mucosa showing weak expression of c-Met. (D) Invasive colorectal carcinoma with strong c-Met expression. Tissues were counterstained with haematoxylin. Bar, (A and C) 57 μm; (B and D) 69 μm.

starved overnight. Part of the cells were treated with heparitinase as mentioned above and activated with 100 ng/ml wild type HGF/SF or mutant HGF/SF (HP1) in 0.5 ml serum free, pre-warmed medium for 10 min at 37°C. Cells were washed once with cold PBS and were immediately cooled on ice. 500 ul lysis buffer was added and cells were harvested. c-Met was precipitated and c-Met phosphorylation was analyzed by Western blotting.
RESULTS

Co-expression of CD44-HS and c-Met in colorectal cancer. Previous studies have shown that heparan sulfate forms of CD44 (CD44-HS) are splice variants containing exon v3. (Jackson et al., 1995; van der Voort et al., 1999). To explore the expression of CD44-HS during colorectal tumor progression, we compared CD44v3 levels in normal colon mucosa, adenomas, and carcinomas (Table 1; Fig. 1). In the normal colon mucosa CD44v3 expression was low to intermediate and strictly confined to the base of the crypts. By contrast, in all adenomas and in 70% (38/54) of the invasive carcinomas, an intermediate to high expression of CD44v3 was observed (Table 1).

For c-Met, enhanced expression along the adenoma-carcinoma sequence was also observed. Whereas the epithelium of the normal colon mucosa showed a low to intermediate expression, c-Met expression in adenomas and carcinomas was intermediate to high and high, respectively (Table 1; Fig. 1).

Colorectal carcinoma cell lines co-express CD44v3 and c-Met. To strengthen the observation that colorectal carcinomas co-express CD44v3 and c-Met, the presence of these molecules was assessed by FACS on a panel of colorectal carcinoma cell lines. On all of these carcinoma cell lines, i.e., colo 320, HT29, SW480, SW620, colo 201, colo 205, and colo320 both CD44v3 and c-Met are clearly expressed (Fig. 2).

Taken together, the above expression studies show that co-expression of CD44v3 and c-Met is common in both primary colorectal adenomas and carcinomas and in colorectal carcinoma cell lines.

CD44v3 on colorectal cells is decorated with heparan sulfate. To verify whether the glycosylation machinery of colorectal cancer cells indeed decorates CD44v3 with HS-chains, we studied CD44v3 immunoprecipitates from the colorectal cancer cell lines SW480 and HT-29 on Western-blot with mAb 3G10. This mAb recognizes the ‘HS-stubs’ that remain on HS-proteoglycans after heparitinase treatment (David et al., 1992). Hence, prior to immunoprecipitation, the tumor cells were treated with heparitinase, or as controls, were sham treated or chondroitinase treated. As is shown by staining
Figure 2. Expression of CD44v3 and c-Met by colon carcinoma cell lines. (A) FACS analysis of the expression of CD44v3 on the colon carcinoma cell lines colo 320, HT-29, SW480, SW620, colo 201, colo 205, and colo320. Wild-type and CD44v3-10 transfected Namalwa cells are shown as negative or positive controls, respectively. Expression was analyzed with mouse anti-CD44v3 (filled histogram) or an isotype-matched control antibody (empty histogram), followed by RPE-conjugated goat anti-mouse. (B) FACS analysis of the c-Met expression on the colon carcinoma cell lines shown in (A). Wild-type or c-Met transfected Namalwa cells are shown as negative and positive controls, respectively. Expression was analyzed with mouse anti-c-Met (filled histogram) or an isotype-matched control antibody (empty histogram), followed by RPE-conjugated goat anti-mouse.
Figure 3. CD44v3 isoforms on colon carcinoma cell lines are decorated with heparan sulfate. CD44v3 was immunoprecipitated from the colon carcinoma cell lines SW480 and HT-29, and, as a positive control, from Namalwa cells transfected with CD44v3-10, with mouse anti-CD44v3. Prior immunoprecipitation, the cells were treated with either PBS (-), 30 mU/ml heparitinase (HT), or 30 mU/ml chondroitinase ABC (CH) at 37°C for 3.5 h. The Western blot of the precipitates was stained with the anti-pan CD44 mAb Hermes-3, stripped, and re-stained with the mAb 3G10 that detects ΔHS-stubs after treatment of HS with heparitinase. CD44v3 isoforms decorated with HS are indicated with arrows.

with an anti-pan CD44 mAb (Fig. 3), one major CD44v3 species of approximately 200 kDa was precipitated from SW480 cells whereas two species of approximately 150 and 200 kDa were precipitated from HT-29. The size of the latter CD44 variant was identical to that from a control cell line
(Namalwa) expressing a single CD44 isoform containing v3-10 (van der Voort et al., 1995; 1999), whereas the shorter species most probably contains a shorter variable domain. Staining of the blots with the 'anti-HS-stub' mAb revealed the presence of bands corresponding to those obtained after staining with the anti-pan CD44 mAb. These bands were specifically present in the precipitates of the heparitinase treated cells, but not in the precipitates of sham- or chondroitinase-treated cells (Fig. 3). Hence, CD44v3 isoforms on colorectal cancer cell lines are HS-decorated.

Expression of HGF/SF in colorectal cancer tissue samples. To explore whether the c-Met ligand HGF/SF is also expressed within the colorectal carcinoma microenvironment, HGF/SF mRNA expression was measured by RT-PCR in paired samples of normal and neoplastic colon tissue from 5 patients. As is shown in Figure 4, HGF/SF mRNA expression was readily detectable in all colorectal carcinoma samples. Although we did not quantify the PCR products, the expression of HGF/SF in the tumor samples was clearly increased, compared to the normal mucosa.

Heparan sulfate on colorectal cancer cells promotes ligand induced c-Met phosphorylation. To investigate whether HS-chains on colorectal cancer cells are able to present HGF/SF to c-Met and promote signaling, the tyrosine phosphorylation of c-Met was studied in HT-29 cells that 1) were either or not treated with heparitinase prior to HGF/SF stimulation; 2) were stimulated with HP1, a non HS-binding mutant form of HGF/SF (Hartmann et al., 1998). As is shown in Figure 5A, heparitinase treatment of HT-29 cells led to an approximately 5-fold reduction in the HGF/SF phosphorylation of c-Met. Also, the HGF/SF mutant HP1 was significantly less potent in inducing c-Met phosphorylation (Fig. 5B). These findings indicate that interaction of HGF/SF with HS-proteoglycans expressed on the surface of colorectal cancer cells facilitates c-Met signaling.
Table 1. Expression of CD44v3 and c-Met in colorectal tumorigenesis.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>expression level*</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD44v</td>
<td>c-Met n (%)</td>
</tr>
<tr>
<td>normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>crypt base</td>
<td>negative/low</td>
<td>1 (17)</td>
</tr>
<tr>
<td></td>
<td>intermediate</td>
<td>4 (67)</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>1 (17)</td>
</tr>
<tr>
<td>upper crypt</td>
<td>negative/low</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>intermediate</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>0</td>
</tr>
<tr>
<td>polyp</td>
<td>negative/low</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>intermediate</td>
<td>4 (67)</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>2 (33)</td>
</tr>
<tr>
<td>carcinoma</td>
<td>negative/low</td>
<td>16 (30)</td>
</tr>
<tr>
<td></td>
<td>intermediate</td>
<td>19 (35)</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>19 (35)</td>
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</tbody>
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* Negative/low, intermediate, high: expression on <10%, 10-50% or >50%

Figure 4. HGF/SF mRNA expression in normal colon mucosa and colorectal carcinomas. RT-PCR was performed on total RNA isolated from five pairs of normal colon (N) and primary colorectal carcinoma (T), on water (none), and on a plasmid containing full-length human HGF/SF cDNA (pHGF/SF). Primers used were HGF/SF-specific or, as a control, glyceraldehydephosphatase dehydrogenase (GAPDH)-specific.
CD44v3 expression is related to poor prognosis. Previous studies from our own and other laboratories have shown that CD44 splice variants containing v6 and v8-10 are unfavorable prognosticators in colorectal cancer. Expression of these variants on the primary tumor predicts metastatic disease and tumor related death (Mulder et al., 1994; Yamaguchi et al., 1996; Wielenga et al., 1998; Ropponen et al., 1998). In view of the ability of CD44v3 to present growth factors, which may promote metastasis, we now studied whether expression of CD44v3 also predicts prognosis. CD44v3 was assessed in a study group of 54 colorectal cancer patients with a long term (6.5-9.5 years) and complete follow-up. Details on this study group have been published previously (Mulder et al., 1994; Wielenga et al., 1998). As is depicted in Table 2 and Figure 6, CD44v3 expression on the primary tumor indeed predicts tumor related death. CD44v3 expression on the tumors was strongly correlated to expression of CD44v6 (data not shown).

Table 2. Prognostic significance of CD44v3 expression.

<table>
<thead>
<tr>
<th>Level of CD44v3 expression**</th>
<th>Number of patients n (%)</th>
<th>Median survival in days</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative/low</td>
<td>16 (30)</td>
<td>1577</td>
<td></td>
</tr>
<tr>
<td>intermediate</td>
<td>19 (35)</td>
<td>1167</td>
<td>0.03</td>
</tr>
<tr>
<td>high</td>
<td>19 (35)</td>
<td>294</td>
<td></td>
</tr>
</tbody>
</table>

* Log-rank test from a univariate analysis. ** Negative/low, intermediate, high: expression on <10%, 10-50% or >50% of the tumor cells, respectively.
Figure 5. Efficient phosphorylation of c-Met in HT29 cells requires interaction between HGF/SF and HS moieties. (A) Comparison of HGF/SF induced c-Met phosphorylation in HT29 cells with or without heparitinase treatment (HT). Cells were either (+) or not (-) activated with 100ng/ml HGF/SF. c-Met was immunoprecipitated and phosphorylation of the β-chain of c-Met was visualized on Western blot with an anti-PY mAb. The blot was stripped and re-stained with rabbit anti-c-Met followed by HRP-conjugated goat anti-rabbit (lower panel). (B) Comparison c-Met phosphorylation in HT29 cells upon stimulation with wild-type HGF/SF, or HP1, a non HS-binding mutant form of HGF/SF.
DISCUSSION

Deregulation of c-Met signaling can initiate and promote tumor growth and dissemination. The present study shows that c-Met is strongly expressed on primary colorectal adenomas, carcinomas, and on colorectal cell lines, while HGF/SF mRNA is present at increased levels within tumor tissue. In addition, it demonstrates that a subset of colorectal carcinomas with unfavorable prognosis strongly express CD44-HS. Since CD44-HS can bind and present HGF/SF, and promotes signaling through c-Met (van der Voort et al., 1999), our observations suggest a role for functional collaboration between CD44-HS and the HGF/SF - c-Met pathway in colorectal tumorigenesis.

Our observation that c-Met is strongly expressed by both colorectal adenomas and carcinomas (Table 1; Fig.1) confirms previous studies, documenting overexpression of c-Met in colorectal tumors (Di Renzo et al., 1991, 1995; Liu et al., 1992). We extend these findings by demonstrating...
that, in parallel, HGF/SF is expressed in colorectal tumor tissue. HGF/SF mRNA levels in tumor tissue were consistently higher than in the normal mucosa (Fig.4). These observations indicate that paracrine or autocrine HGF/SF- c-Met interaction is likely to take place within the colorectal carcinoma microenvironment, promoting tumor growth and motility. At present, the mechanism of c-Met and HGF/SF overexpression in colorectal cancer is largely unknown. Di Renzo and colleagues (1995) reported that c-Met overexpression is associated with amplification of the c-met gene in approximately 10% of primary colon carcinomas and 50% of metastases. However, since high c-Met levels were present in all carcinomas tested (Table 1) other mechanisms must also be involved. As c-Met overexpression occurs from an early stage of colorectal tumor progression onwards, c-met might, like c-myc (He et al., 1998), be regulated by the Wnt-signaling pathway. For HGF/SF the mechanism of overexpression and the precise site of production within the tumor microenvironment remain to be defined. HGF/SF expression by epithelial tumor cells, with autocrine c-Met stimulation, has been reported in human breast cancer (Yamashita et al., 1994; Tuck et al., 1996). Alternatively, fibroblasts within tumor stroma present a potential paracrine source of HGF/SF (Nakamura et al., 1997). Paracrine stimulation may also promote the outgrowth of metastases, as HGF/SF is produced at the two major sites of colorectal carcinoma metastasis, i.e., the liver (Noji et al., 1990) and lymphoid tissue (van der Voort et al., 1997).

CD44v3 isoforms were detected on colorectal adenomas, on a major subset (70%) of invasive colorectal carcinomas, and on all carcinoma cell lines studied (Table 1; Fig.1 and 2). Analysis of CD44v3 immunoprecipitates showed that these isoforms were decorated with heparan sulfate, and thus are HS-proteoglycans (Fig.3). Interestingly, interaction of HGF/SF with HS-moieties on HT-29 cells was found to promote c-Met phosphorylation (Fig. 5). Although the precise contribution of CD44-HS versus other HS-proteoglycans, such as the syndecans (Steinfeld et al., 1996) remains to be explored, our findings suggest a role of HS-proteoglycans in c-Met signaling in colorectal cancer. We have recently identified CD44-HS as a functional co-receptor for HGF/SF. Binding of HGF/SF to CD44-HS promotes signaling through c-Met leading to phosphorylation of several downstream proteins and to of
overactivity of the MAP kinases ERK1/2 (van der Voort et al., 1999). ERK1/2, which have been implicated in the processes of cell motility and invasion (Klemke et al., 1997), are also activated by Tpr-Met and by oncogenic c-Met mutants associated with human papillary renal carcinomas (Jeffers et al., 1998). The enhancing effects of CD44-HS on signal transduction were critically dependent on HGF/SF interaction with HS moieties, suggesting that CD44-HS promotes the action of HGF/SF through concentration of HGF/SF on the cell surface and by presenting it to the high affinity receptor c-Met. Similar mechanisms were proposed for the role of high and low affinity receptors in fibroblast growth factor (FGF)-2 functioning (Yayon et al., 1991; Maccarana et al., 1993; Aviezer et al., 1994; Rapraeger et al., 1991; Schlessinger et al., 1995; Spivak-Kroizman et al., 1994; Klagsbrun et al., 1991). CD44, c-Met and HGF/SF are also expressed in embryonal tissues, including intestine (Sonnenberg et al., 1993; Wielenga et al., 1999b). Presumably, they play a role in mesenchymal-epithelial interactions regulating differentiation and morphogenesis. Interestingly, we have recently shown that CD44 is present in normal mouse embryonal intestine but absent in that of mice with a disrupted Wnt-signaling pathway (Wielenga et al., 1999b). Loss of CD44 in these Tcf-4 mutant mice occurred in the context of a phenotype characterized by the absence of a proliferative stem cell compartment. Binding to CD44-HS of mesenchymal derived growth factors, including HGF/SF and WNT-factors, may be required for normal intestinal stem cell differentiation. In a study by Sherman et al. (1998), CD44-HS was shown to present FGF-2 in embryonal limb bud formation.

Several studies have reported a strong correlation between CD44 expression in invasive colorectal carcinomas and tumor related death (Mulder et al., 1993; Yamaguchi et al., 1996; Ropponen et al. 1998; Wielenga et al. 1998; Bhatavdekar et al., 1998, reviewed by Wielenga et al., 1999a). In these studies, antibodies recognizing different parts of the CD44 molecule, i.e., CD44v6, CD44v8-10, or CD44s (the constant part of CD44) all gave similar results. We now show that CD44v3 expression also predicts prognosis. This correlation of survival with a number of CD44 domains indicates concerted overexpression of these various CD44 variant domains.

In conclusion, we demonstrate that most colorectal tumors co-express
c-Met and CD44-HS, and that co-expression of these molecules is in invasive carcinomas is associated with an unfavorable prognosis. Our findings suggest that during colorectal tumorigenesis, CD44-HS overexpression may enhance signaling through the HGF/SF-c-Met signaling pathway, promoting tumor growth and the development of metastatic disease.

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REFERENCES


activated T cell extravasation into an inflammatory site. Science 278: 672-675, 1997


expression in colorectal adenomas is an early event occurring prior to K-ras and p53 gene mutation. Arch Biochem Biophys 310: 504-507, 1994


54. Stamenkovic I, Amiot M, Pesando JM, Seed B: A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell 56:1057-1063, 1989


56. Taher TEI, Smit L, Griffioen W, Schilder-Tol EJM, Borst J, Pals ST:


73. Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz D: Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64: 841-848, 1991