Reduced Growth, Increased Vascular Area, and Reduced Response to Cisplatin in CD13-Overexpressing Human Ovarian Cancer Xenografts

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ABSTRACT

Purpose: Expression of aminopeptidase N/CD13 can be detected in several solid tumor types. Thus far, the role of CD13 in ovarian cancer has not been studied. We have investigated the expression pattern and biological function of CD13 in ovarian cancer.

Experimental Design: First, we studied the expression of CD13 in ovarian cancer tissue of 15 patients representing three different histological types (5 patients each) by immunohistochemistry. We then stably transfected the IGROV-1 human ovarian cancer cell line with a CD13 expression vector and examined the biological effect of CD13 in vitro and in vivo.

Results: The expression of CD13 in ovarian cancer was associated with the histological subtype: CD13 expression in tumor cells was observed in 80–100% of the patients with a serous or mucinous carcinoma and in only 20% of the clear cell carcinoma patients. In all patients’ tumor samples, CD13-positive blood vessels were present. CD13 overexpression in IGROV-1 cells did not affect in vitro cell growth and sensitivity to doxorubicin, cisplatin, or gemcitabine. CD13 overexpression reduced invasion in Matrigel, which appeared to be independent of the aminopeptidase activity of CD13. Furthermore, the growth rate of IGROV-1/CD13 xenografts was reduced. The area of the vessel lumens was enlarged in a small percentage of vessels in the CD13-overexpressing xenografts. In addition, the CD13-overexpressing tumors were less sensitive to cisplatin.

Conclusions: CD13 is expressed in tumor as well as endothelial cells in human ovarian cancer. Our results suggest that CD13 overexpression affects ovarian cancer growth, vascular architecture, and response to chemotherapy. Further elucidation of the mechanism of the observed effects of CD13 is warranted to better understand its role in the pathophysiology of ovarian cancer.

INTRODUCTION

Ovarian cancer is the most important cause of death among the gynecological malignancies. This is caused mainly by the late diagnosis of the tumor. The biological behavior of the tumor is associated with clinicopathological parameters, such as International Federation of Gynecologists and Obstetricians (FIGO) stage, grade, and tumor type (1). Recently, new molecular markers with potential diagnostic use in ovarian cancer have been identified in patients. CD24, a ligand of P-selectin, as well as maspin, a noninhibitory member of the serpin family, have been identified as adverse prognostic factors (2, 3). Conversely, in a mouse model, dipeptidyl peptidase IV (CD26) expression in SKOV-3 cells significantly decreased dissemination and increased survival time (4).

Aminopeptidase N (CD13; EC 3.4.11.2) is a transmembrane ectopeptidase of Mr 150,000 that is highly expressed on cells of the myeloid lineage and on nonhematopoietic tissues, such as fibroblasts, epithelial cells of the kidney and liver, and pericytes in the brain (5–7). High expression of CD13 has been detected in various solid tumors (6–8). The expression level of CD13 was found to correlate with increased malignant behavior in prostate cancer and colon cancer (9, 10). In contrast, in renal cell cancer, CD13 expression has been reported to decrease with malignant progression (11). In different studies, a role for CD13 in degradation and cellular invasion of the extracellular matrix (ECM) has been established (9, 12, 13). It has been shown for melanoma cells that overexpression of CD13 significantly increased invasion in Matrigel, possibly related to an enhanced degradation of collagen type IV (14). Recently, CD13 has been associated with angiogenesis, the formation of new blood vessels that is required for tumor growth. CD13 expression in tumor microvascular endothelial cells was shown to be induced by angiogenic cytokines and hypoxia. Inhibition of CD13 activity reduced endothelial cell tube formation on Matrigel and inhibited angiogenesis in various in vivo model systems (9, 15, 16).

We have recently found that the activity of a soluble form of CD13, probably originating from shedding of the membrane protein, was elevated in malignant ascites from ovarian cancer patients (17). Given the lack of information on CD13 expression in human ovarian cancer, we set out to study its expression by immunohistochemistry in ovarian cancer samples. In addition, as a preclinical model, we developed a CD13-overexpressing human ovarian cancer cell line (IGROV-1) to study the effects of CD13 on tumor biology and vascular development in tumors grown in nude mice.
MATERIALS AND METHODS

Cells. IGROV-1 human ovarian carcinoma cells (18) and stably transfected derivatives were cultured in DMEM (Bio-Whittaker) with 20 mM HEPES (Bio-Whittaker), 10% FCS (Invitrogen), 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

Plasmid Constructs and Transfection. The pZIP-CD13-SV(x)1 vector and the pZIP-neo-SV(x)1 vector were a kind gift of Dr. L. H. Shapiro (University of Connecticut Health Center, Farmington, CT). IGROV-1 cells were transfected with pZIP-CD13-SV(x)1 or with control vector pZIP-neo-SV(x)1 by the calcium phosphate precipitation method (19). After a 6-h incubation period, the cells were washed and left with fresh medium for 16 h. Subsequently, stably transfectants were selected and maintained with Geneticin (450 μg/ml; G418; Life Technologies, Inc.). Resistant clones were isolated (IGROV-1/neo, IGROV-1/CD13-7, and IGROV-1/CD13-9) and characterized for CD13 mRNA expression and CD13 protein expression and activity.

CD13 Detection in IGROV-1 Cells. Expression of CD13 mRNA was examined by reverse transcription-PCR. Total RNA from the cell lines was reversed transcribed to cDNA. For reverse transcription-PCR, the following human-specific primers were used: for CD13, forward primer 5'-GTAATACGACTCACTATAGGGCCAGGGCCTGTAATTTTTA-3' and reverse primer 5'-AATTAACCTCACAAAGGCCACACGCTCGTCTGTCAG-3'; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward primer 5'-ACCAGATGTCATGCCCATAC-3' and reverse primer 5'-TCCACCCACTGTTGCTGTA-3'. The primers were confirmed to be human specific by testing mouse fibroblasts, liver, and kidney. The samples were denatured for 5 min at 95°C; PCR was performed in 36 cycles of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C, followed by 5 min at 72°C.

The expression of CD13 protein was examined with fluorescence-activated cell-sorting analysis and immunofluorescence. For fluorescence-activated cell-sorting analysis, cells of 70% confluence were harvested by short trypsinization, washed, and suspended in PBS containing 1% BSA. Cells (1 × 10^5) were incubated at room temperature for 30 min with FITC-conjugated antihuman CD13 monoclonal antibody WM-47 (5 μg/ml; DAKO) or FITC-conjugated mouse IgG1 (DAKO). The cells were washed and analyzed on a FACScan (Becton Dickinson).

For immunofluorescence, 2 × 10^4 cells were plated on a coverslip and allowed to attach for 16 h. Subsequently, the cells were washed with PBS and incubated with FITC-conjugated antihuman CD13 monoclonal antibody WM-47 (5 μg/ml; DAKO). Fluorescence was visualized on a confocal laser scan microscope (Leica TCS 4D) using a krypton-argon laser (λ_ex of 488 nm and λ_em of 535 nm) and a 4×4 oil lens.

Aminopeptidase Activity Assay. Surface aminopeptidase activity of intact cells was measured by plating 5000 cells/well in a 96-well plate. Cells were allowed to recover for 16 h. Subsequently, the cells were washed with PBS and incubated with 100 μM L-alanine-4-methyl-7-coumarinylamide trifluoroacetate (Fluka) in PBS with 0.5% BSA and 20 mM HEPES (pH 7.2). The release of the fluorescent product 7-amido-4-methylcoumarin was monitored on a Spectrafluor multilaser reader (Tecan; λ_ex of 360 nm and λ_em of 465 nm) every 5 min. The aminopeptidase activity was calculated from the slope of the fluorescence-time curve, using a calibration curve of 7-amido-4-methylcoumarin (Fluka; Ref. 20). The activity-blocking antibody WM-15 (5 μg/ml; PharMingen; Ref. 21) was used to determine the CD13-specific aminopeptidase activity.

Cell Doubling Time. Cells growing in log phase were harvested and plated in duplicate in a 6-well plate at 7.5 × 10^4 cells/well in DMEM +10% FCS. The number of cells was counted at 24, 48, 72, and 96 h. The cell doubling time in log phase was determined in three separate experiments.

In Vitro Drug Sensitivity. The antiproliferative effects of doxorubicin, gemcitabine, and cisplatin were measured in a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Briefly, 3000 cells were plated in a 96-well plate in triplicate, and the cells were exposed to a drug concentration range for 72 h. The number of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and measurement on a Spectrafluor multilaser reader (Tecan; absorbance 540 nm). The results were expressed as IC_50 values, which indicate the concentrations resulting in a 50% reduction in growth as compared with control cell growth. The experiment was repeated three times.

Matrigel Invasion Assay. The Matrigel invasion assay was performed in a 24-well Transwell system (Falcon) with 8-μm pore-size filters (HTS Fluoroblock Insert; Becton Dickinson) coated with 1% gelatin (Merck) on the lower side and Matrigel on the upper side (1 μg/filter; ECM gel; Sigma). Cells were harvested and resuspended in DMEM with 0.1% BSA. From each cell line, 2 × 10^5 cells were added to the upper compartment (in triplicate), and DMEM plus 10% FCS was added to the lower compartment. Bestatin (Sigma) was added to both compartments. The cells were incubated for 18 h at 37°C and 5% CO_2. Invasion was measured by the addition of 5 μM calcine (Molecular Probes) to the lower compartment during the last 30 min of the assay. Fluorescence from the released-acetoxyethyl ester calcine was measured in a Spectrafluor multilaser reader (λ_ex of 492 nm and λ_em of 535 nm). The total number of invaded cells was calculated using a calibration curve of 100–10,000 cells (for each cell line) incubated for 30 min with calcine-AM.

Adhesion Assay. A 96-well plate was coated overnight with 10 μg/ml Matrigel at 4°C. Cells were released from the flask with 5 mM EDTA and resuspended in DMEM +10% FCS. From each cell line, 2 × 10^5 cells (in triplicate) were allowed to adhere for 1 h at 37°C and 5% CO_2. Nonadherent cells were removed by washing with PBS. Adherent cells were quantified with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Real-Time PCR. For the quantitative real-time PCR, total RNA was extracted with RNAzol (Teltest Inc.) from tumor fragments and reverse transcribed into cDNA. For CD13 and GAPDH, the same primer sets were used as described for the reverse transcription-PCR. For vascular endothelial growth factor (VEGF), we used the primer set described previously (22): forward primer 5'-AGCAAGGCCCACAGGGAATTT-3' and reverse primer 5'-ACCGGCTGGGCTTTCAC-3'. The VEGF primer set recognizes both human and mouse cDNA. Two μl of
cDNA (diluted 1:2 with H₂O) was amplified with 2 µl of LightCycler FastStart DNA Master SYBR Green (Roche), 3.3 mM MgCl₂, and 0.5 mM primers in a total volume of 20 µl. The reaction was performed in a rapid PCR amplification consisting of an initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 0 s, annealing at 54°C for 20 s, and amplification at 72°C for 18 s. Specificity of the amplified product was determined from the melting curve of 15 s with a target temperature of 50°C (one peak) and by visualization on gel (one band). The cycling conditions were similar for all primer sets. GAPDH was used as internal standard and reference gene.

Relative mRNA expression was calculated by:

\[(E^{\text{-Cp target gene}})/(E^{\text{-Cp reference gene}})\]

in which \(E\) = efficiency and \(C_p\) = crossing point. The efficiency (GAPDH, 1.77; CD13, 1.89; VEGF, 1.69) was calculated for all target and control genes via a cDNA concentration range. Six tumors from the first and second passage were analyzed at least twice on separate occasions.

**Xenograft Growth.** Cells growing in log phase were harvested by trypsinization. Per cell line, \(1 \times 10^7\) cells were injected s.c. in both flanks of three female nude mice (Harlan, Horst, the Netherlands). On growth, the tumors were measured in three dimensions, and tumor volume was expressed in mm³ as calculated by the following formula: length \(\times\) width \(\times\) thickness \(\times 0.5\). The volumes were measured twice weekly starting at a tumor volume of \(>20\) mm³. The mean tumor volumes from passage 1 were used to draw the growth curves. From the first passage, tumor fragments of 2–3 mm were transplanted s.c. in both flanks of further recipients. The tumors in the second passage were measured twice weekly, and tumor volume was expressed in mm³ as described above. To determine two volume doubling times, tumor growth was expressed by use of the relative tumor volumes. To that end, the first tumor volume measured was designated \(V_0\) on day 0, and the next measurements were set as the ratio \(V_t/V_0\), in which \(V_t\) is the tumor volume at any time. Two tumor volume doubling times were calculated for each individual tumor, defined as the number of days needed for a tumor to grow from a ratio of 1 to a ratio of 4.

**Xenograft Treatment with Cisplatin.** In the second passage of xenograft growth, separate tumor-bearing mice were used for treatment with cisplatin. At a volume of 100–150 mm³ (designated as day 0), treatment started in groups of 3–6 mice (6–12 tumors), whereas the control mice (groups of 3–6 mice; 6–12 tumors) did not receive treatment. Cisplatin (Bristol-Myers Squibb, Woerden, the Netherlands) was given at the maximum tolerated dose of 5 mg/kg, i.v., weekly \(\times 2\) (23). To determine drug efficacy, mean tumor volumes were used, and the formula \(T/C\% = [(\text{mean tumor volume of treated tumors})/(\text{mean tumor volume of control tumors})] \times 100\%\) was calculated on each day of tumor measurement. Growth inhibition was expressed as \(100\% - T/C\%\). Mean tumor volumes were also used to draw the growth curves.

**S-Phase Fraction in Tumor Sections.** Four different xenografts of each cell line out of two independent experiments were analyzed for S-phase fraction. Frozen tumor tissue was manually cut into small pieces in a citrate buffer as described previously (24). After a 10-min centrifugation at 3,000 rpm, the supernatant was removed, and trypsin was added to the pellet for 10 min at room temperature. Subsequently, trypsin inhibitor was added for 10 min at room temperature. After filtering the suspension, diaminophenyl-indole-dihydrochloride and spermine tetra-hydrochloride were added, and the sample was kept in the dark for at least 15 min. All samples were measured in the same session on the same day using a Partec PAS mercury lamp-based flow cytometer (Partec, Münster, Germany). At least 20,000 events were measured for each sample. Trout erythrocytes were used as calibration cells. The resulting DNA histograms were exported to ASCII format for semiautomated analysis of the S-phase fraction with the MultiCycleR program (Phoenix Flow Systems, San Diego, CA) as described previously (25).

**Immunohistochemistry.** Frozen tissue of ovarian carcinoma of patients was obtained from the tumor bank of the pathology department. IGROV-1, and IGROV-1/neo, IGROV-1/CD13-7, and IGROV-1/CD13-9 xenografts in the first and second passage were removed at a size of 300–500 mm³ and frozen immediately in liquid nitrogen. Cryostat tissue sections (4 µm) were mounted on SuperFrostPlus slides (Menzel-Glaser) and stored at \(-20^\circ\text{C}\). The sections were fixed with acetone and subsequently incubated with 10% serum of the animal species from which the secondary antibody was obtained, followed by a 60-min incubation with the primary antibodies (for antibodies, see Table 1) and, after that, a 45-min incubation with a biotin-labeled secondary antibody. For color development, the slides were incubated with peroxidase-labeled streptavidin (DAKO) and the substrate 3-aminog-ethylcarbazole (DAKO). In between all steps, the slides were washed with PBS. Slides were counterstained with hematoxylin. In the negative controls, the primary antibody was replaced with IgG; human placenta was used as positive control. An experienced pathologist (P. J. v. D.) performed scoring of immunohistochemistry in the patients’ tumor samples in a blinded manner. Staining of the xenografts

<table>
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<tr>
<th>Table 1</th>
<th>Antibodies used for immunohistochemistry</th>
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<tr>
<td>Antigen</td>
<td>Antibody</td>
</tr>
<tr>
<td>Human CD13</td>
<td>WM-15</td>
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<tr>
<td>Human CD31</td>
<td>JC70A</td>
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<tr>
<td>Mouse CD31</td>
<td>CD31 (MEC13.3)</td>
</tr>
<tr>
<td>Mouse VEGF³</td>
<td>VEGF (A20)</td>
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<tr>
<td>Mouse bFGF</td>
<td>FGF-2 (147)</td>
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* (m), monoclonal; (p), polyclonal.

³ VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; FGF, fibroblast growth factor.
was semiquantitatively scored as negative, weakly positive, or positive by two independent observers in a blinded manner. For counting of the number of blood vessels in xenograft tissues, three representative fields (hot spots) per section were selected at ×2.5 magnification. Counting of the vessels in the hot spot was performed at ×40 magnification. With a microscope (Leica TCS 4D; ×10 objective) attached to a videocamera (Kappa CF8/1 FMCC), images (5 images/section) of the blood vessels throughout the whole tumor section were randomly taken. For all of the lumen-containing vessels in the image, the area was measured and analyzed with Leica Q500MC/QWin software. Four different xenografts from two independent experiments (first passage) of each cell line were analyzed.

Statistics. All results are presented as mean ± SE. Statistical differences between mean values obtained in the in vitro assays and in vivo tumor growth were calculated with paired t tests. The Mann-Whitney test was used for calculation of statistical differences between the blood vessel areas. The statistical difference for the CD13 expression between tumor subtypes was determined with the Pearson χ² test. Differences were considered significant when P was <0.05.

RESULTS

CD13 Expression in Human Ovarian Cancer. Representative immunohistochemical stainings of frozen ovarian carcinomas are shown in Fig. 1. Four of five serous ovarian carcinomas exhibited membranous CD13 antigen expression on tumor cells (80% positive). Of the mucinous type carcinomas, four patients displayed apical CD13 antigen expression, whereas focal staining of CD13 antigen was observed in one patient (100% positive). Only one of the five clear cell cancers (20%) expressed CD13 antigen on tumor cells. The number of CD13-positive clear cell tumors was significantly lower when compared with that in serous and mucinous tumors (P = 0.02). In all tumors, a small number of blood vessels were CD13 positive. In most tumors CD13-positive macrophages could be detected (Table 2). This result clearly showed that a high percentage of ovarian carcinomas contain CD13-positive malignant cells. Therefore, we decided to study the effect of CD13 expression on tumor and vascular biology in a human ovarian cancer xenograft model.

CD13 mRNA and Protein Expression in IGROV-1 Cells. All cell lines were analyzed for CD13 mRNA and CD13 protein expression (Fig. 2, A and B; Table 3) and aminopeptidase activity (Table 3). Expression of CD13 mRNA or CD13 protein could not be detected in IGROV-1/parent and IGROV-1/neo clones. High expression of CD13 mRNA and protein was present in the CD13-transfected cells. The protein expression in IGROV-1/CD13-7 was heterogeneously distributed (small shoulders in the fluorescence histogram were seen), whereas in IGROV-1/CD13-9, it was more homogeneous. With confocal laser scan microscopy immunofluorescence, the plasma membrane localization of CD13 in IGROV-1/CD13-7 and IGROV-1/CD13-9 was visualized (Fig. 2C). Proper catalytic function of the transfected CD13 protein was verified using a CD13-specific neutral aminopeptidase activity assay (Ref. 20; Table 3). CD13 activity correlated significantly with the CD13 expression measured by fluorescence-activated cell sorting (r = 0.994; P = 0.006). Taken together, these results demonstrate a

![Fig. 1](https://cancergenome.medscape.com/) CD13 expression in human ovarian cancer samples. Frozen tissues of ovarian cancer of patients were stained with an antihuman CD13 antibody (WM15; 10 μg/ml). Representative examples of a serous tumor (A), a mucinous tumor (B), and a clear cell tumor (C) are shown (×10 magnification; black arrow, tumor cell staining; open arrow, blood vessel staining).

### Table 2

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<tr>
<th>Tumor type</th>
<th>Tumor cell Pattern</th>
<th>Vessels</th>
<th>Stromal cells</th>
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<tr>
<td>Serous</td>
<td>80%</td>
<td>Plasma membrane (4)</td>
<td>100%</td>
</tr>
<tr>
<td>Mucinous</td>
<td>100%</td>
<td>Plasma membrane (5); apical (4)/focal (1)</td>
<td>100%</td>
</tr>
<tr>
<td>Clear cell</td>
<td>20%</td>
<td>Plasma membrane (1)</td>
<td>100%</td>
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*a* In 100% of the tumors, >1 CD13-positive blood vessels were observed.

*b* In 20–40% of the tumors, CD13-positive stromal cells were observed.

*c* Significantly different CD13 expression as compared with the clear cell subtype (P = 0.02).
high overexpression of active membrane-bound CD13 in IGROV-1 cells.

High CD13 Expression Does Not Affect Proliferation and Drug Sensitivity in Vitro. For characterization of the transfected cells, cell proliferation was measured by calculating the doubling time of the cells in vitro. As described in Table 4, no alterations in doubling time were observed. This was confirmed by the cell cycle distribution pattern, as measured with propidium iodide, which was similar for all cell lines (data not shown). Additionally, we determined the sensitivity of the cells for doxorubicin, cisplatin, and gemcitabine. No significant differences in in vitro sensitivity were observed for the tested drugs among the cell lines (Table 4).

Tumor Cell Invasion Through Matrigel Is Reduced by High CD13 Expression. In a Matrigel invasion assay (Transwell system), the effect of CD13 on the invasive capacity of the cells was determined. Without the chemoattractant FCS, all cells invaded similarly (909 ± 101 cells). In the presence of FCS, invasion was stimulated 5.6 ± 0.2-fold for IGROV-1/parent and 4.6 ± 0.3-fold for IGROV-1/neo (P = nonsignificant compared with IGROV-1/parent; Fig. 3). The CD13-overexpressing cell lines responded less to FCS. FCS stimulated the invasion of IGROV-1/parent (a) and IGROV-1/neo (b), whereas a clear plasma membrane staining was observed in IGROV-1/CD13-7 (c) and IGROV-1/CD13-9 (d).
High CD13 Expression Slightly Reduces Adhesion to Matrigel. The adhesion of the cell lines to Matrigel was comparable for IGROV-1/parent (0.20 ± 0.02), IGROV-1.neo (0.21 ± 0.02), and IGROV-1/CD13-7 (0.19 ± 0.03). In contrast, the adhesion of IGROV-1/CD13-9 was slightly reduced [0.15 ± 0.02; a 27% reduction (P = 0.04) compared with IGROV-1/parent; Fig. 4]. The adhesion to defined ECM components (vitronectin, fibronectin, and collagen IV) was similar for all cell lines (data not shown).

CD13 Expression Reduces IGROV-1 Xenograft Growth. To examine the effect of CD13 overexpression on tumor growth, all cell lines were injected s.c. into nude mice, and tumor growth was measured in the first and second passage. Fig. 5 depicts the growth of the tumors in the first passage, starting at the inoculation of the tumor cells (thus including the lag phase of the tumors). At day 15 after inoculation, the IGROV-1/CD13-overexpressing xenografts were significantly smaller than the IGROV-1/parent or IGROV-1.neo xenografts. Mean tumor volumes of 122 ± 18 (IGROV-1/CD13-7) and 352 ± 31 mm³ (IGROV-1/CD13-9) for the CD13-overexpressing xenografts and 868 ± 162 and 686 ± 91 mm³ for IGROV-1/parent and IGROV-1.neo, respectively, were calculated. Because the tumor growth rate appeared less variable between individual tumors in the second passage, two volume doubling times of the tumors were calculated in this passage. Similar doubling times (of at least six independent tumors) were determined for the IGROV-1/parent (5.7 ± 0.7 days) and IGROV-1.neo (4.4 ± 0.6 days), whereas the doubling times of the CD13-overexpressing clones were significantly prolonged [IGROV-1/CD13-7, 15.0 ± 2.9 days (P = 0.023 compared with IGROV-1/parent); IGROV-1/CD13-9, 12.2 ± 0.8 days (P < 0.001 compared with IGROV-1/parent)]. The volume doubling times of the two CD13-overexpressing clones were not significantly different.

In addition to the volume doubling times, the S-phase fraction, which is a measurement of the number of cells that are actively dividing, of the xenografts was determined. The percentage of cells in S phase was similar in the IGROV-1/parent and IGROV-1.neo xenografts (21.5 ± 1.5% and 22.1 ± 1.3%, respectively). A slightly lower percentage of cells in S phase was detected for the IGROV-1/CD13-7 (18.3 ± 1.4%) and the IGROV-1/CD13-9 xenografts (18.4 ± 1.0%). The difference was of borderline significance toward the control transfectants (P < 0.04 for IGROV-1/CD13-9 and P < 0.06 for IGROV-1/CD13-7, unpaired t test).

CD13 Expression in IGROV-1 Tumors. Real-time PCR with human-specific primers showed that CD13 mRNA was virtually absent in the xenografts of the IGROV-1/parent and IGROV-1.neo cells [relative mRNA in arbitrary units (×10⁻⁴), 2.2 ± 1.0 and 1.9 ± 0.4, respectively], whereas high mRNA expression was detected in the IGROV-1/CD13-7 (738 ± 152) and IGROV-1/CD13-9 xenografts (4875 ± 1085). Similarly, immunohistochemical staining revealed prominent heterogeneous expression of CD13 protein in IGROV-1/CD13-7 tumors and homogeneous expression in IGROV-1/CD13-9 tumors (Fig. 6, A–D).

IGROV-1/CD13-9 Is Less Sensitive to Cisplatin in Vivo. Because cisplatin is an important drug for treatment of ovarian cancer, we studied the sensitivity of the IGROV-1 xenografts in the second passage. We measured a maximum inhibition of

<table>
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<th>Table 3</th>
<th>CD13 expression and activity in IGROV-1/parent and IGROV-1 transfected cells</th>
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<tr>
<td>CD13 expression (mean fluorescence)</td>
<td>CD13 activity (fmol/10⁴ cells/min)</td>
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<tr>
<td>IGROV-1/parent</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>IGROV-1.neo</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>IGROV-1/CD13-7</td>
<td>662.7 ± 57.2</td>
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<tr>
<td>IGROV-1/CD13-9</td>
<td>867.0 ± 108.2b</td>
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* The isotype IgG antibody displayed a mean fluorescence of 3.1 ± 0.1 and 3.5 ± 0.2, respectively, for IGROV-1/parent and IGROV-1.neo.

* Significantly different from the IGROV-1/parent (P < 0.001).

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<tr>
<th>Table 4</th>
<th>In vitro doubling time and the sensitivity for drugs (expressed in IC₅₀) of IGROV-1 and IGROV-1 transfected cells</th>
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<tbody>
<tr>
<td>Doubling time (h)</td>
<td>Doxorubicin (nm)</td>
</tr>
<tr>
<td>IGROV-1/parent</td>
<td>20.0 ± 3.5</td>
</tr>
<tr>
<td>IGROV-1.neo</td>
<td>20.2 ± 3.8</td>
</tr>
<tr>
<td>IGROV-1/CD13-7</td>
<td>21.5 ± 2.9</td>
</tr>
<tr>
<td>IGROV-1/CD13-9</td>
<td>20.5 ± 4.2</td>
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Fig. 3 Expression of CD13 on IGROV-1 cells reduces invasion through Matrigel. Cellular invasion was measured in a Transwell system with 10 µg/ml Matrigel (18 h). The FCS-stimulated invasion was significantly less for the CD13-transfected cells as compared with the control cells (*, P < 0.05; **, P < 0.01). Results are given as the mean ± SE of three experiments.
tumor growth with cisplatin treatment of 67% (day 14; n = 6) for IGROV-1/parent and 61% (day 14; n = 6) for IGROV-1/neo, whereas cisplatin was not effective in the IGROV-1/CD13-9 (maximum growth inhibition of 23% at day 12; n = 12; Fig. 7).

CD13 Expression Affects the Mean Vessel Area but not Vessel Density. Because it has been reported that CD13 activity, when expressed in endothelial cells, affects microvessel formation in tumors (16), we analyzed whether a more generalized overexpression of CD13 in tumor tissue has effects on microvessel formation. No significant differences were observed in microvessel density for all tumors (calculated from CD31 staining; data not shown). By visual examination, however, some of the microvessels in the IGROV-1/CD13-9 tumors exhibited a clearly enlarged lumen. To a much lesser extent, these were also observed in IGROV-1/CD13-7, IGROV-1/parent, or IGROV-1/neo xenografts (Fig. 6, E–H). Fig. 6H illustrates a representative example of an enlarged vessel in IGROV-1/CD13-9. Quantification of the vessel area revealed a significant increase in vessel area of the IGROV-1/CD13-9 tumors (mean area of the lumen was 7.87 ± 1.59 μm²; P < 0.0005 compared with IGROV-1/parent), whereas no difference in vessel area was observed for IGROV-1/CD13-7 (3.51 ± 0.50 μm²), IGROV-1/parent (3.79 ± 0.46 μm²), and IGROV-1/neo (2.47 ± 0.26 μm²) tumors (Fig. 8). Calculation of the percentage of blood vessels with a large lumen area (>15 μm²) further illustrated the increase in vessel area; 12 ± 3% of the blood vessels of the IGROV-1/CD13-9 were >15 μm², whereas this value was only 1–2% for the xenografts derived from the other cell lines.

VEGF and basic fibroblast growth factor are not induced in CD13-overexpressing xenografts. VEGF mRNA real-time PCR in six xenografts revealed a nonsignificant trend toward a higher VEGF mRNA expression in CD13-overexpressing tumors relative to control tumors (Fig. 9). However, basic fibroblast growth factor protein did not reveal any obvious difference in VEGF or basic fibroblast growth factor expression among the tumors.

DISCUSSION

In this study, we demonstrated that CD13 is highly expressed in tumor cells of the most frequently occurring human ovarian cancers. We showed in a human ovarian cancer xenograft model that CD13 overexpression reduced the growth of the xenografts. In addition, the vascular architecture and response to chemotherapy were altered in the CD13-overexpressing xenografts.

The expression of CD13 antigen in ovarian cancer cells was associated with the most common subtypes (serous and mucinous). Importantly, CD13-positive tumor cells displayed a very prominent plasma membrane staining comparable with the staining observed in the CD13-overexpressing xenografts. Thus far, the biological or pathophysiological role of CD13 expression in ovarian cancer is unknown. No peptides relevant for the progression of ovarian cancer have been identified as a substrate for CD13, but these might be sought in growth-regulating peptide growth factors (described below). In line with recent reports showing an up-regulation of CD13 antigen in tumor angiogenic vessels (16), we found in all tumors a small number of CD13-positive blood vessels that were not specifically associated with one tumor type or with CD13 expression in tumor cells.

To obtain data on the possible pathophysiological role(s) of CD13 in ovarian cancer, we have generated a CD13-overexpressing IGROV-1 ovarian cancer cell line (18) and studied some important cell biological properties. An important observation was the significantly reduced invasion of CD13-overexpressing IGROV-1 ovarian cancer xenografts. VEGF mRNA real-time PCR in six xenografts revealed a nonsignificant trend toward a higher VEGF mRNA expression in CD13-overexpressing tumors relative to control tumors (Fig. 9). However, basic fibroblast growth factor protein did not reveal any obvious difference in VEGF or basic fibroblast growth factor expression among the tumors.
Fig. 6 Immunohistochemical staining of CD13 and CD31/platelet/endothelial cell adhesion molecule 1 in xenografts. Frozen sections from xenografts were stained for CD13 (WM15; 10 μg/ml) and CD31/platelet/endothelial cell adhesion molecule 1 (MEC13.3; 5 μg/ml). CD13 expression in tumor cells was only observed in the CD13-transfected tumors (×10 objective; inset, ×40 objective; A–D). The insets in C and D show membrane staining of CD13. In the CD13-overexpressing tumors, some extremely large vessels were visible that were hardly observed in the parent or the neo control tumors (×10 objective; E–H). A representative example of an enlarged vessel in IGROV-1/CD13-9 is shown (H).
pressing cells into Matrigel as compared with that of the non-CD13-transfected cells. This is in contrast to data published by Fujii et al. (14), who have reported an enhanced migration by CD13-overexpressing A375M melanoma cells through Matrigel (14). This discrepancy suggests that the balance of proteolytic and/or adhesive activities, which determine the invasive potential of these cell lines, differs qualitatively or quantitatively with regard to the effect of CD13 activity. Moreover, a high concentration of the aminopeptidase activity inhibitor bestatin did not reverse the effect of CD13 overexpression on invasion of IGROV-1 cells. It is unlikely that the absence of an effect of bestatin is due to its inability to completely inhibit the high levels of CD13 activity in the transfected cells. In our previous work with the HT-1080 fibrosarcoma cell line, which also expresses high levels of CD13, we have shown that bestatin can inhibit >80% of CD13 enzymatic activity. Moreover, the invasion of HT-1080 cells was inhibited up to 50% on bestatin treatment (20). Therefore, we conclude that it is not likely that the CD13 catalytic activity is directly responsible for the reduction of the FCS-stimulated invasion of the IGROV-1 cells. In this respect, our results strongly resemble those of a recent study by Kajiyama et al. (4), who have shown a decreased migration of dipeptidyl peptidase IV/CD26-overexpressing ovarian cancer cells through Matrigel that was also independent of aminopeptidase activity (4). An explanation for the reduced invasion of CD13-overexpressing cells might be a decreased adhesion of the cells, which is required for proper invasion in the ECM (Ref. 26; also reviewed by Skubitz (27)). Because we found only a moderate reduction in adhesion to Matrigel (30% reduction), it is questionable whether this explains the less invasive phenotype.

The fact that overexpression of an aminopeptidase results in a decrease of the invasion, rather than an increase, is apparently paradoxical. Such conflicting data, however, have also been reported for SPARC, a matrix glycoprotein that modulates cellular interaction with the ECM (28). SPARC has been linked to an invasive phenotype when expressed by melanoma and glioma cells, whereas expression of SPARC in ovarian cancer cells was inversely correlated with tumor growth (28).

It is possible that cooperation of CD13 with other aminopeptidases, as has been proposed by Riemann et al. (29), may affect intracellular signal transduction pathways involved in adhesion and motility of the cells. The possibility of such a cooperation is based on, among others, the finding that CD13 and other peptidases such as CD10 and CD26 colocalize in

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membrane caveolae in different cell types (30, 31). Furthermore, CD13 (as well as other ectopeptidases) may associate with other proteins into specific membrane regions (e.g., with certain integrins), thereby enabling CD13 to affect signaling processes, such as cell adhesion or apoptosis (32). Similar mechanisms have been described for many non-receptor tyrosine kinases present in caveolae (33). Alternative explanations might be sought in CD13-induced up- or down-regulation of adhesion proteins and/or (metallo)proteases resulting in a decreased invasive capacity. Interestingly, such a mechanism has been described very recently for CD26; overexpression of CD26 in the ovarian cancer cell line SKOV-3 was related to an increased expression of E-cadherin and β-catenin and/or decreased expression of certain matrix metalloproteinases (34). It will be very interesting to study the effects of introducing CD13 in SKOV-3 or, vice versa, CD26 in IGROV-1 to evaluate in more detail the significance of these aminopeptidases for ovarian cancer in general.

One of the most remarkable findings of this study was the significantly reduced tumor growth rate of the CD13-overexpressing clones in vivo. The IGROV-1/CD13-7 xenografts, which have a heterogeneous CD13 expression, grew more slowly than IGROV-1/CD13-9 xenografts in the first passage. In the second passage, however, the number of days to double in volume did not differ significantly between IGROV-1/CD13-9 and IGROV-1/CD13-7 tumors. A slower tumor growth of CD13-overexpressing xenografts may seem inconsistent with the expression data from the patient tumors because, in general, serous tumors (high CD13 expression) grow faster than clear cell tumors (low CD13 expression). However, because in the present study we did not analyze other factors that may affect ovarian cancer growth, more extensive patient studies will be required to answer this type of question. Because there was no direct effect of CD13 expression on the growth of the cells in a monolayer in vitro, CD13 overexpression apparently indirectly affects the growth and/or differentiation properties of the IGROV-1 cells in an in vivo environment. Because of the high CD13 expression in the transfected tumors, it is reasonable to assume that the aminopeptidase activity is elevated in vivo as well. As has been suggested for the role of CD13 in normal ovarian cells, active CD13 may catabolize biologically active peptides adjacent to the cell surface, such as tyrosine kinase-activating growth factors (epidermal growth factor and hepatocyte growth factor) and cytokines [interleukin 8 (35, 36)]. Thus, CD13 might contribute to local regulation of the concentration and/or activity of one or more of these peptides [reviewed by Fujiiwara et al. (37)] and, in this way, may affect the growth rate of the tumors (38, 39). Additional studies should include transfection of CD13 in other ovarian cancer cell lines as well as studies with catalytically inactive CD13. The search for in vivo alterations in signal transduction pathways involved in cellular migration, invasion, and cell growth is an additional way to proceed with further elucidation of the mechanisms involved.

The IGROV-1/CD13-9 xenografts exhibited a reduced sensitivity for cisplatin in vivo. Because we did not observe a change in the sensitivity to cisplatin or other cytotoxic agents in vitro, it is unlikely that CD13 directly induced resistance against cisplatin in the IGROV-1 cells. Instead, the reduced sensitivity for cisplatin may be sought in the slower growth of these tumor cells when grown in vivo. Based on the results of Kolfschoten et al. (23), who have studied the relationship between the S-phase fraction and cisplatin sensitivity in a panel of 14 ovarian xenografts, the small reduction in S-phase fraction observed in our CD13-overexpressing xenografts is at most partly responsible for the resistance against cisplatin. Other drug resistance features, such as impaired drug delivery to the tumors (e.g., due to altered vessel structures), have to be considered. It will be quite interesting to investigate the in vivo sensitivity to other cytotoxic agents to elucidate whether the observed resistance is drug specific, or whether this is a more generalized property of CD13-overexpressing ovarian xenografts.

An intriguing observation was the enlarged vascular area in the IGROV-1/CD13-9 xenografts. As can be observed in Fig. 6G, which is a representative picture of IGROV-1/CD13-7 xenograft tissue, differences in vascular area are present in the tumors of this clone, but it has to be noted that overall, no significant difference was found for the specific parameter calculation as used here when compared with the vasculature of the IGROV-1/parent. Given these considerations, it is likely that CD13 overexpression in IGROV-1 has effects on the development of tumor vasculature. Again, these results display similarity to those of the SPARC study mentioned earlier. Breken et al. (28) have also described that the vessel area was enlarged in SPARC+/- mice compared with SPARC-/- mice, without affecting the microvessel count.

There was no change in the expression level of angiogenic factors (VEGF and basic fibroblast growth factor) in the IGROV-1/CD13-9 tumors that could account for the change in vessel morphology. Several groups have suggested factors that can contribute to the enlargement of the vessel lumen, such as (a) a compensation for a local reduction in the number of blood vessels and blood volume (40), (b) a higher blood flow rate in a specific area that increases the diameter of the vessel (41, 42), and (c) a reduction of the interstitial pressure as well as a reduction in tumor cell density, leading to decompression of the vessels (43, 44). To evaluate whether any of these alternatives apply to CD13-overexpressing tumors and whether differences in vascular function occur in these tumors, additional studies are needed. Treatment of mice bearing CD13-overexpressing tumors with bestatin or other aminopeptidase inhibitors may give more insight into the biological and vascular effects of CD13.

Given that CD13 is abundantly expressed in tumor and endothelial cells in human ovarian cancer and our findings that CD13 overexpression affects the growth of ovarian cancer xenografts, the vascular architecture, and response to chemotherapy, further elucidation of the biochemical effects of CD13 and its role in the pathophysiology and treatment of ovarian cancer is required.

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