Tumor development of colon cancer in rat liver

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Chapter 4

Adhesion molecules in experimental colorectal cancer metastasis in rat liver

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In preparation.
Abstract

Steps involved in colon cancer progression and metastasis are increased protease expression, cell motility and altered expression of adhesion molecules. For cancer cells to metastasize, they need to detach from the primary tumor and attach at a secondary site. Adhesion molecules play a role in these processes. The role of adhesion molecules as regulators of cellular processes is well established now. We have previously shown that upon administration of colon cancer cells to the portal vein of rats, endothelial cells in the liver retract rapidly and cancer cells undergo interactions with hepatocytes directly. In the present study, we investigated adhesion molecules expressed by cancer cells as possible candidates for the observed interactions between cancer cells and hepatocytes. Cancer cells were found to be negative for the integrin subunits αL, β2 and β3. It is therefore unlikely that the integrins αLβ2, αδβ2, αmβ2, ααβ2, αvβ3 and αIIbβ3 are used by cancer cells. On the other hand, αv, α6 and β1 integrin subunits and CD44 and CD44v6 were expressed on cultured cancer cells. Cancer cells in livers at 1, 2 and 3 days after their administration were found to be positive for α6, CD44 and CD44v6. Trypsinization of cultured cancer cells resulted in loss of CD44 and CD44v6 staining but staining of α6 and β1 was more resistant to trypsinization and therefore it is not likely that CD44 or its splice variant is involved in early interactions between cancer cells and hepatocytes. It is concluded that αv, α6 and β1 are potential candidates for the observed early interactions between cancer cells and hepatocytes. However, more information on the precise localization of these integrin subunits should be obtained with electron microscopy.

Introduction

Colorectal cancer patients mainly die of metastatic disease rather than the primary tumor. Key events in metastasis are increased protease production, increased cell motility and altered expression of adhesion molecules (1). On the one hand, adhesion molecules play a significant role in development of tissue structures and maintenance of tissue morphology. On the other hand, adhesion molecules are essential in processes such as cell migration, inflammation and repair of damaged tissue. However, expression patterns are different in these cases (2).
Cell adhesion molecules can be classified into five major groups (3, 4): integrins, selectins, cadherins, immunoglobulin-like molecules and other molecules. Numerous reports suggest their involvement in cancer progression and metastasis. Specifically two types of adhesion molecules, the integrins and the CD44 hyaluronic acid receptors, are of specific interest with respect to the development of colon cancer and its metastasis (5, 6).

Integrins are a diverse family of glycoproteins that form heterodimeric receptors for extracellular matrix (ECM) molecules and some non-ECM proteins. The family can form at least 25 heterodimers of its 18 α-subunits and 8 β-subunits (2). A common motif in (extracellular matrix) proteins that is recognized by integrins is the Arg-Gly-Asp (RGD) sequence. Binding of integrins seems to be partly dependent on recognition of this sequence, but specificity is determined by the combination of the α- and β-subunit. Administration of synthetic RGD peptides to the circulation of animal models of colon cancer metastasis have shown to suppress metastasis of colon cancer in the liver (5).

Adhesion molecules such as integrins are also involved in signal transduction. For example, Gu et al. (7) identified an extracellular signal–regulated kinase-2 (ERK2)-binding site on the β6 integrin subunit. Interactions between β6 and ERK2 have been shown to be responsible for induction of the expression of matrix metalloproteinase-9 (MMP-9) in colon cancer cells (7). Furthermore, it has been shown that invasiveness of colon cancer cells is mediated by the unique C-terminal cytoplasmic tail of the β6 integrin subunit that is linked to the protein kinase C (PKC) signalling pathway (8). Therefore, MMP-9 expression can be upregulated via the unique cytoplasmic tail of β6 that can bind ERK2 and thus induces PKC-mediated signalling. Furthermore, interactions of urokinase-type plasminogen activator (uPA) with the uPA receptor (uPAR) results in ERK activation (9). Later it was shown that β1 associates with uPAR and in that way it is responsible for transmission of the signal inside cells (10). Furthermore, integrin αvβ3 has been shown to be involved in pericellular localization of active MMP-2 (11). So it seems that integrin expression is involved in a number of processes in invasion and metastasis of cancer.

The CD44 family is a group of glycoproteins encoded for by a total of 20 exons. The most commonly expressed form is CD44s, the 85 kD standard form. There is a diversity of forms and functions of CD44 proteins due to alternative splicing and different degrees of glycosylation. CD44 isoforms contain additional peptide domains inserted in the membrane-proximal extracellular portion of the molecule, and are expressed by lymphocytes, fibroblasts
and epithelial cells. The major ligand of CD44 is hyaluronic acid (HA), but CD44 also has affinity for collagens, fibronectin and laminin (12). Its major function is related with homing and activation of T-lymphocytes, probably by binding to HA attached to endothelial cells (13). CD44 also has a signal transduction function. Stimulation of CD44 has been described to induce expression of the integrin leukocyte function-associated antigen-1 (LFA-1), hepatocyte growth factor (HGF) (14) and MMP-2 (15). Colon carcinoma cells express high levels of CD44 (13). CD44s expression is correlated with malignancy and metastatic potential of gastric and colon cancers (6, 16). The v6 splice variant is expressed by activated T-lymphocytes and cancer cells and exhibits greater HA-binding than the standard form (17). Again, apart from adhesion, CD44v6-HA interactions also mediate signal transduction, causing increased levels of cytosolic calcium ions (17). It has been found to play a role in various proliferation and differentiation processes, such as embryogenesis, angiogenesis and carcinogenesis (18, 19). For example, normal liver parenchymal cells express CD44v6 during liver regeneration upon partial hepatectomy (20). In colorectal carcinoma, the relationship between the expression of CD44 splice variants and the degree of malignancy is controversial. In various studies, CD44v6 expression was correlated with metastatic potential of colorectal cancers (6, 20-22) or poor prognosis (23), whereas other studies reported no such correlation (6, 24).

We have previously shown that colon cancer cells administered to the portal vein of rats undergo molecular interactions with hepatocytes rather than with endothelial cells (25). Therefore, we investigated molecules that may be involved in these interactions by evaluation of the expression of various adhesion molecules on cultured rat colon cancer cells and in tumors of colon cancer in different stages of development in rat liver.

Materials and Methods

Animals

For all experiments, male syngeneic WAG-Rij rats of 200-220 g (Broekman, Someren, The Netherlands) were used, kept under constant environmental conditions with food and water ad libitum. Animal care was performed in accordance with the guidelines of the University of Amsterdam.
CC531s cancer cell line, culture and cytospins

An established colon carcinoma cell line, CC531s, transfected with the eGFP gene (CC531s-eGFP) was described previously (25). The cells were cultured at 37°C as monolayers in RPMI-1640 Dutch Modification without L-glutamine (GIBCO/BRL, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 IU penicillin/ml and 100 mg streptomycin/ml. Cells were washed with phosphate-buffered saline (PBS) and after detachment with trypsin (0.05% w/v) and ethylenediaminetetraacetic acid (EDTA) (0.02% w/v) in PBS and centrifugation (250 g, room temp, 10 min), single cell suspensions were obtained with a viability of at least 95% (26). To investigate the effects of trypsinization, cytospins of cancer cells were made by centrifugation of 250 µl cell suspension onto clean glass slides with a Nettich 1502 centrifuge (Nettich Zentrifugen, Tüv, Germany) at 400 g. Furthermore, in vitro cell-cultures of 1, 2 and 4 h were made by culturing cancer cells on sterile clean glass slides for the specified number of hours. For longer lasting cultures, cell suspensions were brought onto clean round glass slides, and grown for up to 3 days. After gentle washing with PBS, the cultures were air-dried for 1 h and stored at -20°C.

Induction of tumors in rat liver

To induce tumors in livers of rats, the animals were anesthesized by intraperitoneal injection of FFM mix (1 ml Hypnorm, 1 ml Midazolam and 2 ml water; 0.27 ml per 100 g body weight) and after a small midline incision, single cell suspensions of $2.5 \times 10^6$ CC531s-eGFP cells in 0.5 ml PBS were injected into the portal vein. The animals were sacrificed with an overdose of sodium pentobarbital after 4 h, 1, 2, 3 days or 3 weeks after injection of the cancer cells. The livers were removed immediately, and tumor-containing liver blocks were dissected and snap-frozen in liquid nitrogen for storage at -80°C until further use.

Cryostat liver sections

Serial sections (8 µm thick) of liver specimens with different stages of tumor growth were cut with a motor-driven cryostat with rotary retracting microtome (Bright, Huntingdon, UK), at a cabinet temperature of -24°C. Sections were collected on clean glass slides at room temperature, and stored at -20°C until use.
Western blotting

For Western blotting, homogenates of scraped cultured CC531s-eGFP cells, and 3-weeks-old liver tumors were prepared. The samples were sonificated for 3 x 5 sec at 14 A, in 10 µl Eekhout buffer (1 M NaCl, 0.01% (v/v) Triton X-100 and 1 µM ZnCl₂ in 10 mM sodium cacodylate buffer, pH 6.0) per mg wet tissue weight, and stirred overnight at 4°C. After brief centrifugation at 10 000 g, 1 part of 3x Laemni loading buffer (30% (v/v) glycerol, 6% (w/v) SDS, 0.3% (v/v) brome fenol blue (BFB), 10 mM dithiothreitol (DTT) in 150 mM Tris/HCl, pH 6.8) was added to 2 parts supernatant. The samples were heated to 56°C for 30 minutes or to 100°C for 5 minutes and electrophorezed on a 10% SDS-PAGE gel at 30 mA. Proteins were blotted onto nitrocellulose membranes (Schleicher & Schuel, Dassel, Germany) and the blots were immunohistochemically stained for various adhesion molecules according to standard procedures. Prior to antibody incubations, nonspecific binding was blocked with 5% protifar in PBS-Tween (PBST) for 1 h. Incubations were performed in the presence of 2.5% protifar in PBST.

Immunohistochemistry and immunocytochemistry

Sections of livers containing tumors and cultured cells were air-dried for at least 1 h before fixation in acetone or 4% formaldehyde (in PBS) at room temperature for 10 minutes. After acetone fixation, sections were air-dried for 10 minutes before incubation with the primary antibody (Table 1). All incubations were performed in PBS, containing 0.2% bovine serum albumin (BSA) and 1% normal rat serum (NRS) to block nonspecific binding, in a moist dark chamber at room temperature for 60 minutes.

Peroxidase-labelled secondary antibodies were visualized using 3-amino-9-ethylcarbazole (AEC) as peroxidase substrate (20 mg AEC in 5 ml dimethylformamide (DMF) and 95 ml acetate buffer, pH 4.9, containing 0.01% hydrogen peroxide). The peroxidase reaction was performed for 10 min at room temperature. Sections were rinsed in water, counterstained with haematoxylin and mounted in glycerin-gelatin. The result was evaluated using standard light microscopy. Fluorescence-labelled secondary antibodies were visualized with a Leica DM IRBE confocal laser scanning microscope or with a Leica DMRA widefield fluorescence microscope (Leica, Mannheim, Germany). Nuclei were counterstained with DAPI (1 µg/ml).
Table 1. Primary and secondary antibodies used in immunostaining and Western blotting procedures.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Origin</th>
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<tr>
<td>αL (CD11a)</td>
<td>mouse</td>
<td>1:50 IH</td>
<td>Instruchemie, Hilversum, The Netherlands</td>
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<td>αv (CD51)</td>
<td>Arm. hamster</td>
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<td>kind gift dr. Wijnands, NKI (25)</td>
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<td></td>
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<td>Amsterdam, The Netherlands</td>
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<td>Pharmingen</td>
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<td>β2 (CD18)</td>
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<td>Instruchemie</td>
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<td>β3 (CD61)</td>
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<td></td>
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<td>kind gift dr. Sleeman, Institut für</td>
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<td></td>
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<td></td>
<td>Germany</td>
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<td>1:200 IH</td>
<td>Dakopatts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1000 WB</td>
<td></td>
</tr>
<tr>
<td>Goat-anti-Arm. hamster IgG</td>
<td>PO</td>
<td>1:120 IH</td>
<td>Jackson, Baltimore, MA, USA</td>
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<td></td>
<td></td>
<td>1:500 WB</td>
<td></td>
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<td>Goat-anti-mouse IgG</td>
<td>FITC</td>
<td>1:100 IH</td>
<td>Jackson</td>
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<td>1:100 IH</td>
<td>Jackson</td>
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<td>PO</td>
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Abbreviations: Arm. hamster, Armenian hamster; UEA-1, Ulex europaeus agglutinin-1; FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate; IH, immunohistochemistry and cytochemistry; WB, Western blotting; PO, peroxidase.
Results

Western blotting of cultured cells and tumor homogenates

Homogenates of scraped cultured cells and homogenates of metastases collected from rat livers at 3 weeks after induction of the tumors were investigated for expression of the integrin subunits αv, β1, CD44 and CD44v6.

The subunits were all expressed both in cultured cells and in tumors. Blotting of α6 was not possible with the antibody available, whereas blotting of αL, β2 and β3 was not performed because immunohistochemistry did not reveal any positivity on CC531s-eGFP cells (see below). The anti-αv antibody revealed a band of 150 kD in both samples. In homogenates of cultured cells but not in homogenates of tumors, a second band of lower molecular weight was present (Fig. 1, lanes 1 and 2) indicating expression of an αv-isoform by cancer cells in culture that is lost during tumor progression. Western blots stained with anti-β1 antibodies revealed a 130 kD band both in cultured cells and tumors. In contrast to αv, tumor homogenates revealed an extra band of β1 staining that was absent in cultured cells (Fig. 1, lanes 3 and 4). This could be either due to co-expression of an altered β1 molecule by CC531s-eGFP cancer cells in tumors or, more likely, stromal cells that synthesize a different β1-subunit than cancer cells. Staining of CD44 revealed in both cultured cells and metastases an 85 kD band showing the presence of the CD44s form, and two isoforms of approximately 180 kD (Fig. 1, lanes 5 and 6). The anti-CD44v6 antibody recognized the isoforms only showing specificity of the antibody for the v6 domain. It shows that both CD44 isoforms contain the v6 domain (Fig. 1, lanes 7 and 8).

![Figure 1](image.png)

Figure 1. Western blots of homogenates of tumors (lanes 1, 3, 5 and 7) and cultured cancer cells (lanes 2, 4, 6 and 8) for the integrin subunits αv (1 and 2), β1 (3 and 4), CD44 (5 and 6) and CD44v6 (7 and 8). In culture, cancer cells express an αv isoform which is lost during tumor progression, whereas in tumors a β1 isoform is expressed which is absent in cultured cells.

Immunolocalization
At 3 weeks after intraportal injection of CC531s-eGFP cells, moderately-differentiated liver tumors of 2-3 mm in diameter were present. Cancer cells in acinar structures and stromal cells can be clearly distinguished (Fig. 2). The α6 and β1 integrin subunits as well as CD44 and the v6 isoform were all abundantly present in these tumors (Fig. 2).

The antibodies against αv did not allow immunolocalization. The subunits αL, β2 and β3 could not be detected on cancer cells, whereas β1 and CD44 showed similar staining patterns with strong stromal and pericellular staining of cancer cells. Endothelium of larger hepatic vessels was positive for CD44. Staining for CD44v6 was restricted to cancer cells showing a pericellular localization pattern. Liver sinusoidal endothelial cells also expressed the β1 integrin subunit. Possibly, part of β1 staining in tumor stroma is present on endothelial cells of newly formed vessels, although CC531s-eGFP tumors are not rich in blood vessels (unpublished observations). Expression of α6 in tumors was restricted to the basal side of cancer cells. Tumor stroma was negative for α6. In liver parenchyma, α6 expression was found on epithelium of bile ducts, and only weakly on endothelial cells of larger vessels.

### Cultured cell preparations and cytospins

CC531s-eGFP cells cultured for 3 days did not stain for the integrin subunits αL, β2 which are the α and β chain of LFA-1 and β3 (Table 2). Sections of rat spleen were used as positive control for specificity of the β3 antibody, and revealed intense staining of lymphocytes. The αL and, β2 antibodies stained leukocytes. CC531s-eGFP cells were found to express the integrin subunits β1 and α6 and CD44, and its CD44v6 splice variant (Fig. 3). All adhesion
molecules were localized in a similar way on the cancer cells. Staining intensities were rather homogenous, with clusters of cells staining slightly more intense than others. Antigens were localized pericellularly, suggesting localization of the adhesion molecules on the cell membrane. This was confirmed by confocal laser scanning microscopy. Various adhesion molecules were localized only at the cell surface without intracellular staining (Fig. 4).

Figure 3. Cultured colon cancer cells stained for β1 (A; bar = 20 μm), α6 (B; bar = 50 μm), CD44 (C; bar = 40 μm) and CD44v6 (D; bar = 40 μm). All antigens are localized at the plasma membrane of cancer cells.

Figure 4. Optical section obtained with confocal microscopy of a cultured colon cancer cell stained for CD44. Localization of the antigen is restricted to the cell surface. Bar = 40 μm.

Cytospins of freshly-harvested cells were also stained with these antibodies. Harvested single cancer cells were mildly positive for the integrin subunits β1 and α6 (Fig. 5A, B and Table 2), and after 2 h of culture, staining was stronger as compared with cytospins.
As expected, harvested cancer cells were negative for integrin subunits αL, β2 and β3 (Table 2) but were also negative for CD44 (Fig. 5C and Table 2) and its v6 splice variant.

![Figure 5. Cytospins of freshly harvested CC531s-eGFP cells. Intense staining of the plasma membrane is obtained in cells stained for integrin subunit β1 (A; bar = 20 μm), moderate staining is obtained with cells stained for α6 (B; bar = 30 μm), whereas CD44 antigen is absent (C; bar = 25 μm).](image)

Table 2. Antigen staining in cytospins and different stages of cell culture.

<table>
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<tr>
<th>Antibody</th>
<th>Cytospin</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>3 days</th>
</tr>
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<tbody>
<tr>
<td>Anti-αL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Anti-α6</td>
<td>±</td>
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<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Anti-β1</td>
<td>+</td>
<td>++</td>
<td>+</td>
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</tr>
<tr>
<td>Anti-β2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>Anti-β3</td>
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<tr>
<td>Anti-CD44</td>
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<td>-</td>
<td>-</td>
<td>±</td>
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</tr>
<tr>
<td>Anti-CD44-v6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>+++</td>
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</table>

Positivity for antigen staining in cytospins and at 1-4 h and 3 days after culture. -, No staining; ±, heterogeneous staining; + to +++, mild to abundant staining.

Liver metastasis at 4 h, 1, 2, and 3 days after induction

Double staining of cancer cells with the lectin UEA and antibodies against integrin α6 or CD44 and CD44v6 was possible at 1, 2, and 3 days after induction of tumors. At 4 h after administration of cancer cells, only faint staining of α6 was achieved. It was impossible to visualize any β1 labelling of cancer cells, if present, because of surrounding β1-expressing sinusoidal endothelial cells at all time points studied. At 2 days after administration of cancer cells, α6 staining was found to be restricted to and to colocalize with cancer cells, particularly localized at the periphery of small cancer-cell clusters. This polarized appearance of α6 expression was even more pronounced at 3 days (Fig. 6A-C), and was similar with the periacinar localization found in tumors at 3 weeks after induction.
Expression of CD44 was found to be largely restricted to cancer cell clusters, with occasional ‘clouds’ of non-malignant CD44-positive cells in the vicinity of cancer cells, probably immune cells (Fig. 6D-F). Staining of CD44v6 was restricted to cancer cells at 1, 2 and 3 days after administration of cancer cells (Fig. 6G-I).

Discussion

We have previously shown that colon cancer cell arrest in rat liver sinusoids is due to size restriction rather than adhesion to endothelium. After cancer cell arrest, endothelial cells retract rapidly and cancer cells have direct interactions with hepatocytes (25). In the present study, we evaluated possible candidate molecules involved in the interaction between cancer
cells and hepatocytes. Adhesion molecules are likely involved and therefore, expression of different integrin subunits and CD44 and CD44v6 was investigated on cultured cancer cells and in livers containing tumors.

Leukocyte function-associated antigen-1 (LFA-1) molecules that interact with ICAM play a role in T-cell and B-cell cancers (27). Furthermore, it has been shown that cancer cells of epithelial origin can express leukocyte-associated adhesion molecules. For example, melanoma cells express very late antigen-4 (VLA-4) that can interact with VCAM (28). It is hypothesized that epithelial cancer cells may use similar mechanisms that are used by white blood cells. However, in the present study, neither cultured cancer cells nor colon cancer tumors in rat liver at different stages of development expressed either subunits αL or β2 of LFA-1 or VLA-4. It is therefore unlikely that these cancer cells use “immune cell” adhesion molecules including αLβ2, αdβ2, αmβ2 and αxβ2. Furthermore, β3 was not expressed on cultured cells or tumors at any stage of development. This suggests that the integrins αvβ3 and αIβ3 are not expressed on these cancer cells either. On the other hand, αv, α6, β1, CD44 and CD44v6 were expressed on cultured cancer cells and in 3 weeks old metastases as shown with Western blotting and immunohistochemistry. Expression of several integrin subunits or dimers have been correlated with enhanced malignancy in various human tumors. For example, in vivo selected colon cancer metastases revealed upregulated expression of, among others, β1 integrin and CD44v6 (29), whereas α6 expression was correlated with enhanced malignancy of prostate cancer (30) and breast cancer (31, 32). Decreased expression of α6 was shown to be involved in tumor progression of skin carcinoma (33) and was also observed in more aggressive colon tumors (34).

In the present study, we were interested in adhesion molecules that may play a role in the initial stages of tumor development i.e. the first hours to days after arrest of cancer cells in the liver. Therefore we also evaluated the presence of α6, β1, CD44 and CD44v6 on cancer cells in the state as when they are administered to rats, namely after trypsinization of cancer cells cultures. It was found that α6 and β1 were still present at the plasma membrane after trypsinization whereas CD44 and CD44v6 protein could not be detected. Staining of CD44 and CD44v6 reappeared weakly after 4 h of culture (Table 2). Apparently, the integrin subunits α6 and β1 are more resistant to trypsinization than CD44 and CD44v6. This implies that CD44 and CD44v6 are less likely candidates for interactions between hepatocytes and cancer cells after initial cell arrest.
In conclusion, in the present study, we have investigated a number of adhesion molecules that may be involved in interactions between cancer cells and hepatocytes. It was found that the integrin subunits $\alpha v$, $\alpha 6$ and $\beta 1$ are expressed at the protein level by cultured and harvested colon cancer cells whereas $\alpha L$, $\beta 2$ and $\beta 3$ were not detectable, even not on cultured cells. CD44 and CD44v6 were expressed on cultured colon cancer cells but were lost after trypsinization. Therefore, $\alpha v$, $\alpha 6$ and $\beta 1$ are potential candidates for the observed initial interactions between cancer cells and hepatocytes. Localization of these integrin subunits at the EM level should provide more information on the precise localization of the integrin subunits. These studies are currently in progress.

References
