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Interactions between colon cancer cells and hepatocytes in rats in relation to metastasis

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Abstract

Adhesion of cancer cells to endothelium is considered an essential step in metastasis. However, we have shown in a previous study that when rat colon cancer cells are administered to the vena portae, they get stuck mechanically in liver sinusoids. Then, endothelial cells retract rapidly and cancer cells bind to hepatocytes. We investigated the molecular nature of these interactions between colon cancer cells and hepatocytes. Cancer cells in coculture with hepatocytes became rapidly activated with distinct morphological changes. Cancer cells formed long cytoplasmic protrusions towards hepatocytes in their close vicinity and these protrusions attached to microvilli of hepatocytes. Then, adhering membrane areas were formed by both cell types. Integrin subunits αv, α6 and ß1 but not αL, ß2, ß3 and CD44 and CD44v6 were expressed on the cancer cells. In conclusion, colon cancer cells show an active behaviour to bind to hepatocytes, likely involving the integrin subunits αv, α6 and ß1, indicating that early events in colon cancer metastasis in liver are distinctly different than assumed thus far.

Keywords: colon cancer • metastasis • liver • hepatocyte • adhesion • integrin • CD44

Introduction

Colorectal cancer patients mainly die of metastatic disease rather than the primary tumour. Key events in metastasis are increased proteolytic activity, increased cell motility and altered expression of adhesion molecules [1–5]. Cell adhesion molecules can be classified into five major groups [3, 6]: integrins, selectins, cadherins, members of the immunoglobulin superfamily and other molecules. They all have been suggested to be involved in cancer progression and metastasis. Two types of adhesion molecules, the integrins and the CD44 hyaluronic acid receptors, are of particular importance with respect to the development of colon cancer and metastasis [7–13]. It is a generally accepted concept that adhesion of cancer cells in the capillary bed of a distant organ is based on interactions between the endothelium and cancer cells [1, 14–17]. The contrasting concept is that of mechanical entrapment of cancer cells in the first capillary bed that the cells encounter [18–21]. We have evidence that both concepts are at least partly correct because we have previously shown that colon cancer cells (CC531s) administered to the portal vein of rats bind to hepatocytes and not to sinusoidal endothelial cells [18]. It was observed that the cancer cells are arrested abruptly in sinusoids, endothelial cells retract locally within 30 min and specific molecular bridges are then formed between cancer cells and hepatocytes [18]. In the present study, we investigated these molecular interactions between these cell types *in vitro* and *in vivo* and focused on the involvement of integrins and CD44 variants.

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

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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 was cultured at 37°C as monolayers in RPMI-1640 Dutch Modification without L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 100 IU penicillin/ml and 100 mg streptomycin/ml (all from Invitrogen). Cells were washed with phosphate-buffered saline (PBS) and after detachment with the use of trypsin (0.05% w/v; Invitrogen) and ethylenediaminetetraacetic acid (EDTA) (0.02% w/v) in PBS and centrifugation (250 g, room temp, 5 min), single cell suspensions were obtained with a viability of at least 95% [22].

To investigate effects of trypsinization on surface molecules of the cells, cytospins of cancer cells were made by centrifugation of 250 µl cell suspension onto clean glass slides with a Hettich 1502 centrifuge (Hettich Zentrifugen, Tüv, Germany) at 400 g. Short-term *in vitro* cell cultures of 1, 2 and 4 hrs were made by culturing cancer cells on sterile clean glass slides. Long-term cancer cell cultures were made on clean round glass slides for up to 3 days. After gentle washing with PBS, cells were air-dried for 1 hr and stored at –20°C.

Cancer cells cultured on glass slides for 3 days were incubated in the presence of isolated hepatocytes [23]. After 1 hr, non-adhering cells were removed by washing with PBS. Then, attached cells were prepared for electron microscopy. Freshly isolated hepatocytes do not adhere to glass slides. Therefore, we could not perform the experiments the other way around as would be more closely resembling the *in vivo* situation.

Induction of tumours in rat liver

To induce tumours in livers of rats, the animals were anaesthetized by intraperitoneal injection of a mixture of 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 2.5×10^6 CC531s-eGFP cells in 0.5 ml PBS were injected into the portal vein. The animals were sacrificed at 4 hrs, 1 day, 2 days, 3 days or 3 weeks after injection of the cancer cells. The livers were removed immediately, and tumour-containing liver blocks were dissected and snap-frozen in liquid nitrogen for storage at -80°C until further use [18, 22].

Serial sections (8 µm thick) of liver specimens containing colon cancer tumours 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.

Electron microscopy

CC531s cells cultured on glass slides in the presence of hepatocytes were fixed with 4% (v/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, for 2 hrs at 4°C. After fixation, cells were rinsed with 100 mM cacodylate buffer, pH 7.4, for 40 min and post-fixed with 1% OsO_4 (Merck, Darmstadt, Germany) in 100 mM cacodylate buffer, pH 7.4, for 1 hr at 4°C. Afterwards, samples were thoroughly rinsed with bidistilled water, dehydrated and embedded in epoxy resin LX-112 (Ladd, Burlington, VT) according to standard procedures. Ultrathin sections (80 nm thick) were cut on an Ultracut E ultramicrotome (Leica Microsystems, Wetzlar, Germany) perpendicular to the glass slides. The ultrastructure was studied with an EM 420 transmission electron microscope (Philips, Eindhoven, The Netherlands). Areas of adhesion between colon cancer cells and hepatocytes were investigated with electron tomography. Electron tomographic reconstructions were made from 200 nm-thick sections, using two perpendicular tilt series (+55°/-55°, increment 5°) [24, 25].

Western blotting

For Western blotting, CC531s cells were scraped to avoid the use of trypsin and were homogenized. Homogenates of 3-weeks-old liver tumours after being dissected from surrounding liver tissue were used as well. The samples were sonicated for 3 x 5 sec. at 14 A, in a concentration of 0.25 g per ml Eekhout buffer (1 M NaCl, 0.01% (v/v) Triton X-100 and 1 µM $ZnCl_2$ in 10 mM sodium cacodylate buffer, pH 6.0), and stirred overnight at 4°C. After brief centrifugation at 10,000 g, 1 part of 3x concentrated Laemli loading buffer (30% (v/v) glycerol, 6% (w/v) sodium dodecyl sulphate, 0.3% (v/v) brome phenol blue, 10 mM dithiothreitol in 150 mM Tris/HCl, pH 6.8 was added to two parts supernatant. The samples were heated for 30 min. at 56°C or for 5 min. at 100°C and electrophoresis was performed on 10% SDS-polyacrylamide gel. Proteins were blotted onto nitrocellulose membranes (Schleicher & Schuel, Dassel, Germany) and the blots were stained immunohistochemically for various adhesion molecules (Table 1) according to standard procedures. Non-specific binding was blocked with 5% protifar in PBS-Tween for 1 hr. Incubations with primary antibodies were performed in the presence of 2.5% protifar in PBS-Tween. Control incubations were performed in the absence of primary antibodies.

Immunohistochemistry and immunocytochemistry

Sections of livers containing tumours and colon cancer cells after culture were air-dried for at least 1 hr before fixation in acetone or 4% paraformaldehyde in PBS for 10 min at room temperature. After acetone fixation, sections were air-dried for 10 min before incubation with primary antibodies (Table 1). All incubations were performed in PBS, containing 0.2% bovine serum albumin and 1% normal rat serum to block non-specific binding, in a moist dark chamber for 60 min at room temperature.

Visualization of the antibodies bound to liver sections and cancer cells was performed either by coloured final reaction product to produce permanent preparations or by fluorescence for confocal microscopy.

Peroxidase-labelled secondary antibodies (Table 1) were visualized using 3-amino-9-ethylcarbazole (AEC) as peroxidase substrate (20 mg AEC in 5 ml dimethylformamide 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 (Table 1) were visualized with a Leica DM IRBE confocal laser scanning microscope SP2-AOBS or with a Leica DMRA wide-field fluorescense microscope (Leica). Nuclei were counterstained with 4,6'-diamidino-2-phenylindole (DAPI; 1 µg/ml; Sigma).

Table 1 Primary and secondary antibodies used for immunostaining and Western blotting

Primary antibodies			
Antigen	Isotype	Dilution	Origin
αL (CD11a)	Mouse	1:50 IH	Instruchemie, Hilversum, The Netherlands
αv (CD51)	Arm. hamster	1:250 WB	Pharmingen, San Diego, CA
α6; C28 (CD49f)	Arm. hamster	1:10 IH 1:60 WB	Kind gift Dr. Wijnands, NKI Amsterdam, The Netherlands
ß1 (CD29)	Arm. hamster	1:200 IH 1:1000 WB	Pharmingen
ß2 (CD18)	Mouse	1:50 IH	Instruchemie
ß3 (CD61)		1:50 IH	Pharmingen
CD44	Mouse	1:1000 IH 1:4000 WB	Pharmingen
CD44v6; 1.1ASML	Mouse	1:300 IH 1:800 WB	Kind gift Dr. Sleeman, Institut für Toxikologie und Genetik, Karlsruhe, Germany
Ulex Europaeus agglutinin 1 (UEA-1)	Lectin	1:100 IH	Dakopatts, Glostrup, Denmark
UEA-HRP	Lectin	1:70 IH	Dakopatts

Secondary antibodies			
Antibody	Label	Dilution	Origin
Rabbit-anti-mouse IgG	PO	1:200 IH 1:1000 WB	Dakopatts
Goat-anti-Arm. hamster IgG	PO	1:120 IH 1:500 WB	Jackson, Baltimore, MA
Goat-anti-mouse IgG	FITC	1:100 IH	Jackson
Goat-anti-Arm. hamster IgG	FITC	1:100 IH	Jackson
Swine-anti-rabbit IgG	TRITC	1:40 IH	Dakopatts
Rabbit-anti-mouse	TRITC	1:200 IH	Dakopatts
Rabbit-anti-UEA-1	PO	1:60 IH	Dakopatts

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.

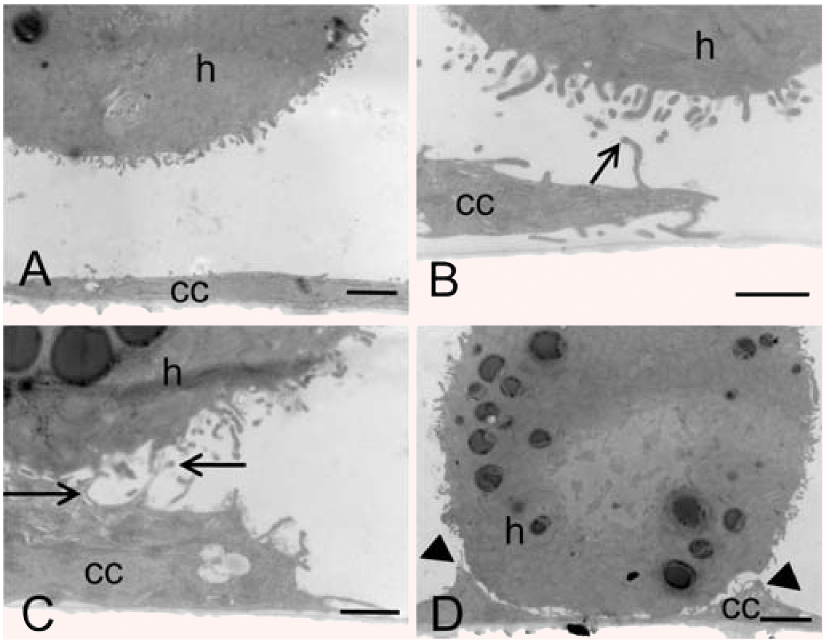

Fig. 1 Electron micrographs of colon cancer cells and hepatocytes in coculture for 1 hr. CC531s cells were cultured on glass for 3 days and suspensions of hepatocytes were added. When hepatocytes were not in the vicinity, cancer cells (cc) were flattened with few small protrusions only and hepatocytes (h) were rounded (**A**). When hepatocytes were in close vicinity of cancer cells, cancer cells rapidly became bulky and formed protrusions in the direction of the hepatocytes (**B**). Contact between cancer cells and hepatocytes was established between cancer cell protrusions and microvilli of hepatocytes (arrows; **B, C**) and then stretches of parallel-running membranes were made (arrow heads; D). Bars = 2 µm (**A, C**) or 1 µm (**B, D**).

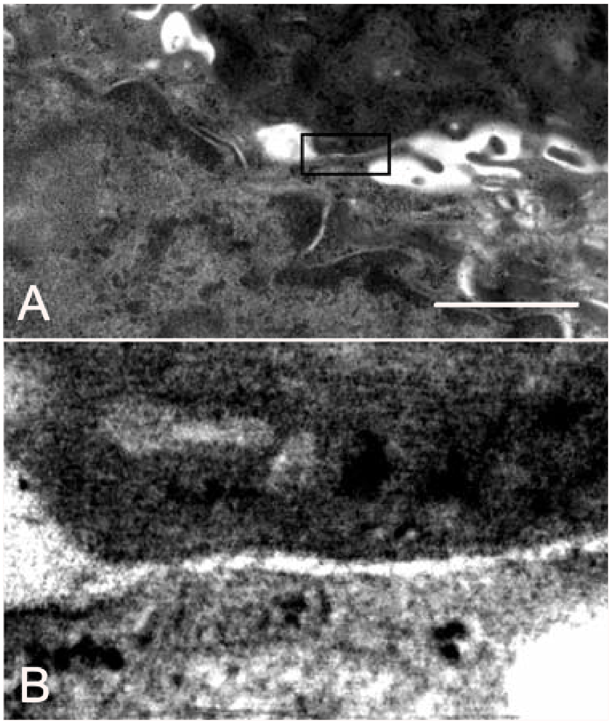

Fig. 2 Electron micrograph (**A**) and electron tomographic reconstruction (**B**) of the intercellular space between a cultured colon cancer cell and a suspended hepatocyte after 1 hr coculture showing electron-dense molecular contacts. The reconstructed area (0.45 µm × 0.28 µm) is indicated. Thickness of the ultrathin section was 120 nm. Bar = 1 µm.

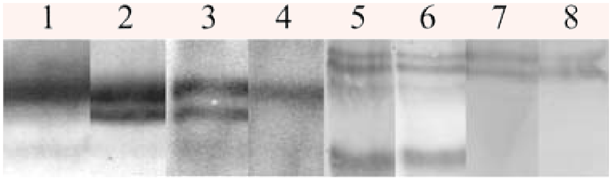

Fig. 3 Western blots of homogenates of 3-weeks-old tumours (lanes 1, 3, 5 and 7) and cultured cancer cells (lanes 2, 4, 6 and 8) for the integrin subunits αv (1 and 2), ß1 (3 and 4), CD44 (5 and 6) and CD44v6 (7 and 8). In culture, cancer cells express an αv isoform which is lost during tumour progression, whereas in tumours a ß1 isoform is expressed which is absent in cultured cells.

Results

Electron microscopy

Colon cancer cells cultured on glass slides were flat with few pseudopodia (Fig. 1A). The morphology of colon cancer cells hardly changed when hepatocytes were not in the direct vicinity. When hepatocytes were approaching cancer cells, cytoplasmic protrusions of cancer cells in the direction of hepatocytes were formed rapidly (within an hour) and cancer cells became more bulky (Fig. 1B). Contacts between cancer cells and hepatocytes started as contacts between protrusions of cancer cells and microvilli of hepatocytes (Fig. 1C) and resulted in large areas of parallel running plasma membranes (Fig. 1D).

Electron tomography revealed electron dense molecular contacts between cancer cells and hepatocytes (Fig. 2) similar to the

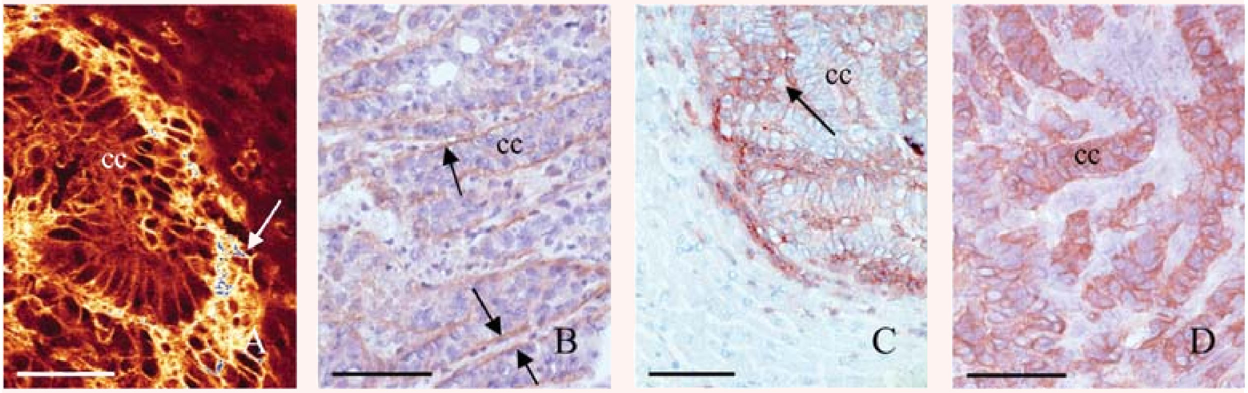

Fig. 4 Localization of ß1 (**A**; bar = 40 μm), α6 (**B**; bar = 60 μm) integrin subunits and CD44 (**C**; bar = 150 μm) and its splice variant CD44v6 (**D**; bar = 60 μm) in cryostat sections of rat liver containing 3-weeks-old tumours. The ß1 integrin subunit is localized at the plasma membrane of cancer cells (cc) and is abundantly present in tumour stroma (arrow) (**A**). Localization of the α6 integrin subunit is restricted to the basal side of cancer cells in acini (arrows) (**B**). CD44 is localized at the plasma membrane of cancer cells (cc) and in tumour stroma (arrow) (**C**), whereas CD44v6 is restricted to cancer cells (**D**).

contacts that have been observed previously *in vivo* [18]. These molecular contacts were present between the ridges of parallel running membranes (Fig. 1D), but not between pseudopodia of cancer cells and microvilli of hepatocytes (Fig. 1C).

Western blotting of liver metastases and cultured colon cancer cells

Western blotting showed that cancer cells that were scraped after culture and 3-weeks-old tumours expressed integrin subunits αv and β1, and CD44 and its splice variant CD44v6 (Fig. 3). Blotting of α6 was not possible with the antibody available, whereas blotting of αL, ß2 and ß3 was not performed because immunohistochemistry did not reveal any positivity on CC531s cells. Control incubations in the absence of primary antibodies were always negative.

The anti-αv antibody revealed a band of 150 kD in both cultured cancer cells and tumours. A second band with a lower molecular weight was present in homogenates of cultured cells but not of tumours indicating expression of an αv-isoform by cancer cells in culture that is lost during tumour progression. Western blots stained with anti-β1 antibodies revealed a 130 kD band both in cultured cancer cells and tumours. Tumour homogenates showed an extra ß1 band that was absent in cultured cells. This was likely expressed by stromal cells. Staining of CD44 revealed in both cultured cells and tumours an 85 kD band indicating the CD44s form, and two isoforms of approximately 180 kD. These two 180 kD bands and not the 85 kD band were stained with the anti-CD44v6 antibody indicating that 180 kD isoforms contained the v6 domain (Fig. 3).

Immunohistochemistry of colon cancer tumours in liver

At 3 weeks after intraportal administration of CC531s cells, moderately-differentiated colon cancer tumours of 2–5 mm in diameter were present in the livers. Cancer cells were arranged in acinar structures surrounded by stromal cells (Fig. 4). The α6 and β1 integrin subunits as well as CD44 and CD44v6 were abundantly present in these tumours. The subunits αL, β2 and β3 could not be detected on cancer cells. The αL and β2 (which are the α and ß chain of lymphocyte function-associated antigen-1 (LFA-1)) antibodies stained leukocytes (data not shown). Sections of rat spleen were used as positive control for specificity of the β3 antibody, and revealed intense staining of lymphocytes (data not shown). Immunolocalization of αv did not succeed with the antibodies available.

CD44 adhesion molecule showed similar staining patterns as the ß1 integrin subunit (Figs. 4A, C and 5F) in stroma and pericellularly on cancer cells. Staining of CD44v6 was restricted to cancer cells showing a pericellular localization pattern (Figs. 4D and 5I). Expression of α6 was restricted to the basal side of cancer cells (Fig. 5C). Tumours at 4 hrs to 3 days after cancer cell inoculation showed similar staining patterns.

Immunocytochemistry of cultured colon cancer cells

CC531s cells expressed α6 and ß1 integrin subunits but not αL, β2 and β3 subunits (Table 2). CD44 and its CD44v6 splice variant were expressed only after longer periods of culture (Fig. 6). Apparently, trypsin treatment during cell harvest resulted in the proteolytic removal of the CD44 and CD44v6 epitope. Staining intensities were rather homogenous, with clusters of cells staining slightly more intense. Antigens were localized pericellularly, suggesting localization of the adhesion molecules at the plasma membrane (Fig. 7).

Discussion

We have previously shown that colon cancer cell arrest in rat liver sinusoids is due to size restriction rather than adhesion to

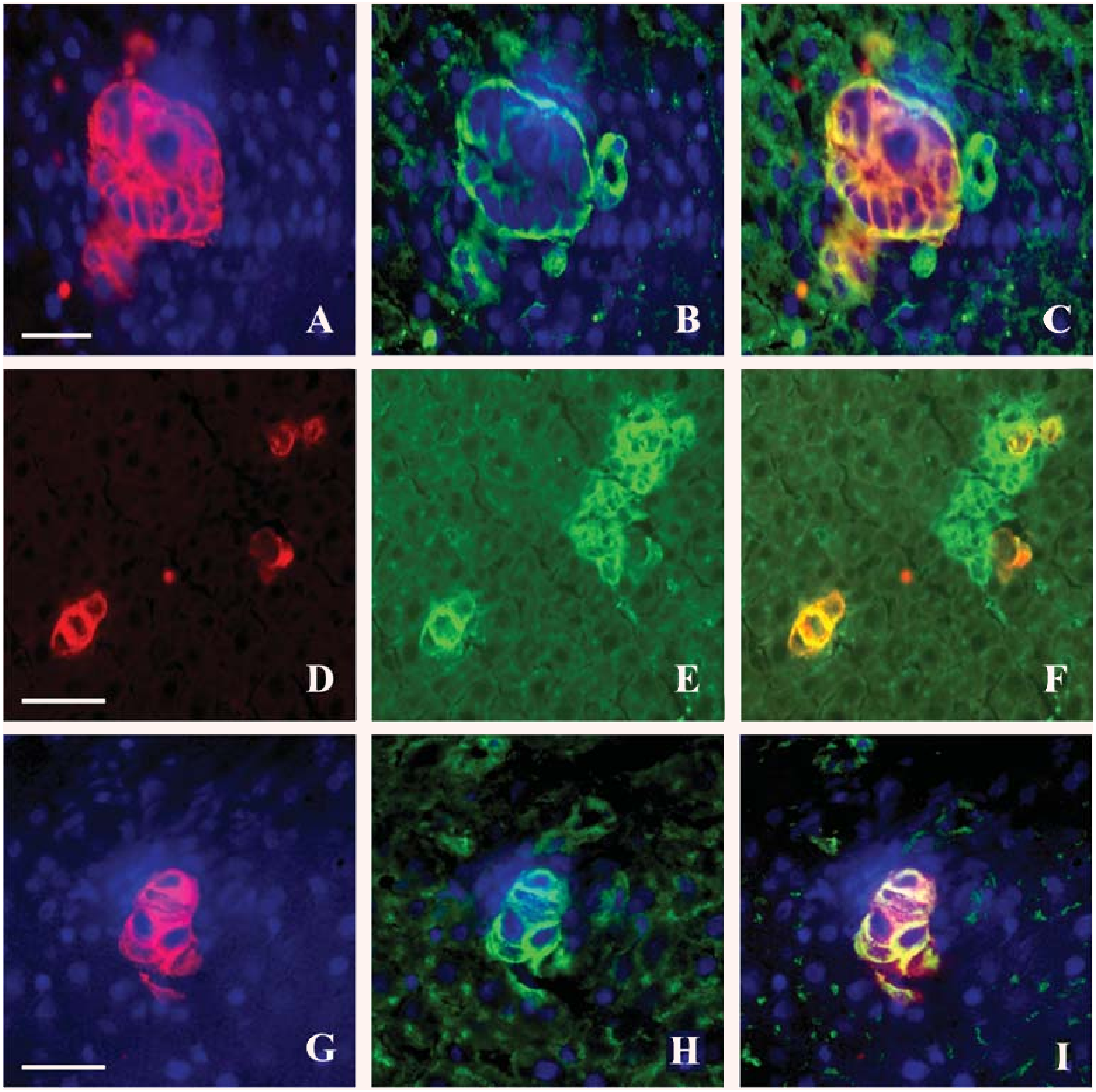

Fig. 5 Double staining of colon cancer cells using Ulex Europaeus agglutinin (UEA) staining (**A**; bar = 30μm, **D**; bar = 50 μm and **G**; bar = 30 μm) and α6 (**B**), CD44 (**E**) and CD44v6 (**H**) staining and their respective overlays (**C, F** and **I**) in 3-days-old tumours in rat livers. Yellow represents colocalization of green and red. Cancer cells express α6 at the basal side (**C**), CD44 is expressed by cancer cells and stromal cells in tumours (**F**), and CD44v6 is exclusively expressed by cancer cells (**I**).

endothelium [18]. We did not find a single cancer cell in the perfusate of livers collected from the hepatic vein indicating that entrapment in the sinuoids was absolute. This would not happen when arrest was due to selective adhesion to the sinusoidal endothelium [26, 27].

After cancer cell arrest, endothelial cells retract and cancer cells have direct molecular interactions with hepatocytes *in vivo* [18]. Retraction of endothelium occurs rapidly (within 30 min) after cancer cell arrest. Other studies have shown that cancer cells can disrupt the endothelial barrier in capillaries either by

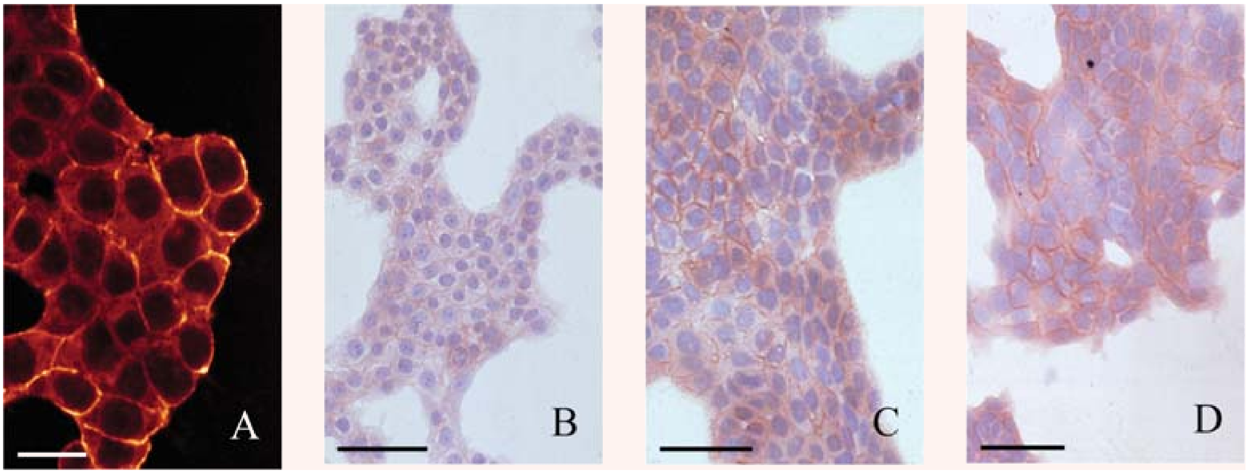

Fig. 6 Colon cancer cells cultured for 3 days stained for ß1 (**A**; bar = 20 µm), α6 (**B**; bar = 50 µm), CD44 (**C**; bar = 40 µm) and CD44v6 (**D**; bar = 40 µm). All antigens were localized at the plasma membrane of cancer cells.

Table 2 Immunohistochemical staining of integrin subunits and CD44 isoforms on rat CC531s colon cancer cells in cytospins and after various periods of culture

Molecule	Cytospin	1 hr	2 hrs	4 hrs	3 days
αL	-	-	-	-	-
α6	±	+	+	+	+++
ß1	+	+	++	++	+++
ß2	-	-	-	-	-
ß3	-	-	-	-	-
CD44	-	-	-	±	+++
CD44v6	-	-	-	±	+++

-, No staining; ±, heterogeneous staining; + to +++, mild to abundant staining.

induction of apoptosis [26–31], or secretion of Vascular endothelial growth factor (VEGF) [32], reactive oxygen species [33, 34], arachidonic acid metabolites such as 12(S)-hydroxyeicosatetraenoic acid [35, 36] or the induction of inflammatory processes [37]. However, VEGF, reactive oxygen species and arachidonic acid metabolites are secreted by cancer cells after adhering to endothelial cells and apoptosis is not induced within 30 min [38]. Inflammatory responses and/or interactions between cancer cells and leukocytes or platelets [37] have never been observed either in our previous study [18, 38] or in the present study. Therefore, in our model endothelial retraction is likely due to mechanical stress caused by the cancer cells arrested in the sinusoids. Endothelial cells are very sensitive to mechanical stress [39].

The Electron Microscopical (EM) analysis of cocultures of colon cancer cells and hepatocytes *in vitro* shows an active behaviour of cancer cells rapidly induced by hepatocytes that are in close vicinity. This active behaviour ultimately results in large membrane areas of cell–cell contacts. These membrane areas are characterized by protein bridges that closely resembled the structures that have been observed between cancer cells and hepatocytes *in vivo* [18]. Adhesion molecules are likely involved. It has been hypothesized that colon carcinoma cells may use similar mechanisms that are involved in invasion and migration of leukocytes [2, 3]. In the present study, neither cultured cancer cells nor colon cancer tumours in rat liver at different stages of development expressed either subunits αL or ß2 of LFA-1. Therefore, it is unlikely that these cancer cells use ‘immune cell’ adhesion molecules including αLß2, αdß2, αmß2 and αxß2 for their interactions with hepatocytes. It cannot be excluded that very late antigen-4 (VLA-4, also known as integrin α4ß1) was involved. VLA-4 has been shown recently to play an essential role in the formation of pre-metastatic niches by bone marrow progenitor cells [40].

Furthermore, ß3 was not expressed on cultured cancer cells or in the tumours at any stage of development. This suggests that the integrins αvß3 and αIIbß3 are not involved either. On the other hand, αv, α6, ß1, CD44 and CD44v6 were expressed on cultured cancer cells and in metastases as shown by immunohistochemistry and Western blotting. Involvement of α6, and/or CD44v6 in cancer cell adhesion has been reported previously [12, 13, 41–45].

We evaluated expression of α6, ß1, CD44 and CD44v6 on cancer cells as they were administered to rats, namely after trypsinization. It was found that α6 and ß1 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 hrs of culture (Table 2). Apparently, the integrin subunits α6 and ß1 were resistant to

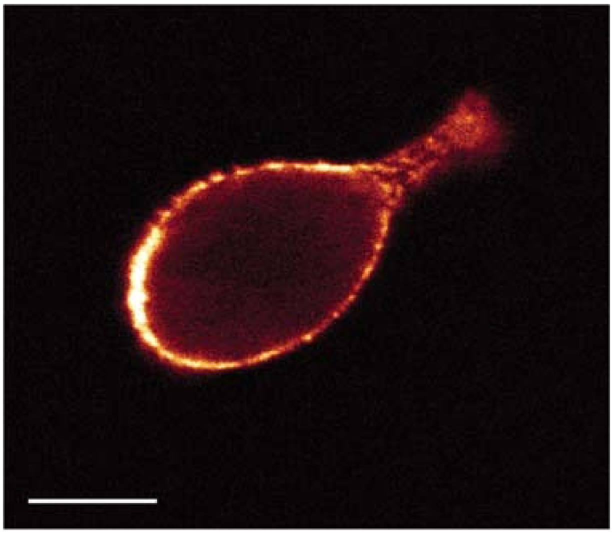

Fig. 7 Optical section obtained with confocal microscopy of a cultured colon cancer cell stained for CD44. Localization of CD44 was restricted to the cell surface. Bar = 40 µm.

trypsinization and CD44 and CD44v6 were not. This implies that subunits α6 and ß1 and not CD44 and CD44v6 can be involved in immediate interactions between hepatocytes and cancer cells after initial cancer cell arrest in the sinusoids. Enns *et al.* [14, 15] showed that α6β1, α6β4, but not αvβ3 integrins are crucial for cancer cell adhesion in liver sinuoids. Others did find a role for αvß3 in liver metastasis [46] and ß1 integrins in the binding of colon cancer cells to laminin [47]. It remains to be established whether αv, α6 and ß1 integrin subunits are directly involved in the interactions between colon cancer cells and hepatocytes but integrins can participate in cell–cell interactions, for example, when members of the immunoglobulin superfamily or Extracellular Matrix molecules are attached to the hepatocytes such as heparins [48] or fibronectin [49]. As a consequence of binding of αv, α6 and ß1-containing integrins, other processes involved in metastasis such as proteolysis and cell motility can be initiated [4, 5, 50, 51].

In conclusion, we have previously found evidence for colon cancer cell dissemination in liver *in vivo* due to size restriction in the sinusoids and subsequent adhesion between cancer cells and hepatocytes and not the endothelium. In the present study, we further elucidated these molecular interactions between cancer cells and hepatocytes *in situ* and *in vitro*. Our findings provide evidence that both concepts for cancer cell arrest in a capillary bed, mechanical entrapment due to size restriction and arrest due to adhesive interactions are correct and are not mutually exclusive.

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