Chapter 7

Mechanisms of colon carcinoma cell binding to components of the extracellular matrix

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Submitted

ABSTRACT

Interactions between colon cancer cells and the extracellular matrix (ECM) play a major role in intravasation and subsequent extravasation during metastasis in colon cancer.
In a human in vitro model the adhesion of the HT29 and Caco2 colon carcinoma cell lines to the main components of the ECM, collagen type I and IV, fibronectin and laminin, was investigated. This adhesion was compared to the adhesion to the overlying endothelium and mesothelium.
Inhibition assays with the use of monoclonal antibodies were performed to study the role of adhesion molecules in tumour cell - ECM interactions.
We showed that different colon cancer cell lines strongly adhere to the ECM, although they express different adhesion patterns to these components. Lower adhesion was seen to the endothelium and mesothelium. Knowledge of the integrins involved in tumour cell - ECM interactions may lead to new therapeutic strategies to prevent metastasis.

INTRODUCTION

Despite the development of modern treatment strategies for colon cancer, recurrences after intentionally curative surgery remain a major problem. Recurrence rates up to 40% have been reported and preferential sites are locally, as well as liver and lungs [1-4]. The majority of death of colorectal cancer patients is caused by metastatic disease rather than the primary tumour [5].

Key events in the metastasic cascade comprise of invasion in tumour stroma, intravasation of the circulatory system at the primary site, survival from defence mechanisms, extravasation at the secondary site and outgrowth of new tumours.

At the secondary site, binding to mesothelial or endothelial cells results in retraction with discontinuity of the cell layer, thereby exposing the underlying extracellular matrix (ECM) [6]. Binding to components of the ECM stimulates the production of matrix metalloproteinases which degrade the ECM [7]. In this way, the tumour cells can enter the underlying tissue, proliferate and grow out to form a metastasis. Tumour cells show a higher binding affinity to the ECM compared to the overlying endothelium or mesothelium [8,9]. This difference in binding affinity causes a gradient for the tumour cells into the underlying tissue. In the case of trauma to the mesothelial or endothelial cell layer, it is likely that more circulating tumour cells are able to adhere and successfully form a metastasis. Trauma may consist of direct trauma like the disruption of the mesothelium in case of abdominal surgery or indirect trauma like inflammation.

Major components of the ECM are collagen type I (C I) and IV (C IV), fibronectin (FN) and laminin (LN). Therefore, these components are thought to play an important role in cancer metastasis.

Binding to these components is accomplished by adhesion molecules on tumour cells. Both the cellular adhesion molecules ICAM and VCAM and the integrins may play a role in this interaction. The very late activation (VLA) antigens form a major subclass of integrins which are transmembrane heterodimers consisting of an α and β_1 chain. It is known that VLA-1 (α1β1), VLA-2 (α2β1) and VLA-3 (α3β1) are able to bind to C I and IV. FN act as a ligand for VLA-3 till VLA-5 and LN for VLA-1, -2, -3, -6 and -7 [10,11].

To study the adhesion of colon cancer cells to components of the ECM and to compare this adhesion with adhesion to the covering layer consisting of endothelium or mesothelium we developed a human *in vitro* model. We used two different human colon cancer cell lines, namely the poorly differentiated HT29 cell line and the moderately differentiated Caco2 cell line. The role of the VLA-antigens as well as ICAM-1 and VCAM-1 in binding to particular components was evaluated.

MATERIALS & METHODS

Cells

The human colon carcinoma cell lines HT29 and Caco2 were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, glutamine (2 mM) and penicillin (10^5 U/L) and maintained by serial passage after trypsinization using 0.05% trypsin / 0.02% EDTA (all, except penicillin, obtained from Invitrogen, Breda, the Netherlands; penicillin from Yamanouchi, Leiderdorp, The Netherlands).
Before the adhesion assay, tumour cells were trypsinized and maintained in suspension culture for 2 hours to regenerate cell-surface proteins.
Human microvascular endothelial cells of the lung (EC) were purchased from Cambrex (Verviers, Belgium) at passage 4 and maintained in EGM-2-MV Bullet kit according to the manufacturer at 37°C, 95% relative humidity and 5 % CO_2. Confluent monolayers were passaged by 0.025% trypsin / 0.01% EDTA and cells were used up to passage 8.
Human mesothelial cells (MC) are obtained from omental tissue of patients undergoing elective abdominal surgery. Isolation of the cells was accomplished using modified techniques from Wu *et al* [12]. To detach MC from the omentum, 0.05% Trypsin with 0.02% EDTA is used. Next, MC are resuspended in RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine (all Invitrogen) and penicillin (10^5 U/L) and brought into culture in collagen coated culture flasks at 37°C, 95% relative humidity and 5 % CO_2. Confluent monolayers were passaged by 0.05% trypsin / 0.02% EDTA.

Adhesion assay

To quantify tumour cell adhesion to components of the ECM, to EC and to MC monolayers, a standardised cell adhesion assay was developed according to methods from Catterall *et all* [13]. Flat-bottomed 96 well microtiter plates (Perkin Elmer, Groningen, The Netherlands) were coated with 20 µg/ml C I, C IV (Sigma-Aldrich, Zwijndrecht, the Netherlands), FN or LN (Boehringen-Mannheim, Mannheim, Germany). To block unspecific binding sites, wells were pre-incubated with 1% BSA for 30 minutes. To obtain endothelial and mesothelial monolayers, confluent cells were trypsinized and 2×10^4 EC and 1×10^4 MC were added to each well. The plates were incubated at 37°C, 95% relative humidity, 5% CO_2 and medium was daily replaced by fresh medium. EC reached confluence in 3 to 4 days as determined by light microscopy. For MC, confluence was achieved after 5 days.

To quantify tumour cell adhesion, trypsinized tumour cells (1×10^{6} cells/ml) were labelled with calcein-AM (Molecular Probes, Leiden, The Netherlands) and 3×10^{4} cells per well were added.

Evaluation of the contribution of integrins in the adhesion of tumour cells to components of the ECM was done by pre-incubation of the tumour cells with the function-blocking antibodies anti-α_1 till anti-α_6, anti-β_1 (BD Biosciences, Alphen a/d Rijn, the Netherlands), anti-ICAM and anti-VCAM (Dako Cytomation, Heverlee, Belgium) in a concentration of 1 µg/ml for 30 minutes at room temperature before adding the tumour cells to the wells.

Plates were centrifuged for 1 minute at $80 \times g$ in a Heraeus centrifuge and incubated at 37°C for 1 hour. After this, wells were washed twice with medium to remove non-adherent tumour cells. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using 485 nm excitation and 530 nm emission filters.

Immunocytochemistry of adhesion molecules

Tumour cells were prepared for staining by cytospin preparation, fixed in acetone for 10 minutes and stored at -20°C until use.

The cytospins were incubated for 30 minutes at room temperature with the following primary antibodies: mouse anti-human monoclonal antibodies to α_1 till α_6, β_1 (BD Biosciences, Alphen a/d Rijn, the Netherlands), ICAM-1 and VCAM-1 (Dako Cytomation, Heverlee, Belgium). Negative controls were incubated with PBS. As secondary antibodies, biotinylated goat anti-mouse antibodies were used followed by incubation with Streptavidin-biotinylated alkalin-phosphatase complex. Substrate development was done with New Fuchsine 4%. Cytospins were counterstained with Haematoxiline.

The expression of cell adhesion molecules was quantified by 2 separate observers using semi-quantitative scoring system ranging from no expression (-), weakly positive (±) to positive expression (+).

Statistical analysis

All data were analysed using analysis of variance (ANOVA) to determine overall differences between groups. The Dunnett post-test was carried out to compare between groups. $P \leq 0.05$ was considered to be statistically significant. Experiments (n=6) were performed at least twice with comparable results.

RESULTS

Validation of assay

Labelling tumour cells with calcein-AM did not decrease their viability (>95% using trypan blue). Incubation times not exceeding 60 minutes gave negligible leakage of calcein out of the cells into the medium (data not shown). Longer incubation times gave significant calcein loss resulting in unreliable results. Therefore, we used 60 minutes incubation time in our experiments. After incubation, three times washing resulted in thorough washing away non-adhering tumour cells and leaked calcein from the experimental wells (Fig 1).
Dilution series showed a linear correlation between tumour cell number and measured fluorescence that was used as a standard to calibrate measured fluorescence. In this way the amount of adhered tumour cells in the experimental wells could be determined.

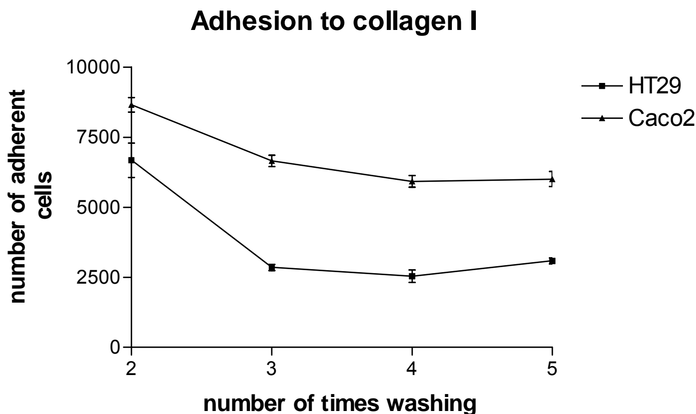

Figure 1. The effect of the number of wash steps after 1 hour incubation of HT29 and Caco2 on collagen I. Means (n=6) ± SEM are shown.

Tumour cell adhesion

Table 1 shows the adhesion of HT29 and Caco2 to the different components of the ECM as well as to endothelial and to mesothelial monolayers. For HT29, highest adhesion after 1 hour incubation was achieved to C I, namely 60.9%, followed by adhesion to C IV with 46.4%, than LN with 39.6% and lowest adhesion was to FN with 20.6%. Caco2 cells showed

a different adhesion pattern with highest adhesion to C IV (51.4%), followed by adhesion to FN with 48.8%, than to C I with 41.8% and lowest adhesion was to LN with 21.9%. To C IV and FN, Caco2 demonstrated a significant higher adhesion compared to HT29 (resp. $p<0.05$ and $p<0.01$). HT29 showed a significantly higher adhesion to C I and LN compared to Caco2 ($p<0.01$). The adhesion to EC was for both tumour cell lines considerably lower compared to the adhesion to the ECM, namely 12.1% of the HT29 and 21.9% of the Caco2 adhered to EC ($p<0.01$). Unlike the endothelial cells, which are acquired from a single donor, are the mesothelial cells obtained from different donors. Therefore, series of adhesion experiments were performed using monolayers of 5 different patients. Highest adhesion was found for Caco2 with 34.1% of the cells adhering. The mean adhesion of HT29 to MC was 21.4% of the cells.
Both cell lines showed low affinity for uncoated wells, with only 2.4% of HT29 cells and 5.7% of Caco2 cells adhering to the plastic of the experimental wells.

Table 1. Adhesion of HT29 and Caco2 to various substrates: components of the extracellular matrix, endothelial cells, mesothelial cells and to empty experimental wells (plastic). Data are expressed as the mean (n=6, % vs total) ± SD.

Substrate	C I	C IV	FN	LN	EC	MC	plastic
HT29	60.9 ± 2.1	46.4 ± 1.4	20.9 ± 2.7	39.6 ± 6.8	12.1 ± 2.0	21.4 ± 5.0	2.4 ± 0.8
Caco2	41.8 ± 4.0	51.4 ± 3.3	48.8 ± 5.7	21.9 ± 1.6	21.9 ± 3.4	34.1 ± 5.0	5.7 ± 0.5

Expression of integrins, ICAM and VCAM

Tabel 2 shows the immunocytochemistry results of the tumour cells. Except for the α4-component, all α-components are expressed on HT29. The α1- and α5-components are only weakly expressed. On Caco2, all α-components are expressed, although the α1-, α4- and α5-components show only weak expression. The β-component is expressed on both tumour cell lines, as well as ICAM-1 and VCAM-1, but the last two are only weakly expressed on HT29.

Table 2. Cell adhesion molecules expressed by HT29 and Caco2 as found by immunocytochemistry.

Ab	α1	α2	α3	α4	α5	α6	β1	ICAM	VCAM
HT29	+/-	+	+	-	+/-	+	+	+/-	+/-
Caco2	+/-	+	+	+/-	+/-	+	+	+	+

Inhibition assays

Inhibition assays using functional blocking antibodies to components of integrins are shown in figure 2 (HT29) and 3 (Caco2). Adding antibodies against the α_2 component gave a significant reduction of 64.9% ($p<0.01$) in HT29 cell adhesion to C I and of 30% ($p<0.01$) to C IV. For Caco2 a reduction of 46.8% was found by adding the α_2-antibody in the adhesion to C I and of 39.9% to C IV. Addition of the α_4-antibody resulted only for Caco2 in a significant reduction in the adhesion to C IV, namely 26.3%. Almost complete inhibition in HT29 cell adhesion to LN was achieved by addition of the α_6-antibody, since this antibody reduces adhesion with 96.1% ($p<0.01$). For Caco2 cells, no significant reduction was observed using the α_6-antibody in the adhesion to LN. Neither antibodies to other α-components, nor antibodies to the β_1-component, ICAM-1 and VCAM-1 gave an inhibition to ECM components.

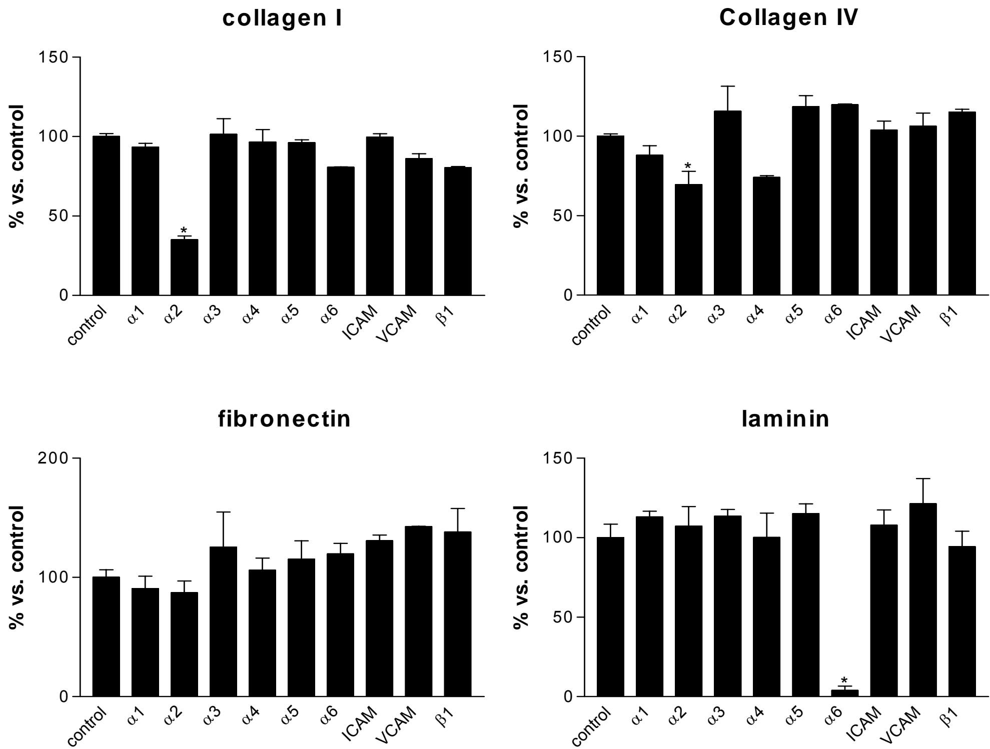

Figure 2. Adhesion of HT29 to different components of the ECM. Tumour cells were pre-incubated with antibodies against α1-6, β1, ICAM-1 and VCAM-1 (1 μg/ml). Data represent means (n=6, % *vs.* control) ± SEM. *, $p < 0.01$.

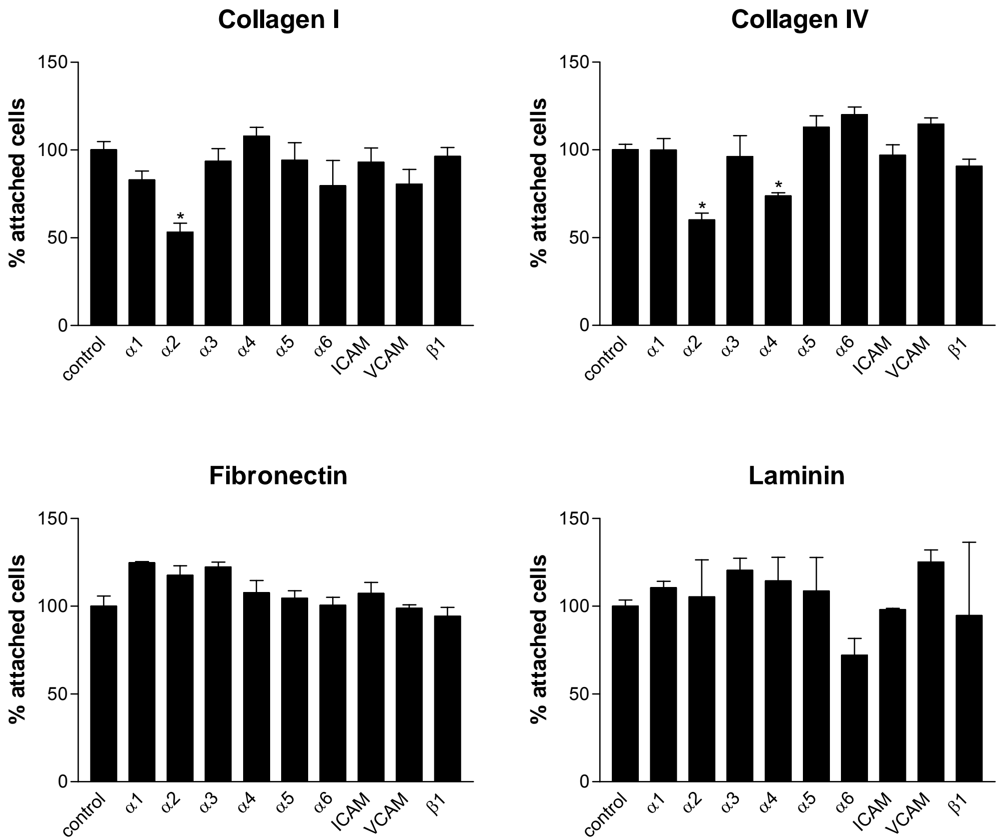

Figure 3. Adhesion of Caco2 to different components of the ECM. Tumour cells were pre-incubated with antibodies against α1-6, β1, ICAM-1 and VCAM-1 (1 μg/ml). Data represent means (n=6, % *vs.* control) ± SEM. *, $p < 0.01$.

DISCUSSION

The capacity for adhesion seems a contradiction in tumour metastasis, in which the first step the detachment of the tumour cell from the primary site is. However, detachment and adhesion are necessary steps and form a delicate balance in metastasis. Interactions between colon cancer cells and components of the ECM are thought to play a major role in intravasation and subsequent extravasation during the metastatic cascade in colon cancer. The purpose of this study was to analyse these interactions.

High adhesion was found for the HT29 and Caco2 colon cancer cell lines to the various components of the ECM, although each cell line displays its own adhesion pattern. For HT29, 60.9% of the cells attached to C I and 40% or more to C IV and LN. Lowest adhesion was seen to FN with only 20.6% of HT29 adhering. More than 50% of the Caco2 cells adhered to C IV, 48.8% and 41.8% of the Caco2 cells adhered to FN and C I respectively. Here, lowest adhesion was to LN with only 21.9% of the cells adhering. This individual adhesion pattern for various types of colon cancer cell lines was also found by Schlaeppi *et al* [14], who compared 4 different colon carcinoma cell lines in their adhesion and invasion to various components of the ECM.

A preceding step in extravasation is adhesion to the mesothelium locoregionally or the microvascular endothelium at secondary sites. To the mesothelium the adhesion varied between different donors with each having their unique characteristics. Here, again a difference was observed between the 2 tumour cell lines, with a significant higher adhesion for Caco2 compared to HT29 to the mesothelium and to the microvascular endothelium.

In this study we found a high adhesion to individual components of the ECM. In order to be able to dissect the individual ECM component, we did not measure the adhesion to a complete ECM consisting of a mixture of the tested components and additional components like hyaluronic acid. Adhesion to a complete ECM may bring about even higher numbers of adherent cells and thereby creates a more pronounced difference in adhesion affinity between the endothelium and ECM and perhaps also between the mesothelium and ECM leading to a stronger invasion gradient.

In our experiments, HT29 shows slightly lower adhesion to both the endothelium and mesothelium and higher adhesion to ECM components compared to Caco2 and therefore the invasion gradient seems more pronounced for HT29, a poorly differentiated colon carcinoma cell line, than for Caco2 which is a more differentiated cell line.

Recently, we investigated the influence of an inflammatory reaction on tumour cell – endothelial cell interactions [15]. In these studies we found that pre-incubation of the endothelium with pro-inflammatory cytokines or reactive oxygen species, that are produced by activated leukocytes, brings about an increase in tumour cell adhesion to the

endothelium. This pre-incubation resulted in a significant increase in apoptosis of endothelial cells. In the early phase of apoptosis the cells are activated with increased expression of adhesion molecules resulting in enhancement of tumour cell adhesion. Eventually, cell death occurs with exposure of the underlying ECM. Therefore, inflammation, but also direct mechanical disruption of the covering monolayer leading to exposure of the ECM may cause enhanced tumour recurrence.

By immunocytochemistry, we showed that α2, α3, α6 and β1 are abundantly expressed on both tumour cells. ICAM, VCAM, α1, α3 and α4 have modest expression on one or both cell lines. These findings are corresponding with the literature [14,16,17]. It is known that the expression of integrins is versatile with pathways of inside-out and outside-in signalling as means of activation [18-21]. Accordingly, the expression of integrins is important, but the functionality is even more important. Consequently, inhibition assays using functional blocking antibodies were performed to get insight in integrins responsible for adhesion to ECM components. These assays clearly display that the binding of HT29 to LN seems almost completely formed by the α6-component, which forms the VLA-6 integrin with β1. The binding to LN exhibit a discrepancy between the two tumour cell lines, since the binding of Caco2 to LN is not significantly inhibitable by the α6-antibody in contrast to the binding of HT29. The α2-component was found to play a major role in the binding of both tumour cell lines to C I and to a lesser extent to C IV. These findings are partly in accordance with the results of Haier *et al* [22]. They used subclones of HT29, namely the poorly liver-metastatic colon carcinoma cell line HT29P and the highly liver-metastatic colon carcinoma cell line HT29LMM. In their study the α2-antibody comparably suppressed the adhesion of both cell lines to C I and IV, but the α6-antibody gave only a minor reduction in the adhesion to LN. Nonetheless, cell lines may not be comparable, since they used HT29 cell lines originating from liver metastases and we from primary tumours and therefore it is possible that these cell lines originate from different subgroups with different characteristics.

The next step in the metastatic cascade after binding to the ECM is invasion through this layer. It is believed that by binding to the ECM matrix metalloproteinases (MMP) are produced or activated which degrade the ECM. As yet, the role of integrins in the degradation of the ECM has to be unravelled.

In summary, we showed that different colon cancer cell lines express a high binding affinity to components of the ECM but that their adhesion pattern varies. At present, the identity of molecules involved in these interactions remains indefinitive, because of the complexity of which the ECM consist and because of the heterogeneity between tumour cells. Knowledge on the selective or more universally expressed integrins involved in tumour cell adhesion to the ECM as well as to the EC and MC may be of help for the development of a cocktail of antibodies aimed at integrins. This cocktail may prove a powerful tool in the prevention of tumour recurrence.

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