Identification of different laminin binding proteins in basolateral cell membranes of human colorectal carcinomas and normal colonic mucosa

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Abstract

The adhesive properties of tumour cells to laminin, the major glycoprotein of basement membranes, play a crucial part in the complex process of tumour invasion and metastasis. We therefore investigated the expression of laminin binding proteins in isolated basolateral cell membranes of human colorectal carcinomas and the adjacent normal colonic mucosa. Cell membrane binding assays and immunoblotting experiments showed appreciable quantitative and qualitative differences in the expression of these proteins in neoplastic and normal tissue. Epithelial basolateral cell membranes of colorectal carcinomas bound five to eight times more radioactive labelled laminin than basolateral cell membranes of the adjacent normal colonic epithelium. The expression of laminin binding proteins with M_r 66 000–69 000 daltons corresponding to the so called 'M_r 67 000 dalton laminin receptor' was three to four times higher in colorectal carcinomas than in normal colonic epithelium. In addition, laminin binding proteins with higher molecular weights, which may be related to the family of integrins, were also increased in colorectal carcinomas. In particular, laminin binding proteins with M_r 180 000 daltons were exclusively expressed on neoplastic epithelial cells of human colorectal carcinomas. Our data suggest that certain classes of laminin binding proteins may be selectively expressed on colonic tumour cells, leading to an increased capacity for migration, invasion, and metastasis.

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Carcinoma of the colon is a leading cause of death from cancer in humans. The incidence rates for colorectal cancer in Western Europe ranges from 25–40 per 100 000 population with 35 per 100 000 in Britain[1]; more than half of these patients are expected to die of this disease.[2] The prognosis for cancer patients is mainly influenced by tumour growth, local infiltration, and metastasis. During the transition from in situ to invasive carcinomas normal or dysplastic epithelial cells resting on a basement membrane are replaced by tumour cells which degrade the subepithelial basement membrane of the crypts and invade the underlying stroma. To enter the circulation, metastasising tumour cells have to penetrate lymphatic walls and vascular basement membranes. Circulating tumour cells are partly arrested in the lumina of capillaries in the target organ. Finally, they penetrate the vascular basement membrane to initiate a metastatic colony. The adhesive properties of tumour cells to

basement membranes are known to play a crucial part in the complex process of metastasis formation,[3] and tumour cell adhesion to basement membranes has been shown to correlate with their ability to metastasise.[4] Basement membranes are electron-dense layers 20–100 nm thick, whose major known components are collagen type IV, heparan sulphate proteoglycan, nidogen, and laminin. Laminin, the major glycoprotein of basement membranes, in its intact form is composed of three chains, including the A chain (440 000 daltons), B1 chain (230 000 daltons), and B2 chain (210 000 daltons).[5 6] In the cross-shaped structure of laminin each of the A and B chains forms one short arm, and the remaining three chains together project down the long arm.[7 8] Laminin has been implicated in many biological processes. With respect to carcinogenesis, including colonic cancer, laminin plays an important part in cell adhesion, mitogenesis, differentiation, and metastasis. Many of the cellular effects of laminin are believed to be mediated through integral laminin binding cell membrane proteins, and two families of laminin binding proteins have so far been identified. One of these is a group of cell membrane proteins that has a molecular mass of 65 000–72 000 daltons and can be found in a variety of tissues and cell lines,[9–11] including a human colonic adenocarcinoma derived cell line.[12] This group of laminin binding proteins, designated as the so called 'laminin receptors,' bind specifically to the pentapeptide Tyr-Ile-Gly-Ser-Arg (YIGSR), a sequence domain of the B1 chain of the laminin molecule. It has recently been shown that these proteins act as a link for the extracellular matrix induced differentiation of endothelial cells.[13]

The second group of laminin binding proteins belongs to the family of integrins. The integrins are composed of a common β chain and one of several α chains. They serve as signal transducers between the extracellular matrix and components of the cytoskeleton and bind specifically to the sequence Arg-Gly-Asp (RGD).[14] This tripeptide has been found in many extracellular and plasma proteins such as fibronectin, vitronectin, collagens, and the von Willebrand factor,[15] and also in the A chain of the laminin molecule.[16] The inhibition of the attachment of cells to laminin by a monoclonal antibody against the integrin $\alpha_3\beta_1$, the so called 'CSAT protein' or very late antigen (human VLA_3), indicated that this integrin acts as a laminin binding protein. Its specific interaction with laminin cannot, however, be readily shown by affinity chromatography or immunoblotting due to their low affinity for laminin.[17 18] Nevertheless, another member of the integrin family, the $\alpha_6\beta$ (VLA_6)

protein, was recently found to show specific high affinity binding to laminin.[19] In addition to these two groups of laminin binding proteins other cell membrane proteins with the capacity to interact with laminin were identified. For example, cell surface proteins with M_r 110 000 and 180 000 daltons were identified in neuroblastoma glioma cells and reported to play an active part in laminin induced neurite outgrowth.[20]

Since little is known about the molecular interaction of epithelial colonic cells and the basement membrane via cell membrane-binding proteins during malignancy and metastasis, we have isolated these proteins from human colorectal carcinomas in an attempt to characterise them and compare their size and quantities with those of the normal colonic mucosa.

Methods

PATIENTS

Surgical specimens of primary human colorectal carcinomas and adjacent normal colonic tissues were obtained from the department of surgery at this medical centre, immediately after their removal. Samples were obtained from patients with colorectal carcinomas that showed differentiation to a high degree in five, medium degree in five, and low degree in seven cases according to the classification of Morson and Dawson.[21] In undifferentiated colorectal carcinomas the epithelial cells were completely unpolarised. However, the ouabain sensitive Na/K-ATPase containing cell membrane fractions (see below) represents that part of the cell membrane which interacts with basement membranes during invasion and metastases. After extensive washing in phosphate buffered saline (pH 7·2), intestinal tissues were placed in liquid nitrogen.

ISOLATION OF BASOLATERAL CELL MEMBRANES

Basolateral cell membranes of human colorectal carcinomas and normal mucosa were prepared as described by Orsenigo et al.[22] Briefly, mucosal scrapings were homogenised in 250 mM sucrose, 0·1 mM phenylmethanesulphonyl fluoride, 10 mM triethanolamine hydrochloride (pH 7·6) (buffer 1) in a Potter homogeniser on ice and centrifuged at 1000 g for 10 minutes. The supernatant was centrifuged for 20 minutes at 19 500 g. The fluffy white upper layer of the double pellet was resuspended in buffer 1, to which 10% Percoll had been added. After centrifugation fo 60 minutes at 48 000 g in a Kontron TST 25·38 fixed angle rotor, basolateral cell membranes were collected as white interfacial bands, diluted by adding a three to fourfold excess of buffer 1, and pelleted by centrifugation at 48 000 g for 30 minutes. The pellet was analysed for ouabain sensitive Na/K-ATPase as a marker for basolateral cell membranes according to Evans.[23] Briefly, activity was determined in 770 µl assay buffer containing 33 mM Tris-HCl, pH 7·4, 33 mM KCl, 3·3 mM $MgCl_2$, and 110 mM NaCl to which 100 µg protein of basolateral cell membranes in 200 µl buffer 1 (see above) was added. The reaction was initiated by the addition of 30 µl 100 mM ATP-Tris (freshly

prepared) and stopped by the addition of 1 ml 25% trichloracetic acid after 30 minutes at 37°C. Liberated orthophosphate was determined in the supernatants after centrifugation. To obtain oubain sensitive Na/K-ATPase activity, the reaction was measured after the addition of 1·1 mM oubain (final concentration) to the assay buffer. The final ratio between the specific ouabain sensitive Na/K-ATPase activities of the purified basolateral cell membranes and the initial homogenates of colorectal carcinomas or normal colonic mucosa (enrichment factor) was (mean (SD)) 7·3 (2·5) and 8·4 (2·9) respectively.

BINDING ASSAYS

For binding assays intact laminin was labelled with [125]I (1 mCi/10 µg antigen) using the chloramine-T method. The specific activity of labelled laminin was approximately $1-2×10^5$ cpm/ng protein. Binding of ^{125}I-labelled laminin to cell membranes immobilised on nitrocellulose filters was performed as described recently.[12] Briefly, purified cell membranes (10–20 µg protein) of colorectal carcinomas and the adjacent normal colonic mucosa were blotted onto nitrocellulose, and incubated in 3% defatted cows' milk in Tris-buffer. Then the strips were incubated with laminin $(2-5×10^5$ cpm) in phosphate buffered saline containing 0·04% Tween 20 (PBS/Tween) for one hour at room temperature followed by washing three times in PBS/Tween and counting in a gammacounter.

AFFINITY CHROMATOGRAPHY

Affinity chromatography was performed basically as described elsewhere.[24] Ten mg of laminin isolated from the EHS-sarcoma[25] was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). During each run, samples of basolateral cell membranes (10 mg protein content) were applied to the laminin-Sepharose column at a concentration of 0·2–0·5 mg/ml (1×6 cm). Samples were recirculated overnight at room temperature, and elution was performed with a linear salt gradient between 0 and 3 M NaCl followed by 0·2 M glycine HCl, pH 2·5. Eluted fractions were lyophilised after extensive dialysis against phosphate buffered saline (pH 7·2) and then against water.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Fractions obtained by affinity chromatography were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis in 10% gels.[26] To identify laminin binding proteins, gel electrophoresed fractions were transferred to nitrocellulose according to Towbin et al[27] and analysed as described recently.[12] Briefly, after saturation of unspecific binding sites with 3% defatted cows' milk in 20 mM Tris-HCl buffer, containing 0·9% NaCl, 20 mM NaN_3 (pH 8·2) (Tris-buffer), nitrocellulose strips with blotted cell membranes were incubated with intact mouse tumour laminin (1 µg/ml) in Tris-buffer containing 0·1% desoxycholate (pH 7·2) (Tris-DOC-buffer) overnight at room temperature. The strips were then washed with Tris-DOC-

Binding of radioactive labelled laminin to isolated basolateral cell membranes from colorectal carcinomas and the adjacent normal colonic epithelium immobilised on nitrocellulose

Case no	Degree of tumour differentiation	Basolateral cell membranes (cpm/10 ng protein)	
		Of carcinomas	Of the adjacent normal colonic mucosa
1	High	5950	950
2	High	6310	1250
3	Medium	8020	1610
4	Low	9220	1590
5	Low	7950	1600
6	Low	7750	1070

Degree of tumour differentiation is explained in the Methods. Values are the mean of triplicate experiments. Standard deviation was less than 10% in all experiments.

buffer and incubated overnight with antilaminin antibodies (1 µg/ml) at 4°C. Bound antibodies were detected with antirabbit IgG antibodies (Janssen, Belgium) coupled to colloidal gold. The quantity and molecular weight distribution of laminin binding proteins was estimated by laser scanning densitometry.

STATISTICAL ANALYSIS
Statistical significance was calculated using the Wilcoxon rank test.

Results

BINDING OF LAMININ TO ISOLATED CELL MEMBRANES
The first evidence of the existence of specific

laminin binding components on the epithelial cell membrane of colorectal carcinomas and the adjacent normal mucosa was obtained from binding assays using radiolabelled laminin. Basolateral cell membranes of colorectal carcinomas bound five to eight times more laminin than basolateral cell membranes of the normal colonic mucosa ($p<0.01$) (see Table). Binding of labelled laminin to isolated basolateral cell membranes from undifferentiated colorectal carcinomas was higher than binding of laminin to cell membranes of highly differentiated colorectal carcinomas. The differences were not significant. Binding of labelled laminin to isolated cell membranes was specific, since the addition of unlabelled laminin successfully inhibited the binding of the labelled molecule (data not shown).

IDENTIFICATION OF LAMININ BINDING PROTEINS IN BASOLATERAL CELL MEMBRANES OF COLORECETAL CARCINOMAS AND NORMAL COLONIC MUCOSA
Laminin binding components in epithelial basolateral cell membranes of colorectal carcinomas were fractionated on laminin-sepharose affinity matrix, separated on sodium dodecyl sulphate-polyacrylamide gel, transferred to nitrocellulose, and identified by immunoblotting. Elution at 0.2–0.3 M NaCl yielded different laminin binding components with M_r of 75 000 to 85 000, 95 000, 100 000, 120 000, 140 000, and 180 000 daltons without reduction of disulphides. At 1 M NaCl a group of laminin binding proteins with molecular weights between 66 000 and 69 000 daltons was eluted, and only small amounts of proteins with M_r 67 000 and 180 000 daltons were obtained by a final elution with 0.2 M glycine HCl, pH 2·5 (Fig 1).

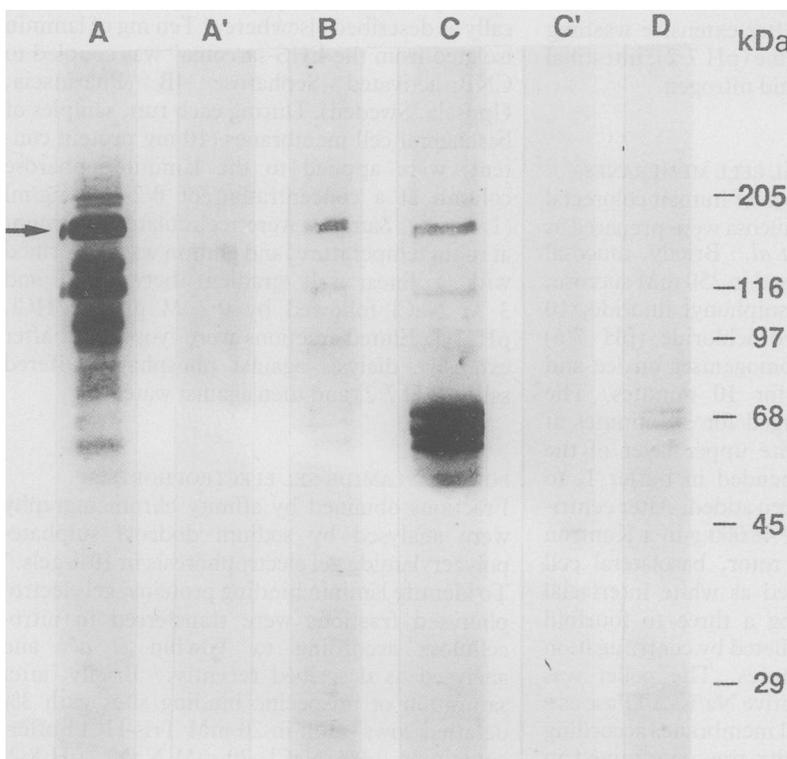After fractionation of basolateral cell membranes prepared from normal colonic mucosa on

Figure 1: Identification of specific laminin binding components of basolateral cell membranes of colorectal carcinomas after fractionation on laminin sepharose and transfer of electrophoresed proteins to nitrocellulose by immunoblotting. Fractionated basolateral cell membranes: lane A, after elution with 0·2–0·3 M NaCl; B, after elution with 0·5 M NaCl; C, after elution with 1 M NaCl; D, after elution with 1·2–3 M NaCl. Lanes A', C': negative control of transferred cell membranes to lanes A, C. Standards are myosin (M_r 205 kDa), β galactosidase (M_r 116 kDa), phosphorylase b (M_r 97 kDa), bovine serum albumin (M_r 68 kDa), ovalbumin (M_r 44 kDa), and carbonic anhydrase inhibitor (M_r 29 kDa).
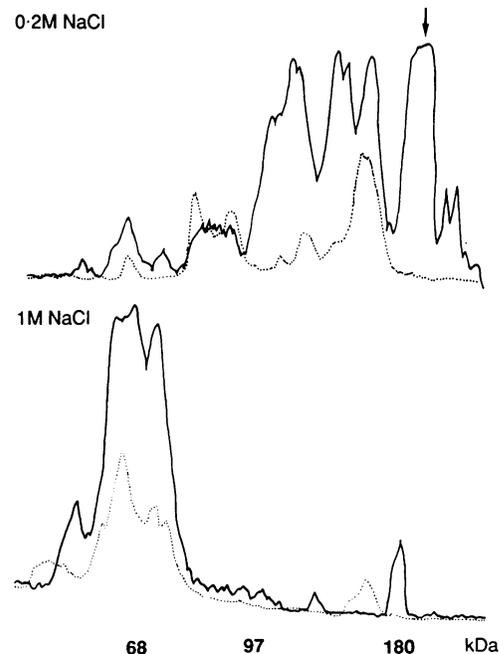

Figure 2: Densitometric analysis of specific laminin binding components in basolateral cell membranes of colorectal carcinomas (—) and normal colonic mucosa (....) after fractionation on laminin-sepharose.

the laminin-Sepharose affinity matrix it was possible to obtain, after elution at 0·2–0·3 M NaCl, an almost identical set of laminin binding proteins with molecular weights of 75 000 to 85 000, 95 000, 100 000, 120 000, and 140 000 daltons, which lacked, however, the 180 000 dalton components. Similarly, elution at 1 M NaCl yielded bands with M_r 66 000 to 69 000 daltons under non-reducing conditions.

Densitometric analysis of the bands detected by immunoblotting showed striking quantitative and qualitative differences between laminin binding proteins from normal colonic mucosa and those from colorectal carcinomas. Calculations of the relative amount of various laminin binding proteins based on an identical enrichment factor for basolateral cell membrane purifications, an equal amount of protein applied to the gels, and identical immunoblotting conditions showed that, in the tumour compared with normal colonic epithelium, expression of laminin binding proteins with M_r 66 000 to 69 000 daltons was three to four times higher and that of laminin binding proteins with M_r 95 000 and 120 000 five to eight times higher (Fig 2). Laminin binding proteins with M_r 75 000 to 85 000 daltons were found in similar amounts in epithelial cells of both colorectal carcinomas and normal colonic mucosa.

Interestingly, very high amounts of laminin binding protein(s) with M_r 180 000 daltons were detected only in basolateral cell membranes of colorectal carcinomas (Fig 2).

Discussion

Analysis of the interaction of colonic tumour cells with the basement membrane is of major importance for understanding tumour cell invasion and metastasis formation. When cells migrate from one tissue to another they must cross the basement membranes.[3] In this study we analysed the expression of laminin binding proteins in isolated basolateral cell membranes of human colorectal carcinomas and normal adjacent colonic mucosa. Our data show appreciable quantitative and qualitative differences between the expression of various laminin binding proteins by human colorectal carcinomas and normal colonic mucosa. On the basis of binding assays we have shown that the binding of epithelial cell membranes of colorectal carcinomas to radioactive labelled intact laminin were five to eight times greater than to epithelial cell membranes of the adjacent normal colonic mucosa.

On closer inspection, using laser densitometric analysis of immunoblots, we could show that the expression of laminin binding proteins with M_r of 66 000–69 000 daltons, most probably corresponding to the so-called '67 000 dalton laminin-receptor,' is three to four times higher in colorectal carcinomas than in normal colonic epithelium. In agreement with our results, Hand et al,[28] using a monoclonal antibody, found higher levels of laminin receptors on the cell surface of colonic carcinomas than on that of benign lesions. In addition, Yow et al have recently shown that the expression of mRNA for the 67 000 dalton laminin receptor is nine times

greater in colon carcinomas than in adjacent normal colonic epithelium.[29] Immunohistological studies on the expression of these laminin binding protein(s) along the crypt-villus axis in the murine small intestine showed that they are increased in basolateral cell membranes of enterocytes in the base of the crypt but not detectable on cells lining the mouth of the crypts and the villi.[30] It is interesting that, in cryostat sections from duodenal biopsy specimens of patients with gluten sensitive sprue, an increased expression of 67 000 dalton laminin receptors was observed in basolateral cell membranes of undifferentiated enterocytes in all hyperplastic crypts extending to the surface epithelium of the flattened mucosa.[31] Recently, Grant et al have shown that the interaction of endothelial cells with laminin through the M_r 67 000 dalton receptor induces differentiation, while laminin binding proteins of high molecuar weights which may be related to the family of integrin receptors (see below) primarily promote cell adhesion.[13] If the M_r 67 000 dalton laminin receptor is an integral transmembrane protein that induces differentiation, it is not clear why epithelial cells with a high density of these proteins are undifferentiated.

In addition to the M_r 66 000–69 000 dalton we found laminin binding proteins with higher molecular weights in basolateral cell membranes of colorectal carcinomas and their normal counterpart. The normal colonic epithelium expresses laminin binding proteins with M_r 95 000, 100 000, 120 000, 140 000 daltons. These proteins were also found in appreciably increased amounts on epithelial cells of colorectal carcinomas (see Fig 2). In view of their molecular weight most of these proteins may be related to the family of integrin receptors.[14] A cell membrane laminin binding protein in a human glioblastoma cell line with M_r 140 000 daltons after reduction was recently identified as a member of the integrin family.[32] In addition, monoclonal antibodies against the VLA_3 protein, a cell membrane dimer with M_r 135 000/130 000 daltons,[15] and the VLA_6 protein, a dimer with M_r 150 000/130 000 daltons,[19] inhibited attachment of cells to laminin, indicating a role of the integrins in cell-matrix interaction. Interestingly, smaller laminin binding proteins with M_r 32 000 daltons, as reported by Yow et al,[29] could not be identified using our techniques.

Laminin, a large multifunctional molecule, influences the behaviour of many cells, including the adhesion of cells to the basement membranes and the induction of morphological changes leading to cellular differentiation.[25] Separate domains of laminin with distinct biological effects have been identified. Therefore it is not surprising that several cell membrane laminin binding proteins exist. It seems that integrins, interacting with the Arg-Gly-Asp sequence in the short arm of the A chain, mediate cell adhesion to laminin,[17] whereas other laminin binding proteins, like cell surface galactosyltransferase,[33] interact with the E8 fragment, a major part of the long arm of the laminin molecule, and may be responsible for cell spreading.[34 35]

The most important finding in this study is probably the identification of laminin binding

protein(s) with M_r 180 000 daltons. This seems to be expressed exclusively on epithelial cells of human colorectal carcinomas. Since metastasising tumour cells have to attach to the extracellular matrix, preferentially to the vascular basement membrane, to induce a (clonal) proliferation in this secondary site, it has been suggested[3] that increased amounts of free laminin binding proteins on neoplastic cells facilitate this type of adhesion. It is not clear, however, why neoplastic cells with a high density of laminin binding proteins detach from the basement membrane and migrate through it.

Our data suggest that certain classes of laminin binding proteins may be selectively expressed on colonic tumour cells, leading to an increased capacity for migration, invasion, and metastasis. Little is known, however, about the functional characteristics of the different laminin binding proteins in relation to epithelial cell differentiation or dedifferentiation. Further investigations on the expression and function of these proteins during invasion and metastasis of transformed cells may give us new tools for studying or even modifying cell-matrix interactions in intestinal neoplasia.

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