Human colonic cancer cells synthesize and adhere to laminin-5. Their adhesion to laminin-5 involves multiple receptors among which is integrin α2β1

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SUMMARY

In the mature gut, laminin-5 is expressed at the basal aspect of the differentiating epithelial cells. In vitro, we show that three more or less differentiated human colonic cancer HT29 cell lines produce and deposit laminin-5; they predominantly synthesize and secrete the 440 kDa form of laminin-5 that comprises the unprocessed 155 kDa γ2 chain, as determined by immunoprecipitation analysis. In contrast, the highly differentiated colon carcinoma Caco-2 cells produce almost no laminin-5.

Using anti-integrin antibodies, we show that adhesion of the two colonic cancer cell lines to laminin-5 is mediated by multiple integrin receptors including those for α3β1, α6β1 and α6β4 integrins like in other cell types. In addition, the implication of integrin α2β1 in this adhesion process is demonstrated for the first time. This has been shown by cell adhesion inhibition experiments, solid phase assays and confocal analysis. Together with previous in situ observations, these data provide a baseline knowledge for the understanding of the regulation of laminin-5 in normal and pathological intestine.

Key words: Intestine, Epithelial cell, Basement membrane, Differentiation, Integrin

INTRODUCTION

Continual renewal of intestinal epithelium is ensured by the proliferation of stem cell populations confined to the crypt region. As cell migration proceeds from crypt base to villus tip, the undifferentiated cells acquire morphological and biochemical features of polarized cells. Among these cells, the absorptive enterocytes carry the digestive enzymes located at the apical brush border membrane. Cell adhesion to the underlying basement membrane is thought to be critical in the regulation of these migration/differentiation events. This hypothesis is strengthened by the observation that changes in the spatial/developmental distribution of basement membrane molecules are concomitant to the morphogenetic processes and compartmentalization in the adult organ (for reviews see Simon-Assmann et al., 1994a; Orian-Rousseau et al., 1996). Among the basement membrane components, laminins display a wide array of biological activities such as cell adhesion, migration and differentiation. It has been shown that a subset of epithelial basement membranes contains the laminin-5 variant (Verrando et al., 1987; Rousselle et al., 1991). This adhesion ligand has been isolated from keratinocytes and squamous carcinoma cells. In these cells, laminin-5 (α3β3γ2) is initially synthesized as a cellular heterotrimeric precursor comprising the 200 kDa (α3 precursor), 155 kDa (γ2 precursor) and 140 kDa (β3) polypeptides (Marinkovich et al., 1992a; Rousselle and Aumailley, 1994). The laminin α3 chain is immunologically related to a distinct 190 kDa laminin α chain interacting with the β1 and γ1 chains to form the laminin-6 variant (Marinkovich et al., 1992b) and with β2 and γ1 chains to form the laminin-7 variant (Champliaud et al., 1996). Laminin-5 and laminin-1 (α1β1γ1) are expressed in the intestine and display a differential localization according to the crypt-to-villus positioning (for review see Simon-Assmann et al., 1998). In particular, laminin-5 presents a gradient of intensity increasing from the base to the tip of the villus (Leivo et al., 1996; Orian-Rousseau et al., 1996). This expression pattern is consistent with that of HD1, a protein of the hemidesmosmal plaque, and integrin α6β4 (Simon-Assmann et al., 1994a; Orian-Rousseau et al., 1996) and suggests a possible role of laminin-5 in differentiation and/or migration of intestinal cells.

To date, models of normal intestinal cell culture displaying the same properties as the enterocytes of the crypt-villus axis, are not available for further in vitro studies. This is the reason...
why we used the Caco2 and HT29 cell lines that derive from human colonic tumors and represent interesting model systems to study various aspects of intestinal cell biology (Chantret et al., 1988; and for reviews see Kedinger et al., 1987; Neutra and Louvard, 1989; Zweibaum et al., 1991). Caco2 cells form apical brush borders and express digestive hydrolases as soon as they become confluent. Their differentiation state can be modulated by exogenous laminin substrates (Vachon and Beaulieu, 1995; Basson et al., 1996). Conversely, confluent cultures of HT29 cells remain mainly undifferentiated (Pinto et al., 1983; Zweibaum et al., 1985). Treatment of the HT29 cells with anti-cancer drugs, 5-fluorouracil or methotrexate, generated HT29-Fu and HT29-MTX clones which are committed to differentiate into various phenotypes (Lesuffleur et al., 1990, 1991). Interestingly Caco2 and HT29 cell lines display a distinct repertoire of integrins, the cell surface receptors that mediate interactions between cells and extracellular matrix (Basson et al., 1992; Simon-Assmann et al., 1994b; Ebert, 1996).

The importance of laminin-5 in the skin has been emphasized by the disastrous consequences of mutations in its constituent chains that give rise to the blistering skin disease Herlitz junctional epidermolysis bullosa (JEB; Aberdam et al., 1994b; Fine, 1994; Uitto et al., 1995). Although pteryloric atresia and various gut alterations are associated with subtypes of JEB (Vidal et al., 1995; Brown et al., 1996; Niessen et al., 1996; Pulkkinen et al., 1997), modifications of laminin-5 have never been analyzed in the gastrointestinal tract of these patients. The analysis of the subepithelial basement membrane in intestinal biopsies taken from children displaying a subtype of intractable diarrhea with villous atrophy, revealed an abnormal laminin deposition (Goulet et al., 1995). These observations point to the need to analyze the characteristics of laminin isoforms in intestinal cells as a baseline to understand their function.

The aim of the present work was to take advantage of the distinct intrinsic properties of Caco2, HT29 and clonal HT29 subpopulations to: (i) define the molecular characteristics and the expression pattern of laminin-5 in intestinal epithelial cells, (ii) determine the adhesion properties of intestinal epithelial cells to exogeneous laminin-5, (iii) define the integrin receptors involved in this process. In this study, we show that the highly differentiated Caco2 cells produce almost no laminin-5 in contrast to the HT29 cells whatever their state or type of differentiation. The molecular form of the intestinal laminin-5 differs from that of keratinocytes or squamous epithelial cells. Furthermore, we demonstrate that colonic cancer cells use specifically the integrin α2β1 to bind to laminin-5 in addition to the receptors described in other cell types.

**MATERIALS AND METHODS**

**Antibodies**

Mouse monoclonal (mAb) and rabbit polyclonal (pAb) antibodies raised against human laminin-5 were: mAb GB3 that recognizes the three constituent chains, α1, β1 and γ1 (Simò et al., 1991) was used. In the cell adhesion assay, mAb 3E1 recognizing the extracellular portion of human integrin β4, mAb P4C10 to the β1 subunit and mAb P1B5 raised against the α3 subunit were purchased from Gibco (France). mAb anti-human integrin α2, P1E6 was from Telios (Pharmaceuticals, Inc.) and 6F1 was a kind gift from Dr R. Bankerts (Roswell Park Cancer Institute, Buffalo, NY, USA). mAb GoH3 specific to the integrin α6β1 was from Immunotech (Marseille, France).

**Colon carcinoma cell lines**

The human colon adenocarcinoma cell line HT29 (HT29p, parental cell line) was established by Fogh et al. (1977). HT29 cells adapted to 10⁻³ M 5-fluorouracil (HT29-Fu) and to 10⁻⁵ M methotrexate (HT29-MTX) were obtained from Dr A. Zweibaum (INSERM Unit 178, Paris, France). The characteristics of the cells have been defined in detail previously (Lesuffleur et al., 1990, 1991). Cells were routinely grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, France) supplemented with 10% heat-inactivated fetal calf serum (Gibco) and 200 µg/ml gentamycin. The human colon adenocarcinoma Caco2 cell line used in this study has also been established by Fogh et al. (1977). Its differentiation properties have been defined previously (Pinto et al., 1983). The cells were maintained in DMEM supplemented with 20% heat-inactivated fetal calf serum, 1% nonessential amino acids (Gibco) and 200 µg/ml gentamycin.

**Immunocytochemical detection of laminin-5**

Detection of intracellular laminin-5 was performed on cells grown on glass coverslips, prefixed 10 minutes with 1% paraformaldehyde and permeabilized 10 minutes with 0.5% Triton X-100 before incubation with mAb GB3 diluted (1/50) in phosphate buffered saline (PBS) for 1 hour at room temperature. After washings in PBS, cells were stained with fluorescein-isothiocyanate-labelled sheep anti-mouse IgG diluted in PBS. Immunodetection of laminin-5 deposited on the glass coverslip was performed following removal of the cells after a one hour treatment with a solution containing 1% Triton X-100, 10 mM EDTA and 25 mM Tris-HCl, pH 7.5. The preparations were mounted under coverslips using the Vectashield mounting medium (Biosys SA, Compiegne, France), observed using an Axiopt microscope (Zeiss) and photographed using HP5 film (Asa 400, Ilford Ltd). On control sections, the primary antibody was omitted.

**Biosynthetic labelling studies and immunoprecipitation experiments**

25×10⁶ cells were seeded on a 100 mm Petri culture dish to obtain confluent monolayers after 6 hours. Cells were then washed and radiolabelled with 100 µCi/ml trans-[^35]S-label™-metabolic labelling reagent containing [^35]S]-methionine and [^35]S]-cysteine (ICN Biomedicals, Orsay, France) in methionine-cysteine-deficient DMEM medium containing 2% fetal calf serum. 24 hours later, the culture medium was collected and 2M NEM, 1 mM PMSF and were added. After 10 minutes centrifugation at 330 g, the medium was either stored at –80°C or used immediately.

For immunoprecipitation, samples were first preincubated with Protein-G-Sepharose or Protein-A-Sepharose CL-4B beads (Sigma) for 1 hour at 4°C. In parallel, the monoclonal and the polyclonal antibodies were incubated with Protein-G-Sepharose and Protein-A-Sepharose beads for 1 hour at 37°C, respectively. The pellets containing the antibodies bound to the beads were washed twice with RIPA buffer (radioimmunoprecipitation assay buffer: 10 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 2 mM cysteine, 2 mM methionine, 250 µM PMSF, 1 mM NEM, 0.5% NP-40, 0.1% SDS, 0.05% Triton X-100, 0.3% sodium deoxycholate, 0.1% BSA, 150 mM NaCl) and incubated with the precleared samples 18 hours at 4°C. Immune
complexes were washed 5 times with RIPA buffer and resuspended in Laemmli buffer containing β-mercaptoethanol. Pellets were boiled and subjected to 6% SDS-PAGE gels. The gels were then dried and exposed to Fuji Medical X-ray film (Fuji) for at least 24 hours at -80°C. Gels were calibrated with 14C-labelled protein standards (Amersham) and apparent molecular masses were determined by linear scanning with a densitometer (Shimadzu, Roucaire). For semi-quantitative analysis, radioactivity was measured in aliquots of each sample using Ultima gold liquid scintillation cocktail (Packard Instrument Company, INC., USA) and a Tri-CARB 1 600 TR liquid scintillation analyser (Packard, USA). When required, the radiolabelled material was pre-incubated 4 hours twice with anti-laminin-1 antibodies before treatment with anti-laminin-5 antibodies. For pulse-chase experiments, attached cells were incubated 1 hour in 100 µCi/ml of [35S]methionine and [35S]cysteine in deficient DMEM medium and then 0, 3, 6, 24, 48 hours in complete DMEM medium for chase. Cells were scraped into a small volume of RIPA buffer, ground and centrifuged at 330 g for 10 minutes. Cell extracts and conditioned medium were then processed for immunoprecipitation.

Immunoblot analysis

Western analysis of cell extracts and conditioned medium was performed at 7 days on confluent cell cultures. Cell extraction was achieved by incubating the cells 1 hour at 37°C with 0.1 M phosphate buffer, pH 7.2, containing 2 N NaBr, 2 mM PMSF, and 10 µg/ml leupeptin, grinding them in 500 µl of 50 mM Tris buffer, pH 7.2, containing 1% SDS, 5% β-mercaptoethanol, 10 mM EDTA, 2 mM PMSF and 10 µg/ml leupeptin. The samples were centrifuged 30 minutes at 100,000 g and the pellet discarded. 10 µg of cleared protein extract and conditioned medium were incubated 5 minutes in Laemmli buffer containing 5% β-mercaptoethanol and 2% (w/v) SDS at 100°C. Proteins were separated on 6% SDS-PAGE gels and subsequently electrotransferred overnight onto nitrocellulose in 25 mM Tris-HCl, 0.192 M glycine, pH 8.2, 20% (v/v) methanol. After transfer, the nitrocellulose was saturated 1 hour at 37°C with 3% BSA in buffer (10 mM Tris, pH 7.4, 0.15 M NaCl containing 1 mM EDTA, 0.1% (v/v) Tween-20 and 0.5% gelatin (v/v), and then incubated 1 hour at room temperature with specific antibodies. After reaction with the secondary antibody, nitrocellulose sheets were treated with streptavidin-peroxidase (Amersham) added at 1:1,500 in TBS, 1 mM MgCl₂ and the pellet discarded. 10 µg of cleared protein extract and conditioned medium were then processed for immunoprecipitation. Western analysis of cell extracts and conditioned medium was performed in duplicate. The number of independent assays performed for each antibody ranged from 2 to 4. Results were expressed as the mean plus or minus one standard error. The paired Student’s t-test was used to analyse the data for statistical significance of differences between means. Differences with a P value of less than 0.05 were considered significant.

For cell adhesion inhibition experiments using anti-substrata antibodies, wells coated with laminin-1/nidogen or laminin-5 were incubated with serial dilutions in PBS of mAb BM 165 against the trimeric laminin-5 molecule for one hour at 37°C as indicated in the corresponding figures. In inhibition assays with anti-integrin antibodies, cell suspensions were mixed with dilutions of antibodies before plating onto the coated wells. Each assay point was derived from triplicate wells.

In vitro binding assay on immobilized laminin-5

α2β1 integrin was purified from detergent extracts of outdated human platelets by affinity purification on collagen-Sepharose as previously described (Kern et al., 1993; Messent et al., 1998). α2β1 integrin binding assays on immobilized laminins were performed as previously described (Tuckwell et al., 1996; Messent et al., 1998). In brief, 96-well plates (Maxisorp, Nunc) were coated with laminin-5 or laminin-1 at indicated concentrations in Tris-buffered-saline (TBS) for 1 hour at room temperature. Unbound substrate was removed and wells washed 3 times with TBS and then blocked with 50 mg/ml bovine serum albumin (BSA) in TBS for 1 hour at room temperature. BSA was removed and biotinylated α2β1 integrin added at 2 µg/ml in TBS containing 2 mM MgCl₂ and 1 mM MnCl₂ for 2 hours at room temperature. Wells were washed 3 times with TBS, 1 mM MgCl₂ and streptavidin-peroxidase (Amersham) added at 1:1,500 in TBS, 1 mM MgCl₂, 1 mg/ml BSA for 15 minutes at room temperature. Wells were washed 3 times with TBS, 1 mM MgCl₂, and bound integrin visualised with 3,3,5,5-tetramethylbenzidine-peroxidase substrate mixed 1:1 with H₂O₂. The reaction was stopped with 2.5 M H₂SO₄ and the OD was read at 450 nm.

Confocal microscopy analysis of α2 integrin subunit

Cells seeded to near-confluency and grown for 2 hours on glass coverslips, were washed once in PBS. The cells were then incubated 1 hour at room temperature with the anti-α2 integrin antibody (mAb P1E6) and then with the anti-mouse secondary antibody. The coverslips were mounted in 0.1% paraphenylendiamine in PBS-glycerol and sealed with varnish. Confocal laser scanning images corresponding to X-Y plane serial sections were taken with a Leica confocal microscope using a TCS4M scanner (Leica Instruments, GmbH, Nussloch, Germany) equipped with a crypton laser. The wells were washed once with Ham F10 and saturated 1 hour at room temperature with 1% bovine serum albumin (BSA, Vth fraction, Sigma Chimie, Saint-Quentin-Fallavier, France). 1-2x10⁵ cells suspended in 0.1 ml medium were then incubated 2 hours and after removal of the supernatants containing the non-adherent cells, washed twice in PBS and fixed at 4°C in 70% ethanol for 15 minutes. The plates were air dried for at least 30 minutes at 37°C and cells were stained 25 minutes at room temperature with 0.1% Crystal Violet (100 µl/well) in distilled water. The wells were rinsed briefly with distilled water and air dried. The stain was solubilized using 10% acetic acid in distilled water (100 µl/well) at room temperature. Colour yields were then measured in an Elisa reader (Dynatech) at 550 nm. A blank value corresponding to BSA-coated wells (25% of maximal cell adhesion) was automatically subtracted. Adherent cells were photographed using a Leica photomicroscope in phase contrast mode. Each assay point was performed in duplicate. The number of independent assays performed for each antibody ranged from 2 to 4. Results were expressed as the mean plus or minus one standard error. The paired Student’s t-test was used to analyse the data for statistical significance of differences between means. Differences with a P value of less than 0.05 were considered significant.

RESULTS

Expression of laminin-5 in colon carcinoma cell lines

Based on the observation that laminin-5 is found in situ in the basement membrane lining the differentiating/differentiated villus enterocytes but not underlying the proliferative crypt cells (Leivo et al., 1996; Orian-Rousseau et al., 1996), we first compared laminin-5 synthesis in undifferentiated HT29p and differentiated Caco2 cell lines. Immunoprecipitation analysis of conditioned medium from both cell lines using anti-laminin-5 mAb GB3 revealed important differences. Three major bands of 165, 155, 140 kDa and faint bands with an estimated mass of 100 kDa were detected in the HT29p culture medium (Fig. 1, lane 1). In Caco2 conditioned medium, none of the major
bands was observed (Fig. 1, lane 4), yet a prolonged exposure of the gel revealed that all subunits were present, the 165 kDa band being fainter than the other chains (not illustrated). The immunocytochemical detection of laminin-5 confirmed the differential expression between HT29 and Caco2 cells (Fig. 2). HT29 cells displayed a regular labelling of laminin-5 in basal patches; some of them were organised in several definite parallel lines (Fig. 2A) particularly obvious when cells were detached from the substratum by EDTA treatment (Fig. 2C). Only tiny spots dispersed within the cytoplasm were present in the differentiated Caco2 cell line (Fig. 2B).

To determine whether this differential expression of laminin-5 is related to the different polarisation/differentiation potential of these cell lines, we analysed the two differentiated HT29-derived cell lines, HT29-FU and HT29-MTX. At confluence, the MTX-adapted cells are almost exclusively of the mucus-secreting type with apical brush borders that strongly express differentiation markers such as dipeptidylpeptidase IV and villin; the HT29-Fu cells form a mixed population of polarised fluid-transporting cells and of goblet cells (Lesuffleur et al., 1990, 1991). Immunoprecipitation with mAb GB3 of medium conditioned by the two differentiated HT29-derived cell lines identified the same bands as those seen in the parental cell line (Fig. 1, lanes 2 and 3, versus lane 1). The intensity of the bands was significantly stronger in the case of HT29-MTX cells and an additional doublet of approximately 200 kDa was also observed (Fig. 1, lane 3).

Immunoprecipitation experiments of conditioned medium from HT29-MTX cells using polyclonal antibodies SE85, SE144 and mAb K140 directed, respectively, to the α3, γ2 and β3 chains (Fig. 3, lanes 1-3) revealed patterns identical to that found with mAb GB3. Interestingly, immunoprecipitation of conditioned medium from tongue squamous carcinoma SCC25 cells with the SE144 antibody identified a 105 kDa product (Fig. 3, lane 4) which was not detected in intestinal epithelial cells (Fig. 3, lane 2). Pulse-chase experiments (Fig. 4) showed that in addition to the major form secreted by the intestinal cells (165, 155 and 140 kDa bands) (Fig. 4 lanes 6-9), the 105 kDa polypeptide was found in very small amounts in the culture medium only after 48 hours of chase (Fig. 4, lane 10). Furthermore, the comparative intensity of the bands showed that the intestinal cells produce a significantly smaller amount of laminin-5 than the SCC25 cells (Fig. 3, lane 4 versus lane 2).

The identity of the polypeptides immunoprecipitated by the various anti-laminin-5 antibodies was confirmed by western blot (Fig. 5). The antibody directed to the α3 chain (pAb SE85) specifically recognised a 165 kDa polypeptide in the culture medium (Fig. 5A, lane 1) and HT29 cell extracts (Fig. 5B, lane 5); in addition, a doublet at approximately 200 kDa was found in cell extracts. These molecular masses correspond to those described for the precursor (200 kDa) and the mature (165...
kDa) forms of laminin α3 in keratinocytes (Marinkovich et al., 1992a). No bands were identified on immunoblots corresponding to the Caco2 cells (Fig. 5A, lane 2, and B, lane 6). In HT29 conditioned medium (Fig. 5A, lane 3) and cell extracts (Fig. 5B, lane 7), pAb SE144 reacted with a major 155 kDa band and to a much lesser extent with a 105 kDa polypeptide which correspond, respectively, to the precursor and mature laminin γ2 chains described in keratinocytes (Marinkovich et al., 1992a) and in SCC25 cells (Fig. 3, lane 4). This antibody also reacted with 155 kDa and 105 kDa bands in culture medium and total extracts of Caco2 cells (Fig. 5A, lane 4, and B, lane 8). Thus, the immunoblot and immunoprecipitation experiments revealed that the 105 kDa γ2 product was barely detectable whatever the intestinal cell line considered and that no α3 chains were found in Caco2 cell extracts or medium. The identity of the 140 kDa band, immunoprecipitated by the anti-α3 or γ2 chain antibodies could not be assessed by this technique because antibodies specific to the denaturated laminin β3 chain are not available.

Immunoprecipitation with anti-α3 chain antibody further detected in HT29 cell culture medium a 220 kDa band that could correspond to the β1 and γ1 chains of laminin-1 (Fig. 6, lane 1). Serial immunoprecipitations were performed with the anti-laminin-1 antibody (lane 2) to deprive the medium conditioned by the cells, from laminin-1 chains. Subsequent immunoprecipitation with mAb BM 165 revealed a drastic decrease in the intensity of the 220 kDa band (Fig. 6, lane 3) indicating that this material was largely due to laminin-1 molecules and not attributable to laminin-5. Since a band in the position of α3 (165 kDa) was precipitated with the anti-laminin-1 antibody (Fig 6, lane 2), it can be speculated that other α3 chain containing-laminins may be present. Laminin-6 (α3β1γ1) and laminin-7 (α3β2γ1) isoforms are good candidates since bands migrating in the position of the β1, γ1 (210, 220 kDa) and α3, β2 (190 kDa) chains can be seen.
Identification of the integrin receptors involved in adhesion of colonic cells to laminin-5

The localisation of laminin-5 in the villus basement membrane (Leivo et al., 1996; Orian-Rousseau et al., 1996), and the observations that laminin γ2 and α3 chains might be involved in migratory processes such as wound repair and invasion (Ryan et al., 1994; Pyke et al., 1994, 1995; Lotz et al., 1997) led us to analyse the integrin receptors involved in the adhesion of HT29p and Caco2 cells to laminin-5. Cell adhesion assays showed that attachment of Caco2 and HT29 cells to increasing concentrations of exogenous laminin-5 was rather similar (Fig. 7). Their adhesion patterns on laminin-5 did not differ from those on laminin-1/nidogen. To assess the specificity of cell adhesion to laminin-5, the culture wells were first coated with laminin-5 or laminin-1/nidogen complex and then with mAb BM165 before addition of the cell suspension. The antibody hampered the adhesion of the cell lines to laminin-5 whereas no effect was observed on adhesion to the laminin-1/nidogen complex (illustrated for HT29p cells in Fig. 8).

In the literature, the putative receptors for laminin-5 described so far are α3β1, α6β1 and α6β4 depending on the cell line (Carter et al., 1991; Delwel et al., 1993, 1994; Niessen et al., 1994; Rousselle and Aumailley, 1994). Caco2 and HT29 cell lines are of particular interest because they display different integrins on their cell surface. Caco2 cells express high levels of integrin α3β1 and α2β1 and lower levels of integrin α3β1 and α6β1; they do not express integrin α6β4 (Basson et al., 1992; Ebert, 1996). In contrast, HT29 cells express integrin α6β4 in addition to integrins α1β1, α2β1 and α3β1 (Simon-Assmann et al., 1994b; Ebert, 1996). To determine the receptor(s) involved in their adhesion to laminin-5, we performed inhibition assays with the anti-α2 (mAb P1E6), anti-α3 (mAb P1B5), anti-α6 (mAb GoH3), anti-β1 (mAb P4C10) and anti-β4 (mAb 3E1) integrin antibodies on HT29p (Fig. 9A) and Caco2 (Fig. 9B) cells. Adhesion of HT29p cells to laminin-5 was totally inhibited by mAb P1E6. Inhibition was dose-dependent and the anti-integrin antibody dilution leading to 50% inhibition was 1/8,650. Monoclonal antibodies P1B5 and GoH3 led, respectively, to 55% and 60% inhibition of HT29p cell adhesion. With antibodies against the integrin β subunits, total inhibition was obtained with mAb P4C10 but no inhibition was observed with mAb 3E1. Therefore, we can conclude that the potential laminin-5 receptors in HT29p cells are integrins α2β1 and α3β1. However, adhesion via the α6β4 integrin could also occur through the α6 subunit. In the case of Caco2 cells, adhesion to laminin-5 was almost totally prevented by the mAb P1E6. No inhibition was observed with mAb P1B5 while 25% inhibition was obtained with mAb GoH3. mAb P4C10 induced inhibition of adhesion whereas no effect was observed with mAb 3E1. Therefore, the α2β1 receptor and to a lesser extent the α6β1 integrin appear to play a role in the adhesion of Caco2 cells to laminin-5.

Similar experiments were performed on cell suspensions seeded on laminin-1 (Fig. 9A and B). The integrin α6β4

Fig. 7. Adhesion of colonic cancer cells to various concentrations of substrata. Multiwell plates were coated with laminin-1 (●), laminin-5 (○) or with fibronectin (▲) at various concentrations. After saturation with 1% BSA, cells were seeded and allowed to attach for 2 hours. Extent of cell adhesion was measured with a colorimetric reaction.
appeared to mediate adhesion to laminin-1 in HT29 cells which lack α6β1 integrin. None of the anti-α subunits antibodies tested had a detectable effect on the adhesion of Caco2 cells to laminin-1 in contrast to the anti-β1 mAb P4C10; thus, the α1β1 integrin present on Caco2 cells, and on HT29 cells, might be a candidate receptor of this ligand.

It is worth noting that a distinct cellular specificity was observed concerning the implication of α2β1 integrin in cell binding to laminin-5. Indeed, normal human keratinocytes isolated as previously published (Rousselle and Aumailley, 1994) do not use this integrin for their adhesion to laminin-5; yet, they use this integrin to bind to laminin-1 (Fig. 9C).

As α2β1 integrin has not yet been described as a putative receptor to laminin-5, further experiments were done to confirm that this integrin is indeed implicated in colonic cancer cells. First, to investigate the direct interaction between integrin α2β1 and laminin-5, the binding of biotinylated purified α2β1 integrin to immobilised laminin-5 was measured. Fig. 10A

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**Fig. 8.** Effect of mAb BM165 on adhesion of HT29p cells to laminin-5. Multiwell plates were coated with laminin-5 (C) or with laminin-1 (●) at 5 and 10 µg/ml, respectively. After saturation with 1% BSA, the wells were incubated with various concentrations of mAb BM 165 for 60 minutes before cell adhesion assays. Extent of cell adhesion depicted in A was measured with a colorimetric reaction and expressed as a percentage of adhesion in the absence of antibodies. The concentration of mAb BM165 leading to 50% of inhibition equals to 0.2 µg/ml. The data were compiled from 2 independent assays performed in duplicate. Attachment and morphology of the cells on laminin-5 (B) and on laminin-5 in the presence of mAb BM165 (C). Bars, 50 µm.

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**Fig. 9.** Effect of anti-integrin subunit antibodies on adhesion of intestinal epithelial cells and keratinocytes to laminin-5 and laminin-1. HT29p (A) and Caco2 (B) cells were seeded on 5 µg/well laminin-5 (LN-5) or on 10 µg/well laminin-1 (LN-1). The antibody concentrations were those that gave maximum inhibition of adhesion: mAb P1E6 against α2 (1/2700) for the HT29 cells and (1/8100) for the Caco2 cells, mAb P1B5 against α3 (1/900), mAb GoH3 against α6 as well as mAb P4C10 against β1 (1/8100), mAb 3E1 against β4 (1/25). Dose-dependent curves were initially performed to determine the optimal concentrations of antibodies. Otherwise, no inhibition was observed whatever the dilution of the antibody. The number of cells that attached in the absence of antibodies was taken as 100% adhesion. Error bars represent s.e.m. values of the average results. Asterisks indicate significant differences between the assay (with antibody) and the control (without antibody); *P<0.05; **P<0.005. (C) Representative experiment for freshly isolated normal human keratinocytes showing an inhibition of cell adhesion by mAb P1E6 against α2 subunit only on laminin-1.
shows that α2β1 integrin supports dose-dependent binding of laminin-5. This molecular interaction was completely inhibited by EDTA and by the anti-α2 integrin antibody 6F1 (5 µg/ml). A comparison is shown of α2β1 integrin binding to laminin-1 (10 µg/ml). In this panel the background binding has been subtracted from the data. In both A and B results are expressed as the mean ± standard error for three wells.

in favour of α2β1 receptor binding to laminin-5 was provided by the confocal microscopy analysis. The distribution of integrin α2 subunit, stained with mAb P1E6, was analysed on HT29p cells cultured on laminin-1 and laminin-5. Preparations through the X-Y section revealed a strikingly different distribution of α2 integrin between the two substrata (Fig. 11A-
F). When seeded on laminin-1, the cells depicted a relatively uniform membrane staining of $\alpha 2$ subunit whatever the section plane (illustrated for one single cell; Fig. 11A-C). On laminin-5, there was a clear-cut accumulation of $\alpha 2$ integrin staining at the basal pole of the cell (Fig. 11D) visualised by a segregation of intensively labelled patches. Concomitantly, there was a decrease in the staining at the lateral and apical part of the cell (Fig. 11E,F). The segregation of $\alpha 2$ integrin on laminin-5 substratum was confirmed by the confocal analysis through the Y-Z section plane of cells (Fig. 11H versus G). In some cases, the cells showed a well spread and flattened morphology parallel to the $\alpha 2$ integrin rearrangement (Fig 11I). It is also important to stress that a clear epithelial resurfacing of the $\alpha 2$ integrin subunit at the ventral surface of the cells paralleled the deposition of laminin-5 by the cells themselves after 5 days of culture (not illustrated).

**DISCUSSION**

In the skin, laminin-5 is known to play a major role in cell adhesion and integrity of the basement membranes in regions of cell-connective tissue interactions (Rousselle et al., 1991). The intestine is characterised by tightly orchestrated cell movements and progressive cell differentiation events during morphogenesis and cell renewal. Linked to these processes, differential expression of basement membrane molecules and receptors such as integrins has been described (for reviews see Simon-Assmann et al., 1995, 1998; Beaulieu, 1997). In the human mature intestine, which is composed of a simple epithelium, the location of laminin-5 at the basal pole of epithelial cells according to an increasing gradient settled from crypt to villus tip (Orian-Rousseau et al., 1996) would argue for a role in differentiation and/or migration.

The present study shows that the highly differentiated colon carcinoma Caco-2 cells express very low amounts of laminin-5, whereas HT29p cells and two derived subclones which are phenotypically related to foetal epithelial cells produce significant amounts of the protein. These observations can be correlated to the cellular origin of laminin-5 described recently (Orian-Rousseau et al., 1996). Indeed, detection of laminin-5 $\gamma 2$ chain with anti-mouse antibodies on hybrid chick/mouse intestines pointed to a dual and developmentally-regulated expression of this chain: synthesis by moderately differentiated epithelial cells during intestinal development, relayed by the differentiated mesenchymal cells at more advanced stages. Of further interest is the finding of an inverse correlation between expression of laminin-5 and of laminin-1. Indeed, HT29 cells lack laminin-6 chain in contrast to Caco-2 cells which express and gradually accumulate laminin-1 as they undergo enterocytic differentiation (De Arcangelis et al., 1994; Vachon and Beaulieu, 1995). Furthermore, using an antisense RNA strategy we demonstrated the direct involvement of laminin-1 $\alpha 6$ chain in the basement membrane assembly and in Caco-2 cell differentiation (De Arcangelis et al., 1996). Although the mechanism of epithelial cell migration in the adult intestine remains an enigma (Heath, 1996) the hypothesis that laminin-5 could be involved in cell migration rather than in cell differentiation is supported by the following observations: (i) laminin-5 $\gamma 2$ chain is preferentially expressed in invasive cells in colon cancers (Pyke et al., 1994, 1995); (ii) the promoter activity of the LAMC2 gene encoding laminin $\gamma 2$ chain is upregulated by the hepatocyte growth factor, known for its effect on cell motility (J. Olsen et al., unpublished data); (iii) laminin-5 $\alpha 3$ chain gene expression is increased in the keratinocytes at the wound sites (Ryan et al., 1994); (iv) a large cell-adhesive protein formerly called ladsin, which is in fact similar to laminin-5, has been shown to stimulate both the chemotactic and chemokinetic migration of the rat liver cell line BRL and of endothelial cells (Kikkawa et al., 1994, 1996); (v) finally, a specific cleavage of laminin-5 $\gamma 2$ chain by matrix metalloprotease-2 has been shown recently to induce migration of breast epithelial cells (Giannelli et al., 1997).

Immunoprecipitation and immunoblot experiments allowed us to identify the laminin-5 molecule produced and secreted by colon cancer cell lines. In all HT29 cell populations, the prominent form of the molecule found in the cells as well as in the media is the 440 kDa heterotrimeric molecule corresponding to the KM1 intermediate form described in the skin by Marinkovich et al. (1992a). It consists of the 165 kDa $\alpha 3$, the 145 kDa $\beta 3$ and the 155 kDa $\gamma 2$ chains. Thus, in colon epithelial cells, the 155 kDa $\gamma 2$ precursor is not efficiently processed into the 105 kDa form to produce the 400 kDa form found in keratinocytes and SCC25 cells (Marinkovich et al., 1992a; Rousselle and Aumailley, 1994). In Caco-2 cells, the major chain synthesized is the $\gamma 2$ chain, while $\alpha 3$ and $\beta 3$ chains are barely detectable; this leads to an intracellular accumulation of the 155 $\gamma 2$ chain in Caco-2 cells. Another interesting feature is the possible expression of laminin-6 ($\alpha 3\beta 1\gamma 1$) and laminin-7 ($\alpha 3\beta 2\gamma 1$) in HT29 cells. These two isoforms have been shown to be enhanced in response to wounding during intestinal restitution (Lotz et al., 1997). Complexes between laminin-5/laminin-6 and laminin-5/laminin-7 have been described in the amniotic membrane in which they are covalently associated through disulphide bridges (Champliaud et al., 1996).

Our present study shows that, in contrast to other cell types such as keratinocytes, colon epithelial cells can use the $\alpha 2\beta 1$ integrin to adhere to laminin-5. This result was unexpected; indeed, the $\alpha 2\beta 1$ complex, clearly identified as a collagen receptor on MDCK cells (Saelman et al., 1995), or as a laminin-1 receptor on Clone A colon cancer cells (Lotz et al., 1990) has never been described as a laminin-5 receptor. Of particular interest is the fact that this $\alpha 2\beta 1$ receptor is not implicated in HT29 and Caco-2 cell adhesion to laminin-1 although in the solid phase assay $\alpha 2\beta 1$ integrin is able to link laminin-1. This observation and the finding that keratinocytes do not adhere to laminin-5 through the $\alpha 2\beta 1$ receptor strengthen the hypothesis that the ligand binding specificity can be determined by the cellular environment. In this regard, Chan and Hemler (1993) showed, by expressing the $\alpha 2$ cDNA in two different cell types, that the $\alpha 2\beta 1$ integrin expressed by these cells displays distinct patterns of functional activity, such as differential binding on laminin-1 or collagen. Apart from the $\alpha 2\beta 1$ integrin, Caco-2 cells use the $\alpha 6\beta 1$ integrin, whereas HT29 cells bind to laminin-5 through $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins. These data are in accordance with those of the literature, showing that the $\alpha 3\beta 1$ (Carter et al., 1991; Delwel et al., 1994; Rousselle and Aumailley, 1994), the $\alpha 6\beta 1$ (Delwel et al., 1993; Rousselle and Aumailley, 1994), and the $\alpha 6\beta 4$ (Niessen et al., 1994) integrins are receptors for laminin-5. For these receptors, interactions with laminin-5 are dependent on
the coil-coiled structure. Accordingly, adhesion of HT29 cells on heat-denatured laminin-5 (in which the cell-binding activity carried by the carboxy-terminal domain of the molecule was impaired) was reduced to 50% (not illustrated). On the basis of the data obtained with HT29 cells, one can postulate that their adhesion to laminin-5 involves a multimerization process (Diamond and Springer, 1994) in which integrin α2β1 exerts a regulatory effect on α6β4 or vice-versa. This type of model, which presumes the existence of integrin molecules that become apposed, is described in other systems (Karenca et al., 1994; Tozeren et al., 1994). Similarly, Carter et al. (1991) have proposed that α3β1 recruits α6β4 into stable anchoring complexes in keratinocytes.

Concerning the correlation between our present data and integrin binding to laminin-5 in the intestine, one can at this point only speculate. While laminin-5 is expressed early during development, integrin α6β4 appears only later on during morphogenesis (Simon-Assmann et al., 1994a) indicating the involvement of the β1 integrin family for laminin-5 binding at this period of time. During villus onset and formation, the expression of α2β1 coincides with that of laminin-5; early expression in the intestinal anlage and gradient with pronounced expression at the tips of villi (Wu and Santoro, 1994; Perreault et al., 1995; Dieckgraefe et al., 1996; Leivo et al., 1996; Orian-Rousseau et al., 1996). Thus, during development, the α2β1 integrin may serve as a receptor for laminin-5. Yet, no obvious correlation can be made between the locations of laminin-5 and α2β1 integrin in the adult organ in which α2β1 integrin is described in the crypts (Wu and Santoro, 1994; Perreault et al., 1995), while laminin-5 is located mostly along the villi (Leivo et al., 1996; Orian-Rousseau et al., 1996). At this stage, one can assume that attachment of cells to laminin-5 in the villus region is mediated by other integrins such as α6β4 or α3β1 which are indeed found in this area. In the human adult intestine, α6β4 integrin is also colocalized with HD1, a hemidesmosomal protein, whereas the BP (bullous pemphigoid) antigens are not detected suggesting the existence of type II hemidesmosomes (Orian-Rousseau et al., 1996). Similarly, HT29 cells only express HD1 and α6β4 integrin which colocalize in basal patches between actin stress fibers (Fontao et al., 1997). It is tempting therefore to speculate that these type II hemidesmosomal structures together with laminin-5 could be implicated mostly in cell migration, which occurs continuously in the adult organ.

Until now, binding sites of the integrins have not been mapped on laminin-5. Pfaff et al. (1994) have reported that the EIXNd fragment in the cross region of laminin-1 was responsible for binding to integrin α2β1, suggesting that the N-terminal region of the laminin α1 chain was implicated; this region is deleted in the laminin α3A chain (Ryan et al., 1994; Galliano et al., 1995). This shorter α3 A chain was the first to be identified by immunological methods and cDNA cloning (Rousselle et al., 1991; Aberdam et al., 1994a). Subsequently, however, multiple α3 cDNAs were identified in human and mouse (Ryan et al., 1994; Galliano et al., 1995). Recently Miner et al. (1997) described a novel full-length α3B form that comprises additional domains, in particular domains V and VI. Therefore interaction of integrin α2β1 with this α3B isoform is possible. Alternatively, the possibility cannot be excluded that integrin α2β1 could bind to another chain of the laminin-5 molecule. In accordance with this are the data of Underwood et al. (1995) who identified a binding sequence on the β1 subunit of laminin-1. More recently, using systematic peptide screening, Nomizu et al. (1997) located an active sequence responsible for interaction with integrin α2β1 on the γ1 chain in a region which is highly conserved with the γ2 chain. Definitive identification of the fragments responsible for the binding of α2β1 integrin will be possible only when small biologically active fragments of laminin-5 will be available.

From the data reported here, it is reasonable to speculate that laminin-5 could exert different physiological functions such as anchorage, cell migration or differentiation depending on the tissue considered and on the receptors used by the cells. The data reported herein on colonic epithelial cells together with the former analysis of laminin-5 expression and origin in the intestinal tissue, give a basic knowledge which will contribute to the understanding of the biological regulation or dysregulation of this molecule in intestinal pathologies.

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