Adhesive properties and integrin expression profiles of two colonic cancer populations differing by their spreading on laminin

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SUMMARY

The mostly undifferentiated parental HT29 (HT29p) human colonic adenocarcinoma cell line and a differentiated subpopulation selected by the anti-cancer drug 5-fluorouracil (HT29-Fu) (Lesuffleur et al. (1991) *Int. J. Cancer* 49, 721-730) display strikingly different behavior when grown on laminin coatings: the former grows as aggregates while the latter grows as monolayers. In an attempt to explain this difference, we performed a comparative study of cell adhesion properties and of expression, involvement and localization of the α_6, β_1 and β_4 subunits constituting the integrin family among the two cell populations.

HT29p and HT29-Fu cells exhibited a similar adhesion pattern to laminin and laminin fragments E8 and P1. In both cell lines, cell adhesion could be blocked at about 90% with anti-α_6 subunit antibodies and around 30-50% with anti-β_1 antibodies; no inhibition of the cell adhesion was obvious when using anti-β_4 antibodies. Immunoprecipitations of iodinated membrane-solubilized proteins and immunoblotting experiments showed that all α_6 chains expressed in both HT29p and HT29-Fu cell populations exist as $\alpha_6\beta_4$ integrins; β_1 subunits are associated with α_2 and α_3 chains. When HT29p or HT29-Fu cells were injected subcutaneously in nude mice, a similar expression pattern of α_6, β_4 and β_1 integrin subunits was noticeable in the resulting tumors: α_6 and β_4 subunits were localized at the basal surface of the tumor cells facing the stromal elements, and to a lesser extent at the cell-cell contacts within the tumor-cell clumps; β_1 subunits were mainly found within the cytoplasm of the tumor cells.

Despite these overall similarities among the two cell lines, the following changes could account for their different behavior on laminin: less proteolytic processing of the β_4 integrin subunit occurred in HT29-Fu cells yielding peptidic fragments of 175 kDa, which are absent from the parental cells; the immunostaining pattern of the various subunits demonstrated a segregation of α_6, β_4 and β_1 integrin subunits on the basal side of the HT29-Fu cells when cultured on laminin to the detriment of their lateral location, a phenomenon that was not obvious in the parental cells.

Altogether, these results suggest that the distinct behavior of the undifferentiated versus differentiated HT29 cell populations on laminin is not related to altered adhesion properties of the cells but rather to a deficient stabilization of the adhesion leading to cell spreading. This difference is highlighted by a variable basal segregation potential of the laminin-binding integrins, which could be due to an altered form of the β_4 subunits.

Key words: integrin, colonic cancer cell, laminin

INTRODUCTION

It is well known that changes in cell-cell and cell-matrix interactions are involved in the process of tumor invasion and metastasis. The metastatic capacity of tumor cells can be correlated to altered cell contacts, basement membrane integrity, and/or cellular receptors to matrix molecules. In order to study the tumor/stromal cell interactions and in parallel basement membrane formation in vitro, coculture models of neoplastic cells and fibroblasts have often been used. This model has enabled us to show that altered deposition of basement membrane molecules occurred concomitantly with perturbation of the heterologous cell interactions when human colonic cancer lines were used (Bouziges et al., 1991). Caco-2 cells grow as a monolayer on top of the fibroblastic cell layer, while HT29 cells do not spread and form aggregates on the fibroblasts. A structured basement membrane, assessed at the electron microscopic level and by immunocytochemical detection of basement membrane molecules, is only present at the heterologous cell interface of the cocultures involving Caco-2 cells. The peculiar behavior of HT29 cells when cultured on fibroblasts has also been observed when they are plated on laminin, whereas on other isolated extracellular matrix components (type I collagen, fibronectin,...) they grow as monolayers.

After confluency, the HT29 cell line is composed mainly of undifferentiated cells with a small percentage of differentiated cells (Lesuffleur et al., 1990). The treatment of the parental HT29 cells with an anti-cancer drug, 5-fluorouracil, leads to the selection of cells (HT29-Fu) that are committed to differ-

entiation (Lesuffleur et al., 1991). We found that the attachment properties of these cells is strikingly different from those of the parental cells, since they were able to spread on a laminin substratum like the well differentiated Caco-2 cells. Several hypotheses can be postulated to explain the different behavior of the two HT29 cell populations on laminin. An altered expression of cell adhesion molecules, of extracellular matrix molecules or of proteases could lead to unbalanced cell-cell versus cell-substratum contacts. Otherwise, the differences between the parental and Fu cells could result from differential expression or distinct properties of cell receptors to laminin. The present study focuses on the latter aspect.

Multiple laminin-binding proteins have been identified (Mercurio and Shaw, 1991), among which are integrins, composed of α and β transmembrane subunits (Hynes, 1992), and non-integrin molecules (Mecham, 1991). The functional diversity of integrins is dictated by their particular α/β subunit composition. The integrins that have been shown to be potential receptors for laminin belong to the β_1 series ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$). Only the $\alpha_6\beta_1$ is specific for the binding of cells to laminin; it recognizes a site in the E8 fragment of laminin localized at the C-terminal end of the molecule (Sonnenberg et al., 1991a). α_1-, α_2- and $\alpha_3\beta_1$ integrins, as well as having an affinity for other extracellular matrix molecules (fibronectin, collagen), can act as potential receptors for laminin. Recently, it has been reported that laminin is the ligand for another receptor, the $\alpha_6\beta_4$ integrin (Lee et al., 1992).

The purpose of the present work was to compare the expression and functional properties of the major integrin receptors to laminin containing the β_1, β_4 and α_6 subunits in the mostly undifferentiated parental HT29 cells with those of the differentiated HT29-Fu subpopulation. For this purpose, cell adhesion to laminin and laminin fragments, as well as involvement of various integrin subunits in adhesion, have been estimated; the identification of the integrins expressed has also been approached by immunoprecipitation experiments and the localization of constitutive integrin subunits has been traced immunocytochemically.

MATERIALS AND METHODS

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^{-5} M 5-fluorouracil (HT29-Fu) were obtained from Dr A. Zweibaum (Unité 178 INSERM, Paris, France). The conditions of treatment of the cells have been defined in detail previously (Lesuffleur et al., 1991). Both HT29p and HT29-Fu cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, France) supplemented with 10% heat-inactivated fetal calf serum (Gibco) and with 200 µg/ml gentamycin.

Antibodies

Rat monoclonal antibodies (GoH3) prepared against the α_6 subunit (Sonnenberg et al., 1987) were a generous gift from Dr Sonnenberg (Blood Transfusion Service, Amsterdam, The Netherlands). Mouse monoclonal antibodies 4E9G8 directed against the cytoplasmic tail (A isoform) of the α_6 subunit were a generous gift from Dr Lissitzky (IBCP, CNRS-UPR 412, Lyon, France) (Rémy et al., 1993). Rat monoclonal antibodies (439-9B) against the human β_4 subunit (Kennel et

al., 1989) were kindly provided by Dr Kennel (Oak Ridge National Laboratory, TN, USA). Rabbit polyclonal anti-integrin β_1 subunit (AB 1938 P) was purchased from Chemicon (Temecula, CA, USA). Mouse monoclonal antibodies (K20) against the β_1 subunit were purchased from Immunotech (Marseille, France). Rat monoclonal anti-α_5 (BIIG2) and anti-β_1 (AIIB$_2$) were kindly provided by Dr Damsky (University of California, San Francisco, USA) (Hall et al., 1990). Mouse monoclonal anti-α_1 (TS2/7; Hemler et al., 1984) and anti-α_4 (B5G10; Hemler et al., 1987) were gifts from Dr Hemler (Dana-Farber Cancer Institute, Boston, MA, USA); mouse monoclonal anti-α_2 (P1E6), anti-α_3 (P1B5) were purchased from Telios.

### Radioiodination of cell membrane proteins

HT29p and HT29-Fu cell membranes were prepared as described by Stallmach et al. (1990). Confluent monolayers (6 days after seeding) of HT29p or HT29-Fu cells were harvested by scraping, after a short wash in Hanks' medium containing various protease inhibitors (2 µg/ml aprotinin, 10 µg/ml leupeptin, 17 µg/ml PMSF, 1 µg/ml pepstatin, 1 µg/ml antipaine, 10 µg/ml benzamidine). After centrifugation (1000 g, 10 minutes), the pellet was resuspended in 0.01 M triethanolamine hydrochloride, pH 7.5 (TEA-buffer), containing 8.5% sucrose, 1 mM PMSF and 1 mM N-ethylmaleimide (NEM) as inhibitors, and homogenized on ice in a Potter homogenizer. The homogenate was loaded on a 41% solution of sucrose in TEA buffer and centrifuged for 1 hour at 105,000 g (rotor TI70). Cell membranes, concentrated as a white interfacial band, were collected, diluted in TEA buffer and pelleted by centrifugation at 105,000 g for 20 minutes. Protein solubilization was achieved by incubating 400 µg of cell membrane with 400 µl of 1% Triton X-100 in TBS solution (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM MnCl$_2$, 0.1 mM NEM and 0.1 mM PMSF), pH 7.4, for 1 hour on ice under agitation, according to Hauri et al. (1985). Non-solubilized particles were removed by ultracentrifugation (100,000 g, 15 minutes, 4°C). Iodination of Triton X-100-solubilized membrane proteins was performed with Na^{125}I (Amersham, 100 mCi/ml) using the lactoperoxidase method (Enzymobeads, Bio-Rad).

Immunoprecipitation

Iodinated proteins were first preincubated for 1 hour at 4°C with Protein A-Sepharose CL-4B beads (Pharmacia) or with rabbit anti-rat IgG-agarose beads (Sigma) for subsequent immunoprecipitation with, respectively, mouse or rat monoclonals. After centrifugation, samples of the precleared fractions were incubated with the anti-α_6 GoH3 (0.5-1 µg per assay), anti-β_4 439-9B (1 µl per assay), anti-β_1 K-20 (2 µg per assay) mAb for 3 hours at 4°C. Then 25 µl of Protein A-Sepharose or rabbit anti-rat IgG-agarose beads was added and incubation was carried on for another 2 hours. Immune complexes were washed three times in TBS-containing protease inhibitors, resuspended in Laemmli buffer with or without 100 mM dithiothreitol (DTT) and heated for 5 minutes at 100°C. The samples were analyzed by SDS-PAGE on 6% gels according to Laemmli (1970). For autoradiography, Hyperfilm MP (Amersham) was used in combination with intensifying screens. Gels were calibrated with ^{14}C-labeled protein standards (Amersham) and apparent molecular masses were determined by linear scanning with a densitometer (Shimadzu, Roucaire).

Western blot analysis

Samples of 200 to 250 µg of cell membrane proteins were incubated in Laemmli buffer containing 2% (w/v) SDS with or without 100 mM DTT at 100°C for 5 minutes. Proteins were separated on 6% SDS-PAGE and subsequently transferred overnight onto nitrocellulose in 25 mM Tris-HCl, 192 mM glycine, pH 8.2, 20% (v/v) methanol. After transfer, the nitrocellulose was saturated with 0.3% BSA in TEA buffer for 1 hour at 37°C, rinsed in TEA buffer containing 0.1% sodium desoxycholate. For immunological revelation the nitrocellulose was incubated overnight at 4°C with the monoclonal anti-β_4 439-9B antibody diluted at 1/200, with the polyclonal anti-β_1 polyclonal
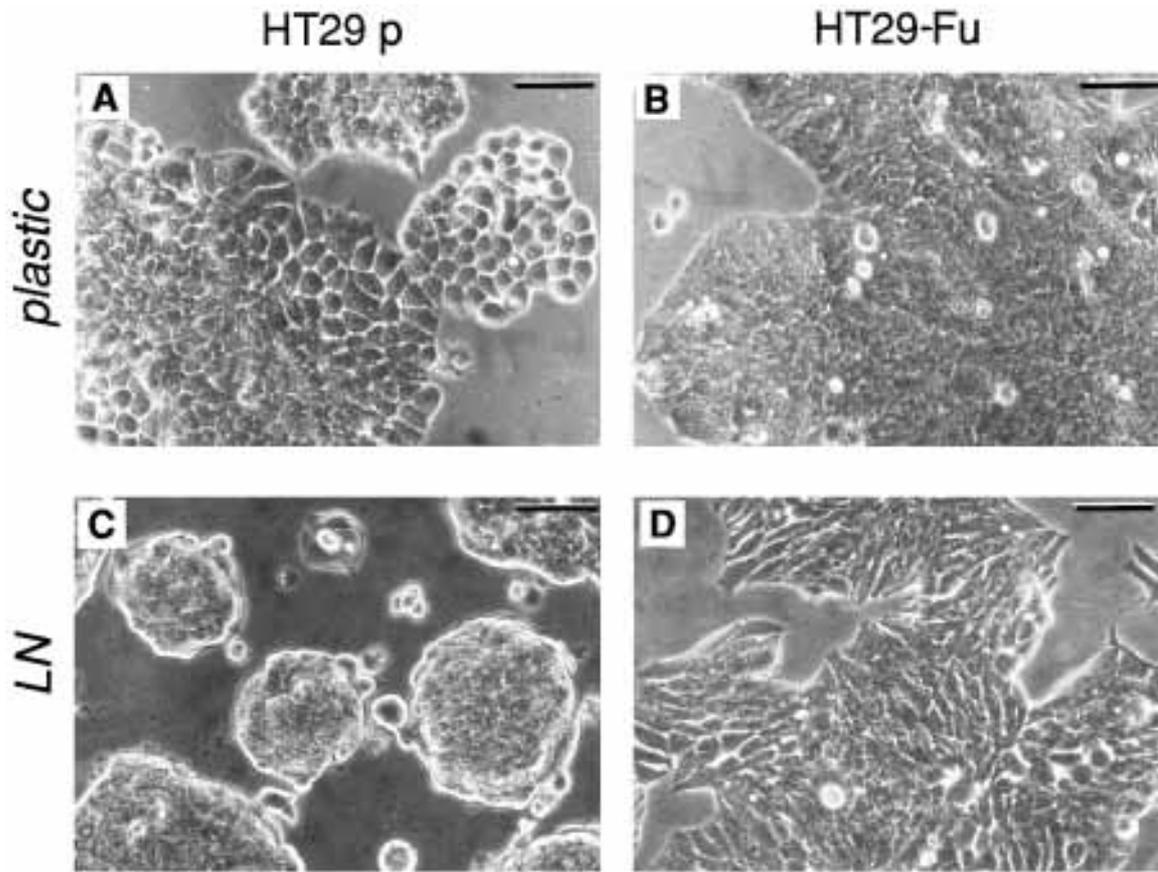
HT29 p

HT29-Fu

Fig. 1. Phase-contrast micrographs of HT29p (A,C) and HT29-Fu (B,D) cells cultured for 7 days on plastic (A,B) or on laminin(LN)-coated (C,D) dishes. Note the peculiar behavior of HT29p cells, which grew as aggregates on laminin. Bar, 50 µm.

AB1938P antibody diluted at 1/100 or with the monoclonal anti-α_6 4E9G8 antibody diluted at 1/500. After several washes, the nitrocellulose was incubated with affinity-purified goat anti-rat IgG (Jackson), anti-rabbit IgG (Bio-Rad) or anti-mouse IgG (Jackson) conjugated to alkaline phosphatase at 1:3000 (v/v). Finally the blot was rinsed and revealed with BCIP/NBT color reagents (Bio-Rad). In some experiments, immunological revelations have been performed on immunoprecipitated material after electrophoretic separation and transfer on nitrocellulose.

Cell attachment and inhibition assays

Twenty-four-well tissue culture plates (Falcon) were coated for 1 hour at 37°C with increasing concentrations (0.6 to 10 µg/cm^2) of laminin dissolved in Ham's F10 medium (Gibco). The laminin used was extracted from mouse Engelbreth Holm Swarm (EHS) carcinoma and purified by affinity chromatography (Simo et al., 1991). The wells were subsequently rinsed and saturated with 1% BSA (Boehringer) for 1 hour at 37°C. Albumin-treated dishes served as controls. Colon carcinoma cells were plated (10^5 cells per well) in DMEM without serum. The cells were counted after 2 hours of incubation at 37°C.

Adhesion assays on laminin fragments were performed in microtiter wells (96-well tissue culture plates, Costar) coated for 1 hour at 37°C with 6 µg E8 or P1 fragments in 100 µl Ham's F10 medium. The subsequent saturation with albumin, plating and counting of the cells were identical to those for laminin coatings. Laminin fragments have been kindly provided by Dr Lissitzky (IBCP, CNRS-UPR 412, Lyon, France).

For the inhibition assays, cells were plated on 6 µg/cm^2 laminin-coated wells in DMEM without serum. GoH3 mAb to α_6 (0.1 to 1 µg/well), K20 mAb to β_1 (2 and 4 µg per well), 439-9B mAb to β_4 (1:500 or 1:250 dilutions), were added at the time of plating; cells were incubated for 2 hours with the antibodies and subsequently counted.

Immunocytochemical detection of integrin subunits

Cells were seeded directly onto glass coverslips or on coverslips coated with 6 µg/cm^2 of purified EHS-laminin or fibronectin (Centre de Transfusion Sanguine, Strasbourg), saturated with albumin, and cultured for 3 to 4 days in standard medium. After a short wash in 0.9% NaCl, cells were fixed with 1% paraformaldehyde in phosphate-buffered saline for 10 minutes. The cells were permeabilized with 0.5% Triton X-100 (10 minutes), incubated for 1-2 hours with the primary antibodies (anti-α_6 GoH3 mAb, 1:50; anti-β_1 AIIB2 mAb, 1:50; anti-β_4 439-9B mAb, 1:100) and for 30 minutes with an anti-rat secondary antibody conjugated with fluorescein isothiocyanate (1:50; Jackson Laboratory). After mounting in a glycerol/PBS/ phenylenediamine solution, cells were observed with a Zeiss-Axiophot Microscope.

Tumors grown in nude mice

HT29p or HT29-Fu cells (10^7) were injected subcutaneously into the backs of nude mice. They formed tumors, which were recovered after one month. The tumors were embedded into Tissue-Tek compound frozen in Freon cooled in liquid nitrogen. The immunocytochemical localization of α_6, β_1 and β_4 integrin subunits was performed on 5 µm cryosections, with the antibodies described in the preceding paragraph.
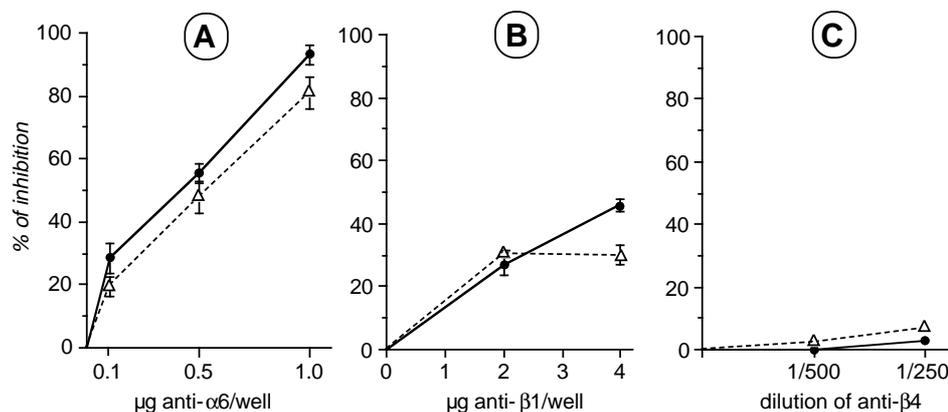
RESULTS

HT29p and HT29-Fu cell attachment to laminin and to laminin fragments

When HT29p cells were plated on laminin (Fig. 1C) they attached to the substratum, and within 3 days formed aggregates of densely packed cells; these aggregates were loosely attached to laminin. On the contrary, HT29-Fu cells grew on laminin, like on plastic, as monolayered areas (Fig. 1D). This difference in the cell behavior could not be attributed to a differential attachment; indeed both cell populations adhered equally well to EHS laminin. Concentration curves (not illustrated) indicated that maximal attachment occurred at 6 µg laminin/cm^2 for both cell populations; at that concentration about 10% of the cells adhered within 15 minutes and by 30 minutes maximal adhesion ($\approx$90% of total cells) was seen (illustrated at 2 hours in Fig. 2A). Cell attachment assays on laminin fragments P1 and E8 revealed (Fig. 2B) that E8 is as efficient for attachment as the entire molecule for both cell populations, while P1 is a less efficient attachment substratum. The percentage of HT29-Fu cells attached on P1 fragment was slightly higher than that of HT29p cells (62% versus 48%).
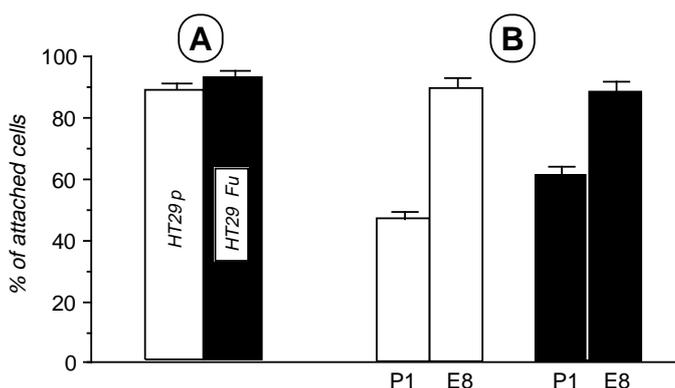
Fig. 2. Adhesion of HT29p (□) and HT29-Fu (■) cells to laminin (A) and to laminin fragments (B). Cells were allowed to adhere to tissue culture wells coated with laminin or with proteolytic fragments of laminin, P1 and E8 for 2 hours at 37°C. Results are expressed as percentage of attached cells relative to the total number of cells seeded in each well. The data were compiled from 10 (adhesion on laminin) and 4 (for the fragments) independent assays. Error bars represent standard errors of the means.
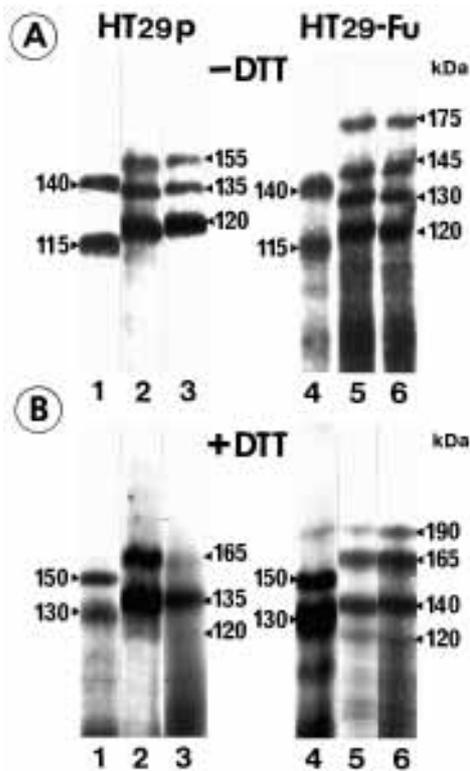
Effects of anti-integrin antibodies on cell adhesion

To examine the functions of α_6, β_1 and β_4 integrin subunits, we tested the ability of relevant monoclonal antibodies to inhibit cell adhesion to laminin (Fig. 3). Antibodies to α_6 subunit (1 µg/well) caused more than 80% inhibition of HT29p and Fu cell adhesion to laminin. Antibodies to β_1 partially blocked adhesion of both cell types to laminin. The inhibition of cell adhesion was similar in both cell lines with the lowest concentration of antibodies used (near 30% for 2 µg anti-β_1 per well). A higher concentration (4 µg/well) of the antibody led to a further inhibition of the HT29p (near 50%) but not of the HT29-Fu cell adhesion to laminin. The anti-β_4 antibodies, 439-9B, did not affect cell adhesion significantly.

Immunoprecipitation of integrins from HT29p and HT29-Fu cells

Various antibodies against subunits of integrins were used to identify the receptors present at the cell surface of HT29p and HT29-Fu cells (Fig. 4 and Table 1).

The β_1 integrin profile was determined by subjecting ^{125}I-labeled detergent-extracted proteins of HT29p or HT29-Fu cells to immunoprecipitation using K20 monoclonal antibodies. In non-reducing conditions two bands migrating at about 115 and 140 kDa were observed in both cell populations (Fig. 4A, lanes 1 and 4). Under reducing conditions, in both cell lines a shift in electrophoretic mobility was observed with the two main bands being found at about 130 and 150 kDa (Fig. 4B, lanes 1 and 4). No distinguishing differences were observed in the electrophoretic mobility or in the relative intensity between the chains (immunoprecipitated with the anti-β_1 antibodies) expressed by the HT29p and HT29-Fu cells. To identify the β_1 subunit, a direct immunoblot analysis was performed using a polyclonal rabbit antiserum raised against a conserved cytoplasmic domain of the β_1 subunit (Chemicon). The antibody weakly recognized a band at 110 kDa without reduction and at 135 kDa under reducing conditions (not illustrated), showing that the immunoprecipitated band having the lowest molecular mass corresponds to the β_1 subunit. The increase in electrophoretic mobility of β_1 subunit under reducing conditions is well known and has already been noted in an other cancer cell line (Lotz et al., 1990). The highest molecular mass band corresponds to α subunits defined below.

Immunoprecipitations using the anti-α_6 GoH$_3$ mAb yielded a pattern of proteins identical to that obtained using the anti-β_4 439-9B mAb (Fig. 4). Analysis of the proteins extracted

Fig. 3. Inhibition assay of HT29p (—) and HT29-Fu (---) cell adhesion to laminin (6 µg/cm^2) by antibodies against integrin subunits. The antibodies were mAb GoH3 directed against the α_6 subunit (A), mAb K20 directed against the β_1 subunit (B) and mAb 439-9B directed against the β_4 subunit (C). Ordinate: percentage of inhibition of cell adhesion after 2 hours at 37°C compared to the control adhesion of the cells in the absence of antibodies; abcissa: concentration or dilution of the antibodies used. The data were compiled from 3 to 4 independent assays; error bars represent standard errors of the means.

Fig. 4. Representative autoradiographs of iodinated cell surface proteins extracted from HT29p (lanes 1-3 in A and B) and from HT29-Fu (lanes 4-6 in A and B) cells, immunoprecipitated by anti-β_1 mAb K20 (lanes 1 and 4), anti-6 mAb GoH3 (lanes 2 and 5) and anti-β_4 mAb 439-9B (lanes 3 and 6). The immunoprecipitated antigens were separated on SDS-PAGE (6%) under non-reducing (−DTT; lanes 1-6 in A) and reducing conditions (+DTT; lanes 1-6 in B). Apparent molecular masses (in kDa) are indicated for the main immunoprecipitated bands.

Table 1. Apparent molecular masses of the various integrin subunits in HT29p and HT29-Fu cells

	−DTT		+DTT	
	HT29p	HT29-Fu	HT29p	HT29-Fu
α_6	133	130	117	121
β_4	–	175	–	188
	153	145	163	164
	118	117	135	138
β_1	114	115	130	131
α^*	142	141	149	150

Mean values of five independent experiments, calculated from immunoprecipitation with the various antibodies after separation on 6% SDS-gels under non-reducing (−DTT) and reducing (+DTT) conditions.

Identity of the bands was determined by immunoblotting experiments with α_6, β_4 and β_1 antibodies.

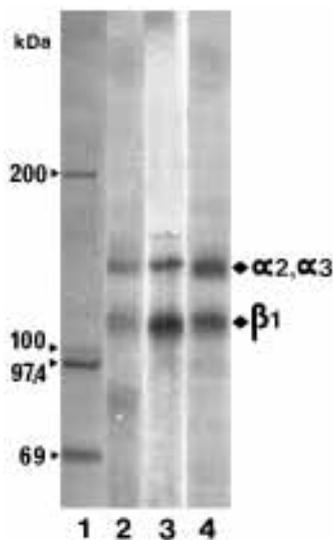α^*, α subunits associated with β_1.

Fig. 5. Immunoblot analysis of the α_6 subunit from HT29p (lane 1) and HT29-Fu (lane 2) cells from immunoprecipitated protein fractions. Samples were analyzed by SDS-PAGE (6%) under non-reducing conditions, transferred to nitrocellulose and incubated with mAb 4E9G8. Molecular mass markers are as indicated in kDa.

from HT29p cells and immunoprecipitated by the GoH$_3$ mAb (Fig. 4A, lane 2) or by the 439-9B mAb (Fig. 4A, lane 3) revealed three bands with estimated sizes at about 155, 135 and 120 kDa without reduction. Under the same conditions, in HT29-Fu cells, a band migrating at 175 kDa was detected in addition to the three bands migrating between 145 and 120 kDa (Fig. 4A, lanes 5 and 6). Immunoblot analysis was performed in order to identify the α_6 proteins using the mAb 4E9G8 directed against the cytoplasmic tail of the α_6 subunit. The antibody strongly reacted with the band migrating around 135 kDa in both HT29p and HT29-Fu cells (Fig. 5). Using anti-β_4 antibody in immunoblotting experiments, only weak immunoreactivity was detected and recovered in two or three bands (not illustrated), therefore enabling us to conclude that the bands migrating at 175, 145 and 120 kDa correspond to the known degradation products of the β_4 subunit (as already observed in numerous cell lines; see Discussion). Upon reduction, the α_6 subunit decreased in size while the β_4 subunit products increased in size, in general (Fig. 4B, lanes 2,3 and 5,6; see in Table 1). It should be noted that from one experiment to the other, there was a marked variability in the relative amounts of the β_4 subunit products.

Thus, from the immunoprecipitation experiments, one can assume that α_6 chains are associated with β_4 rather than β_1

chains. To verify this observation, preclearing experiments were performed. HT29p-detergent cell extracts were precleared with anti-β_4 antibody prior to immunoprecipitation with the anti-α_6 mAb. In the sequential preclearing, three cycles of immunoprecipitation with anti-β_4 were needed to eliminate all the β_4 subunits. Subsequent immunoprecipitation with anti-α_6 antibody did not reveal any band in the α_6 region (not illustrated).

Estimation of the amounts of the various subunits (α_6, β_4, β_1) was performed after immunoprecipitation. It appears that the α_6 subunit represents 1.2% and 1.7% of the total labeled solubilized membrane proteins in HT29p and HT29-Fu, respectively. Interestingly, very similar percentages were recovered for the β_4 subunit. As far as β_1 subunits were concerned, 1% and 1.4% of the labeled proteins from HT29p and HT29-Fu, respectively, were precipitated with the specific antibody.

To find out which subunits are linked to β_1 chains, immunoprecipitations using monoclonal antibodies specific for α subunits that are potentially associated with the β_1 subunit were carried out. The HT29p and HT29-Fu cells were found to express α_2 and α_3 subunits (illustrated for HT29p cells in Fig. 6, lanes 2 and 3, respectively), and very low levels of α_1 subunit (not illustrated). Immunoprecipitation with α_4 or α_5 antibodies did not lead to detectable radioactive bands on the autoradiograms. The apparent molecular masses of the α_2 and α_3 subunits, which strictly correspond to that of the band asso-

Fig. 6. Analysis of the α subunits associated with β_1 chains in HT29p cells. Radiolabeled detergent protein extracts were immunoprecipitated with anti-α_2 P1E6 mAb (lane 2), anti-α_3 P1B5 mAb (lane 3), and anti-β_1 K20 mAb (lane 4). Samples were analyzed on SDS-PAGE (6%) under non-reducing conditions. Lane 1, molecular mass markers are as indicated in kDa.

ciated with the β_1 chains (Fig. 6, lane 4; Fig. 4 lanes 1 and 4), were in the range expected from published studies on other cell types (mean values are 142 kDa for α_2 and 140-150 kDa for α_3 subunits, without reduction).

Immunolocalization of α_6, β_1 and β_4 subunits in HT29p and HT29-Fu cells

The distribution of integrin subunits was analyzed by immuno-fluorescence using anti-α_6, anti-β_4 and anti-β_1 antibodies after permeabilization of HT29p and HT29-Fu cells cultured for 3-4 days on coverslips coated with laminin, and as controls on glass coverslips or on fibronectin coatings.

In HT29p cells, α_6 subunits were localized in two distinct areas whatever the substratum used: at sites of cell-cell contact as a bright linear staining (Fig. 7A,B) and at regions of cell-substratum contact where short linear fibers were present over the whole ventral surface of the cells (Fig. 7A',B'). In HT29-Fu cells, seeded on glass coverslips, the staining pattern at cell/cell contacts was also obvious (Fig. 7C) and the basal pattern appeared generally as long and thin parallel fibers (Fig. 7C'). A distinct localization pattern was observed in HT29-Fu cells cultured on laminin; more often anti-α_6 antibodies stained basal patches that were found mostly at the periphery of the cells; in addition, the intercellular staining was weak (Fig. 7D,D').

Concerning the localization of the β_4 subunit, we observed only a slight immunoreactivity at the boundary between HT29p cells seeded on glass coverslips, on laminin or on fibronectin (Fig. 7E,F). The staining at the basal surface occurred as microfilamentous strands concentrated at the central parts of the cells cultured on glass coverslips (Fig. 7E), or was distributed randomly when the cells were cultured on laminin or on fibronectin (Fig. 7F). In HT29-Fu cells cultured on glass coverslips, labeling of the β_4 subunit was present as basal parallel fibers occupying the whole ventral surface of the

cells (Fig. 7G); when the cells were cultured on laminin, the basal staining was arranged in arrays of short fibers concentrated at the edges of the cells (Fig. 7H') resembling the basal polymorphous patches already described by Marchisio et al. (1991). As for HT29p, in most cases, β_4 was virtually absent from the lateral sides of the HT29-Fu cell population whatever substratum was used (Fig. 7H).

The distribution of β_1 subunits was strikingly different between HT29p cells (Fig. 8A) and HT29-Fu cells (Fig. 8B), but was not modified by the ligand used (glass, laminin or fibronectin). In the parental cell line, β_1 subunits were located mainly at cell-cell boundaries; furthermore, a weak diffuse staining pattern was observed within the cells (Fig. 8A). In HT29-Fu cells, the β_1 labeling was concentrated at the basal surface of the cells, in small focal adhesion plaques usually found at the margins of the cells (Fig. 8B).

Immunolocalization of α_6, β_4 and β_1 integrin subunits in HT29p and in HT29-Fu tumors developed in nude mice

When injected subcutaneously into nude mice, both cell types formed well-delimited and compact tumors surrounded by a conjunctive envelope. The tumor cells were arranged into clumps delineated by stromal areas containing fibroblasts and vessels from the host. The localization patterns of α_6 and β_4 subunits were very similar with one another and among the two types of tumors (Fig. 9A,B,D,E), although the nature of the differentiation pattern between HT29p and HT29-Fu cells was different (Kedinger et al., unpublished observations). α_6 and β_4 labeling was restricted to the tumor cells; none of the stromal elements expressed these integrin subunits. Bright but discontinuous staining was obvious at the interface between the tumor cells and the surrounding connective tissue, and to a lesser extent at the cell-cell contacts within the tumor-cell clumps. The expression pattern of β_1 was somewhat different, but identical in both types of tumors (Fig. 9C,F). β_1 subunit staining was obvious at cell-cell contact sites and within the cytoplasm of the tumor cells, but no preferential deposition was found at the tumor/stroma interface.

DISCUSSION

The purpose of our study was to analyze laminin receptors in a mostly undifferentiated colonic cell line (HT29p) and in a subpopulation (HT29-Fu) of this parental line, selected by adaptation to an anti-tumoral agent 5-fluorouracil, which is fully differentiated (Lesuffleur et al., 1991). A detailed examination of the adhesive properties of both cell populations was initiated by the observation that laminin promoted cell spreading of the differentiated cell line but not of the parental cells. The present work focuses on a comparative analysis of the expression and properties of integrins belonging to the β_1 and β_4 families. We show here that the overall expression and relative involvement in the cell adhesion to laminin of α_6, β_4

Fig. 7. Distribution of α_6 (A-D') and β_4 (E-H') integrin subunits in HT29p (A-B', E, F) and in HT29-Fu (C-D',G-H') cells grown for 3 days on glass coverslips or on coverslips coated with laminin. A'-H' illustrate the ventral surface of the cells. Bars, 20 μm.

and β_1 integrin subunits as well as the exclusive association of α_6 with β_4 molecules are similar in both cell lines. Yet, the following differences among the two cell types could account for their different behavior on laminin: (1) in the HT29-Fu cells, but not in the HT29-p cells, the three subunits display a basal segregation on laminin substratum, to the detriment of their lateral localization; (2) apparent sizes of β_4 integrin fragments exhibit reproducible differences among both cell
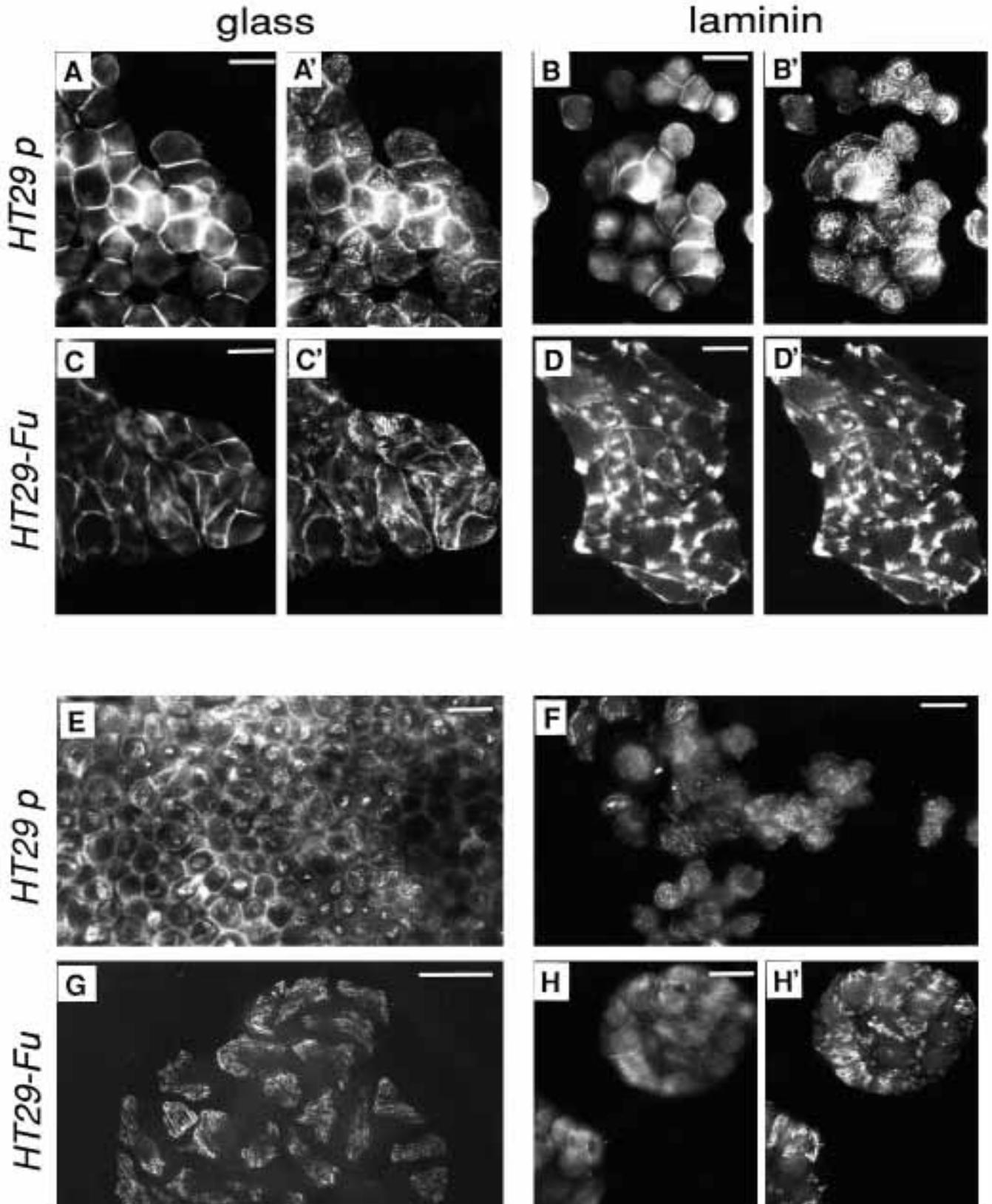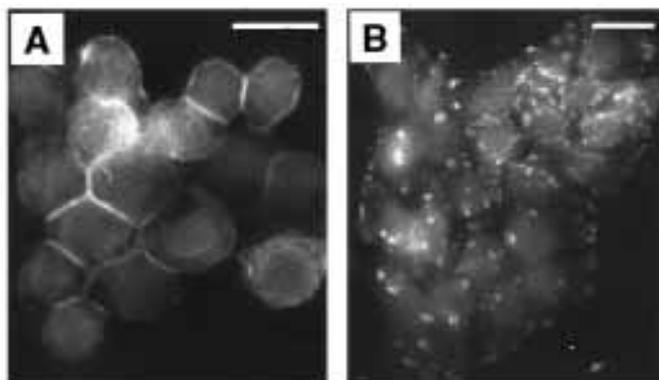
Fig. 8. Distribution of β_1 integrin subunits in HT29p (A) and HT29-Fu (B) cells grown on glass coverslips. Staining pattern is identical when cells are cultured on laminin. Bars, 20 μm.

lines. Our data also show that these modifications do not alter adhesion of the cells to laminin but rather a step further: i.e, stabilization of the attachment allowing cell spreading.

The HT29 colonic cancer cell line displays a large array of adhesive receptors (Stallmach et al., 1990; Phillips et al., 1991; Schreiner et al., 1991; Taichman et al., 1991; Wilson and Weiser, 1992; Del Bufalo et al., 1992). Among those that are laminin-binding molecules, we found that, although the integrin subunits α_6 and β_1 are expressed in both cell populations, they are not co-associated, as shown by the immunoprecipitation experiments. All the α_6 chains being strictly linked to the β_4 chains, the major complex found in HT29 cells is therefore the $\alpha_6\beta_4$ integrin. On the other hand, β_1 is found mostly associated with α_2 and α_3 subunits. Although many cell types contain both $\alpha_6\beta_1$ and $\alpha_6\beta_4$ complexes at variable ratios (Sonnenberg et al., 1990a), α_6 subunits have been found exclusively linked to the β_4 subunits in various colonic cancer cell lines (Lotz et al., 1990; Schreiner et al., 1991; Lee et al., 1992).

No differences in the adhesion of HT29p and HT29-Fu cell populations to laminin were obvious. The finding that antibodies to the α_6 subunit caused an almost complete inhibition of this adhesion strongly suggests that $\alpha_6\beta_4$ is the major integrin complex involved in HT29 cell adhesion to laminin. Yet, the anti-β_4 antibodies used did not significantly inhibit cell adhesion to laminin. Considerable controversy exists in the literature concerning the potential role of the β_4 subunit in cell adhesion and the putative extracellular substratum of the $\alpha_6\beta_4$ integrin (De Luca et al., 1990; Sonnenberg et al., 1990b; Tamura et al., 1990; Lee et al., 1992). This receptor, frequently described in cancer cells, has been shown to be expressed also in various organs, mainly in the skin (Kajiji et al., 1989; Staquet et al., 1990; Stepp et al., 1990; Adams and Watt, 1991; Jones et al., 1991; Sonnenberg et al., 1991b). In this tissue, $\alpha_6\beta_4$ forms the transmembrane root of hemidesmosomes.

The β_4 peptidic bands detected in HT29 cells are characteristic of previously described proteolytic products of the β_4 subunit (Hemler et al., 1989; Giancotti et al., 1992). Interestingly, a different electrophoretic pattern is obvious among the two cell lines, indicating that a less-important proteolytic cleavage occurs in the HT29-Fu cells compared to the parental cells. Indeed, in the former cells, a high molecular mass band migrating at 175 kDa is detected, which is absent in the HT29 parental cell membrane preparations. In order to determine if

there is a definite correlation between the presence/absence of the 175 kDa band and the cellular morphology on laminin, other colonic cancer cell lines were examined. Firstly, Caco-2 cells, which are remarkably well differentiated and spread on laminin, have a completely different integrin repertoire; indeed, these cells only express receptors of the β_1 integrin family (mainly $\alpha_1\beta_1$, $\alpha_2\beta_1$ and weakly $\alpha_6\beta_1$; our unpublished observations; and Basson et al., 1992). Secondly, an additional differentiated subclone of the HT29 cell line (HT29-MTX selected by another anticancer drug, methotrexate; Lesuffleur et al., 1990) that grows in clusters on laminin, like the parental HT29 cells, exhibits the same β_4 profile after immunoprecipitation as the HT29p cells. Nevertheless, complete examination of the adhesion properties of this HT29-MTX subclone reveals that not only $\alpha_6\beta_4$ complexes are involved in the attachment to laminin, since antibodies to α_6 only blocked adhesion to $\approx$50% (not illustrated). Thus, these observations argue in favor of the involvement of the highest proteolytic fragment of β_4 in the stabilization of cell adhesion to laminin. However, it is very difficult to generalize from this conclusion, due to the unique patterns of integrins found in various cell lines. From recent observations (Giancotti et al., 1992), it appears that the proteolytic processing of β_4 observed in cultured cells also occurs in vivo in the skin but not in the cornea, suggesting that β_4 may be regulated in a tissue-specific fashion.

The partial inhibition of cell attachment obtained with an antibody directed against the β_1 integrin subunit indicates that both HT29 cell populations also use a β_1 integrin to adhere to laminin. Immunoprecipitation experiments allowed us to detect mainly $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins, as has also been described elsewhere (Schreiner et al., 1991; Taichman et al., 1991). The $\alpha_2\beta_1$ integrins are known to be mainly receptors for collagen, whereas $\alpha_3\beta_1$ integrins have been defined as promiscuous receptors whose affinity for laminin depends on the cell line tested (for reviews see Ruoslahti, 1991; Hynes, 1992). Thus, $\alpha_3\beta_1$ is a good candidate for participating in the attachment of HT29 cells to laminin. It should be pointed out that the percentage of inhibition of cell adhesion with the anti-β_1 mAb is slightly greater in HT29p than in HT29-Fu cells. Furthermore, immunocytochemical detection of β_1 subunits after several days in culture clearly shows a differential location from cell-cell contacts in HT29p to cell-substratum areas in HT29-Fu cells, regardless of the substratum used. According to Carter et al. (1990), β_1 integrins function in initial adhesive contacts to extracellular matrix molecules and are then relocated in cell/cell junctions. Thus a different membrane redistribution of a β_1 integrin after the immediate adhesion seems to occur among the two cell lines, which might explain, at least partly, their differential spreading.

Our present data are in agreement with those reported by Remy et al. (1993), who did not find any major differences in the production levels of α_6 chains, or in their association with either β_4 or β_1 subunits among two subpopulations of LoVo cells (another human adenocarcinoma cell line), differing in their differentiation pattern and metastatic potency. Yet in both studies devoted to the comparison of the expression and characteristics of the integrins composed of α_6, β_4 and β_1 subunits, the possibility cannot be excluded that non-integrin molecules acting in cell-substratum or cell-cell recognition are additionally involved in modification of cell behavior. This possibility is supported by the observation that in human colon adenocar-
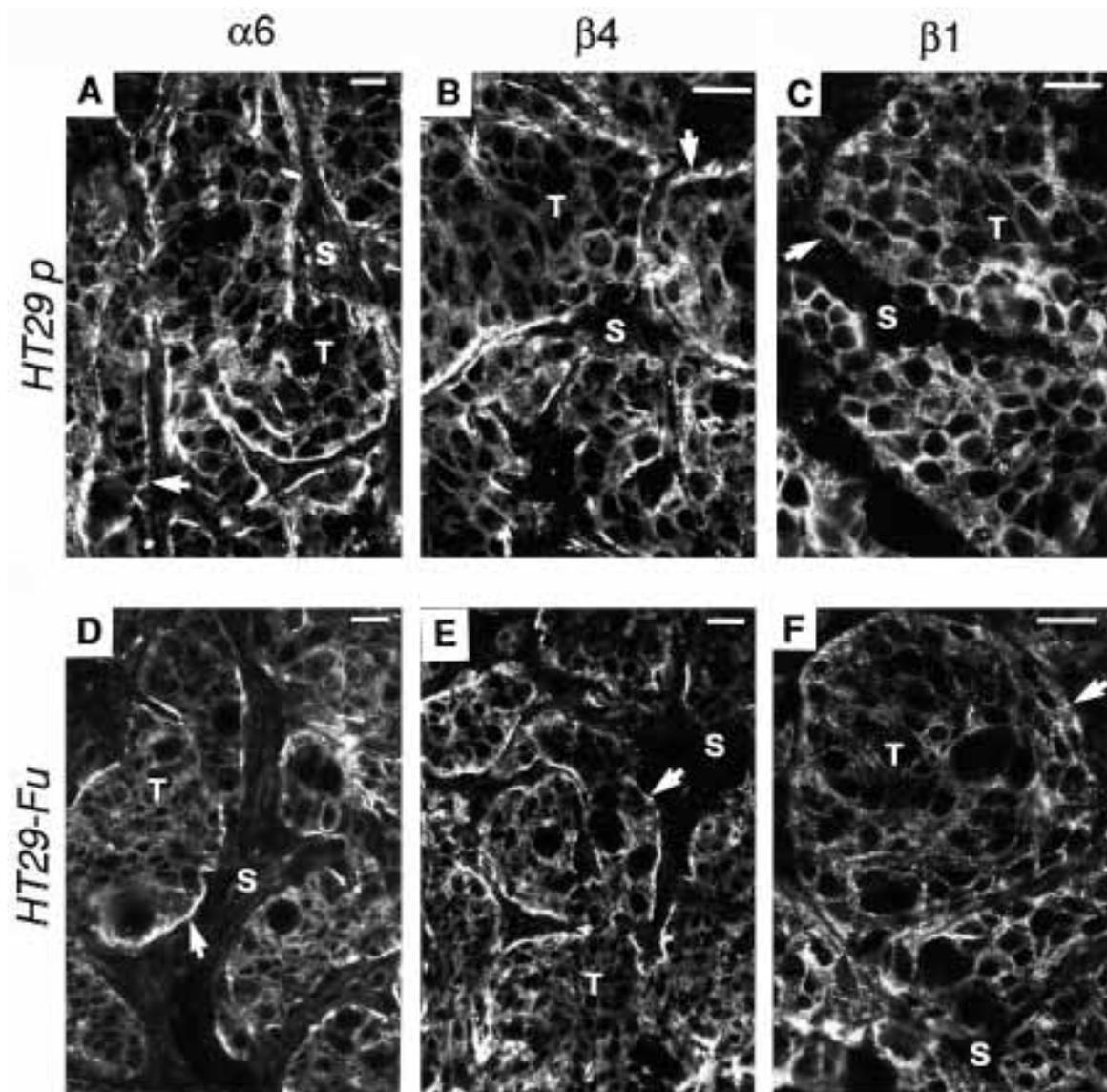
Fig. 9. Immunolocalization of α_6 (A,D) β_4, (B,E) and β_1 (C,F) integrin subunits with anti-α_6 mAb GoH3, anti-β_4 mAb 439-9B and anti-β_1 mAb AIIB2, respectively, in HT29p (A-C) and in HT29-Fu (D-F) tumors developed in nude mice. S, stroma; T, tumor cells; arrows, stroma/tumor cell interface. Bars, 20 μm.

cinomas, an increased expression of the 32/67 kDa laminin receptor (Mafune et al., 1990; Cioce et al., 1991) is obvious, while some integrin subunits are down-regulated in the most poorly differentiated carcinomas (Pignatelli et al., 1990; Koretz et al., 1991; Stallmach et al., 1992).

It is worth noting that the different spreading properties of the two HT29 cell lines can be correlated with altered epithelium-stroma interactions in the parental cells. Indeed, in cocultures of HT29p cells seeded on a confluent monolayer of fibroblasts, there is no deposition of basement membrane molecules at the heterologous cell interface (Bouziges et al., 1991), in contrast to those comprising HT29-Fu cells (unpublished observations). These basement membrane molecules have been shown previously to be produced in a complementary manner and with a precise chronology by both epithelial and stromal cell populations (for references see Simon-Assmann and Kedinger, 1993). In addition, the organization of

these molecules requires actual contact between the cell types. Thus, the altered heterologous cell contact and basement membrane deposition could be linked to a modified expression of integrins; such a relationship has been emphasized in several studies (for a review see Albelda, 1993). As a result of these deficiencies, it is interesting to note that subcutaneous grafting in immunodeprived newborn rats of the parental HT29 cell line led to the development of lung metastasis, which could not be detected in the case of the grafted Fu cells (unpublished observations).

Altogether, these data indicate that, although the overall expression of the integrins studied is similar in the two populations of HT29 cells, the difference in their spreading on laminin could be linked to modifications in the molecular form of the β_4 subunits and to a differential segregation of the α_6, β_4 and β_1 integrin subunits. Whether these changes are responsible for the altered epithelium-stroma cell interactions and

thus for the invasion potential of the parental cell line remains to be analyzed.

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