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Role of αvβ5 and αvβ6 Integrin Glycosylation in the Adhesion of a Colonic Adenocarcinoma Cell Line (HT29-D4)

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Abstract We have previously characterized the expression of the αvβ5 and αvβ6 integrins as major receptors for the human colonic adenocarcinoma cell line (HT29-D4), on vitronectin and fibronectin, respectively [Lehmann et al. (1994): Cancer Res 54:2102–2107]. In the present work we investigated the glycosylation role of these integrins in their adhesive functions. To this end, we used glycohydrolases to show that cell surface integrins were N-glycosylated and sialylated, and that only the αv subunit carried some immature oligosaccharide side chains. To alter the glycosylation state of the cell surface αvβ5 and αvβ6 integrins, we used two oligosaccharide-processing inhibitors: 1-deoxymannojirimycin (dMNJ) and tunicamycin (TM). Following treatment of HT29-D4 cells with dMNJ, cell surface αvβ5 and αvβ6 carried only high-mannose-type sugar chains, while TM-treated cells expressed de-N-glycosylated integrins. Neither α/β heterodimers assembly nor cell surface expression were impaired in the presence of the drugs. Finally, we established that adhesion of dMNJ- or TM-treated cells was altered on both vitronectin and fibronectin substrata, whereas the adhesion of these cells on laminin or collagen type I was virtually unchanged.

Key words: integrins, glycosylation, adhesion, colon, adenocarcinoma

Integrins are a family of integral membrane receptors mediating cell–cell and cell–substratum interactions. Integrin-regulated cell adhesion and migration are likely to be of particular importance in pathological events, such as wound healing, cancer growth, and metastasis. Integrins are noncovalent heterodimeric transmembrane glycoproteins consisting of two subunits (α and β) [Hynes, 1992]. The 16 α and eight β subunits identified combine to form at least 20 different receptors with a large diversity in the cell-type distribution, ligand specificity, and cell signaling mechanisms [Hynes and Lander, 1992; Juliano and Haskill, 1993]. The αv-containing integrin family is constituted to date of five members: αvβ1, αvβ3, αvβ5, αvβ6, and αvβ8 [Moyle et al., 1991]. Initially described as the vitronectin receptor [Pytela et al., 1985], αvβ3 displays a large spectrum of ligand recognition (fibronectin, vitronectin, fibrinogen, osteopontin, von Willebrand's factor . . .). αvβ1 has a more restricted ligand specificity, i.e., fibronectin or vitronectin [Bodary and McLean, 1990; Marshall et al., 1991; Vogel et al., 1990], while αvβ5 and αvβ6 recognize only vitronectin and fibronectin, respectively [Busk et al., 1992; Smith et al., 1990].

All αv integrins mediate their functions by interacting with the Arg-Gly-Asp consensus sequence of the adhesive proteins. The αv subunit is post-translationally cleaved into two polypeptides linked by a disulfide bond. The heavy chain (HC) (Mr 120,000) is probably involved in ligand recognition, while the light chain (LC) (Mr 30,000) supports the membrane anchor of αv and its connection to the cytoplasm. Amino acid sequence of αv subunit revealed 13 potential Asn-linked glycosylation sites; 10 are located on the HC and three on the LC [Susuki et al., 1987].

Abbreviations used: BSA, bovine serum albumin; Endo H, endoglycosidase H; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, Dulbecco's phosphate buffered saline; PNGase F, peptide N-glycanase F; SEM, standard error.

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The importance of glycosylation for α and β integrin function has been extensively studied, essentially for the very late activation antigen (VLA) integrin family [for review, see Akiyama, 1992]. This was particularly well illustrated for β1 integrin functions during cell ontogeny [Wadsworth et al., 1993] and melanoma cells metastasis [Kawano et al., 1993; öz et al., 1989]. Few reports have also described the glycosylation state of the human vitronectin receptor αvβ3 [Polack et al., 1989; Troesch et al., 1990] and the αIIbβ3 integrins [Frachet et al., 1992]. Despite these reports, the role of the N-glycosylation on α and β subunit assembly and integrin function is not completely understood, especially for the αv-containing integrin family [Akiyama, 1992]. A well established approach for studying the plasma membrane protein glycosylation is the use of specific inhibitors of carbohydrate-processing enzymes. This strategy was also employed for the studies of integrin glycosylation [Akiyama et al., 1989; Chammas et al., 1991, 1993; Spiro et al., 1989].

Using the monoclonal antibody 69-6-5 designed by our group, we have previously shown that HT29-D4 cells (a colonic adenocarcinoma cell line) adhere to vitronectin- or fibronectin-coated substratum via αvβ5 and αvβ6 integrins [Lehmann et al., 1994]. The aim of the present work was to determine the role of αvβ5 and αvβ6 glycosylation in α/β assembly, cell surface expression, and cell adhesion on vitronectin and fibronectin substrata. To this end, we used the mannose analog 1-deoxymannojirimycin (dMNJ), a Golgi α-mannosidase inhibitor. dMNJ blocks the trimming of the first mannose residues from N-linked high-mannose chains, and thereby inhibits the addition of terminal sugars which are needed to form complex-type oligosaccharide structures. In order to inhibit the glycosylation of the integrins we used the antibiotic tunicamycin (TM), which prevents the formation of the first lipid-linked oligosaccharide intermediate GlcNAc-P-P-dolichol. Our data evidenced that αv, β5, and β6 subunits are N-glycosylated, and that carbohydrates carried by these receptors play a crucial role in cell adhesion functions.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Grand Island, NY) and Dulbecco's PBS[3] was from Oxoid, Ltd. (Basingstoke, United Kingdom). Fetal calf serum was from Sera-Lab (Crawley Down, United Kingdom). BSA and lactoperoxidase were purchased from Sigma Chemical Co. (La Verpillière, France), as were monosaccharides and concanavalin A-agarose. Tunicamycin and Endo H were purchased from Boehringer (Mannheim, Germany) and deoxymannojirimycin was obtained from Genzyme (Boston, MA). PNGase F was prepared from *Flavobacterium meningosepticum* and was kindly provided by Dr. Karamanos (Limoges, France). Reagents for SDS-PAGE were from Bio-Rad (Richmond, CA). Sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC biotin) was obtained from Pierce (Rochford, IL). ^{125}I-streptavidin (40 μci/μg), Na[^{125}I], ^{125}I-protein A, and D-[2-^{3}H]-mannose (10–20 Ci/*mmol*) were from Amersham (Les Ullis, France). ^{3}H-thymidine (50 mci/mmol) was from I.C.N. (Orsay, France). EHS mouse laminin and human fibronectin were from Sigma. Rat-tail collagen type I was from Calbiochem (Meudon, France). Vitronectin was prepared according to Yatogho [Yatogho et al., 1988]. Anti-β6 polyclonal antibodies 205 and 206 were a gift of Dr. Quaranta (The Scripp Research Institute, La Jolla, CA), and the anti-β5 polyclonal antibodies were gifts of Dr. Hemler (Dana-Farber Cancer Institute, Cambridge, MA) and Dr. Reichardt (University of California, San Francisco). Rat mAb 69-6-5, directed against the αv integrins, was prepared as described [Lehmann et al., 1994]. Anti-β1 mAb (clone K20), anti-αv mAb (clone AMF7), and anti-α6 mAb (clone GOH3) were from Immunotech (Marseille, France). Anti-α3 mAb (clone P1B5) was from Telios Pharmaceutical (San Diego, CA).

Cell Culture

The human colon adenocarcinoma cell line HT29-D4 was routinely cultured as described [Fantini et al., 1986]. For inhibitor treatment, 2 days after seeding, cells were cultured for 3 days in medium in the absence or presence of either 100 μg/ml deoxymannojirimycin or 1 μg/ml tunicamycin. Cell culture media were changed daily. As already described [El Battari et al., 1986; Ogier-Denis et al., 1990], under these conditions dMNJ and TM induced 20% and 40% inhibition of cell growth, respectively, with no cell mortality as determined by Trypan blue exclusion test.

Cell Surface Labeling

Iodination. HT29-D4 cells were surface radioiodinated in the presence of lactoperoxidase [Lehmann et al., 1994].

Biotinylation. Cell surface proteins were biotinylated as described by Garcia and coworkers [Garcia et al., 1991], except that cells were labeled in suspension.

Immunoprecipitation. Labeled cells were lysed in Triton X-100 and extracted proteins were immunoprecipitated using the 69-6-5 mAb (iodinated proteins) or anti-β5 and anti-β6 polyclonal antibodies (biotinylated proteins), as previously described [Lehmann et al., 1994].

Glycosidase Treatments of Denatured Proteins

^{125}I-labeled or biotinylated immunoprecipitated proteins were solubilized with 1% SDS. Soluble proteins were digested either at 37°C by 20 mU/ml PNGase F during 120 min in PNGase F buffer (Tris-HCl buffer, pH 8.6, containing 1% Triton X-100, 0.1% β-mercaptoethanol, 0.25% SDS, and 10 mM orthophenanthrolin) or by 10 mU/ml Endo H for 6 hr at 37°C in Endo H buffer (50 mM citrate-phosphate buffer, pH 5.5, containing 1% Triton X-100, 0.1% β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). Neuraminidase treatment was performed in the presence of 100 mU of the enzyme for 2 hr in Endo H buffer. Reactions were stopped by addition of SDS-PAGE sample buffer 5×, and proteins were analyzed in SDS-PAGE under reducing conditions according to Laemmli [1970].

Slab gels containing ^{125}I-proteins were dried and exposed to Fuji X-ray film with an intensifying screen at −80°C. Biotinylated proteins were transferred onto nitro-cellulose membranes, Hybond C+ (Amersham), according to the supplier instructions. Mr markers were labeled with 1% Ponceau red in 0.2% trichloroacetic acid. Membranes were then blocked with a 0.1 M Tris-buffered solution, pH 8.2, containing 1% BSA and 0.2 M NaCl, and incubated in the presence of 5×10^5 cpm/ml of ^{125}I-streptavidin in the same buffer for 1 hr. Membranes were dried and exposed to Fuji X-ray film with an intensifying screen at −80°C.

Immunoblotting

αv integrins from 2×10^8 cells were immunoprecipitated with 69-6-5 mAb as described above. After separation on 7.5% SDS-PAGE, the proteins were transferred on nitrocellulose membrane. β6 subunits were identified using the anti-β6 polyclonal antibody and ^{125}I-protein A (3×10^5 cpm/ml).

^{3}H-Mannose Labeling and Concanavalin A Chromatography

HT29-D4 cells were labeled using ^{3}H-mannose, and labeled oligosaccharides were prepared and analyzed essentially as described [El Battari et al., 1986].

Flow Cytometry Analysis of Cell Surface Expression of Integrin Subunits

Cell surface expression of αv and β1 integrin subunits in HT29-D4 cells was determined by flow cytometry. Cells were harvested after a 3 day drug treatment and resuspended in medium containing 1% BSA. The single-cell suspension (10^6 cells/ml) was incubated for 90 min at 4°C in the presence of anti-integrin mAbs or non-immune immunoglobulin (10 μg/ml each). Cells were rinsed four times with ice-cold PBS containing 1% BSA, and were then incubated for 45 min at 4°C with excess of appropriate secondary FITC-conjugated anti-mouse or anti-rat antibodies. After washing, cells were fixed in 1% paraformaldehyde and cell bound fluorescence was analyzed by injection on an EPICS flow cytometry device. Gating parameters were settled so that 90% of injected cells were analyzed. Results are expressed as the mean of fluorescence ± SD in arbitrary units.

Cell Adhesion Assay

Cell adhesion assays were performed as described [Lehmann et al., 1994]. Briefly, 96-well flat-bottom microtiter plates were coated for 1 hr at 37°C with fibronectin, vitronectin, laminin, or collagen type I at indicated concentration, or PBS-1% BSA (negative control). Eighteen hours before adhesion assays, 20 μCi/ml of ^{3}H-thymidine was added to the culture medium already containing drugs. Cell adhesion assays were performed in DMEM containing 0.2% BSA. In all assays, whatever the culture conditions, cell mortality did not exceed 5%. Nonspecific cell adhesion on BSA-coated wells was generally below 0.5% of input radioactivity, and was subtracted as background. Assays were performed in triplicate; calculated SEM was routinely below 5%.

Neuraminidase Treatment of Intact Cells

^{3}H-thymidine-labeled HT29-D4 cells were detached as described and incubated for 30 min at 37°C in the presence of neuraminidase (0.2 U/ml) in PBS-Ca^{2+} 0.1 mM/Mg^{2+} 1 mM. Cells were washed three times before adhesion assays.

RESULTS

Biochemical Characterization of αvβ5 and αvβ6 Glycosylation

HT29-D4 cells were surface labeled and proteins from detergent extracts were immunoprecipitated with the 69-6-5 mAb. As recently described [Lehmann et al., 1994], under reducing conditions the 69-6-5 mAb immunoprecipitated three polypeptides displaying Mr of, respectively, 125,000 (a mixture of αv-HC and β6 subunits), 98,000 (β5 subunit), and 30,000 (αv-LC) (Fig. 1, lane 1).

To check for the presence of oligosaccharides N-linked to the αv, β5, and β6 integrin subunits, immunoprecipitated material was treated with three different glycohydrolases: PNGase F, Endo H, or neuraminidase (Fig. 1).

PNGase F treatment of αv integrins generated three polypeptides of Mr 94,000, 80,000, and 20,000, all displaying a higher electrophoretic mobility compared to control (Fig. 1, lanes 1, 2). As shown in lanes 6 and 7 of Figure 1, the Mr 80,000 polypeptide, recovered after PNGase F treatment, corresponded to the de-N-glycosylated β5 subunit, whereas the Mr 94,000 band represented the de-N-glycosylated αv-HC. Anti-β6 antibody immunoprecipitated a single broad band of Mr 115,000–125,000, shifting to Mr 95,000–100,000 after PNGase F digestion (Fig. 1, lanes 9, 10). Under our experimental conditions, the β6 and αv-HC subunits comigrated regardless of their glycosylation state. Finally, as with the 69-6-5 mAb, the αv-LC subunit (Mr 30,000), recovered with either the anti-β5 or anti-β6 antibody, was converted by PNGase F to the Mr 20,000 protein (data not shown). Following Endo H treatment, the molecular weights of the αv-HC and αv-LC were decreased by 4,000 and 5,000, respectively, whereas β5 and β6 subunits remained Endo H resistant (Fig. 1, lanes 3, 8, 11). Neuraminidase-treated αv (heavy and light chains) and β5 and

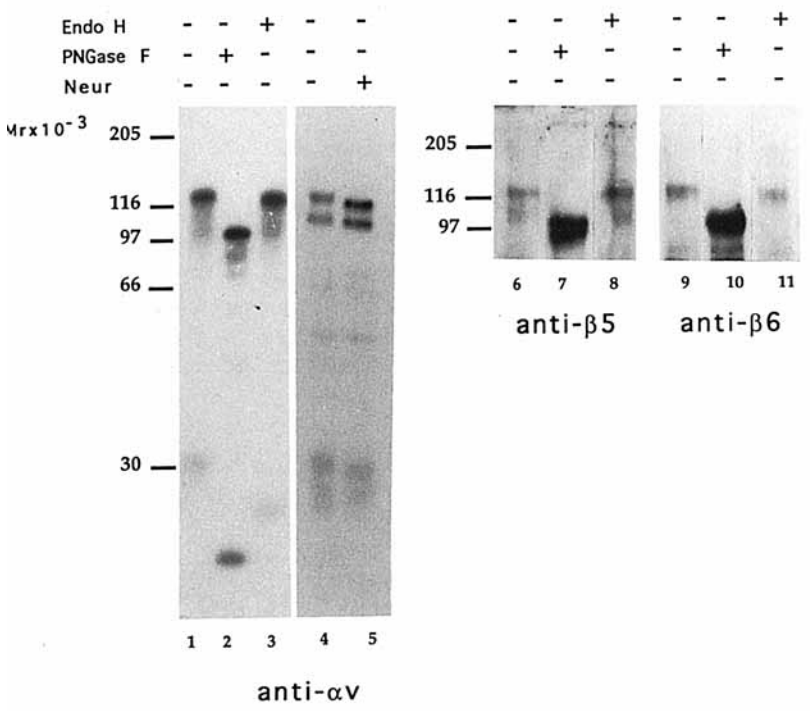

Fig. 1. Glycohydrolase digestion of αvβ5 and αvβ6 integrins from HT29-D4 cells. Labeled cell surface proteins were immunoprecipitated with 69-6-5 anti-αv mAb, anti-β5, or anti-β6 antibodies. Integrins were incubated with Endo H, PNGase F, or neuraminidase (Neur) as indicated. Labeled integrins were then analyzed by SDS-PAGE and revealed as described in Materials and Methods. Positions of molecular size standards are shown on the left side of the autoradiogram.

β6 integrins moved slightly faster on SDS-PAGE (Fig. 1, lanes 4, 5). Taken together, these results indicate that αvβ5 and αvβ6 integrins from HT29-D4 cells contained sialylated complex carbohydrates, and that only the αv subunit carried high-mannose oligosaccharides.

Concanavalin A Chromatography of ^{3}H-Mannose-Labeled Oligosaccharides

Prior to immunoprecipitation experiments, we controlled whether dMNJ completely or partially inhibited carbohydrate processing in HT29-D4 cells. Control and dMNJ-treated cells were first metabolically labeled with ^{3}H-mannose, and labeled oligosaccharides were analyzed by concanavalin A-agarose chromatography. Typically, fractionation of oligosaccharides on a concanavalin A-agarose column gives three peaks. The flowthrough fraction consists essentially of tri- and tetra-antennary complex-type sugars. The weakly adsorbed fraction, eluted by 10 mM α-methylglucoside, is exclusively composed of biantennary structures. Finally, the tightly associated fraction, eluted with 0.5 M α-methylmannoside, is a mixture of hybrid and high-mannose-type glycoproteins. Results presented in Figure 2 revealed that the distribution of ^{3}H-mannose-labeled oligosaccharides in flowthrough (FI), α-methylglucoside (FII), and α-methylmannoside (FIII) fractions greatly differed between control and dMNJ-treated cells. In the presence of dMNJ, almost all the radioactivity was found in the tightly bound fractions (FIII). This result demonstrated that, as expected, most of the glycoproteins from dMNJ-treated cells are of the high-mannose type. Similar results were obtained when concanavalin A-agarose chromatography was performed on cell surface radioiodinated protein extracts (data not shown).

Structural Effects of Deoxymannojirimycin and Tunicamycin on Cell Surface Integrins

We then evaluated the structural consequences induced by the action of the inhibitors dMNJ and TM. HT29-D4 cells were pretreated for 3 days in the presence of dMNJ (100 μg/ml) or TM (1 μg/ml), conditions which inhibited cell growth by 20% and 40%, respectively, but which did not induce any cell mortality. Cell surface labeled proteins were then immunoprecipitated with 69-6-5 mAb, anti-β5, or anti-β6 polyclonal antibodies.

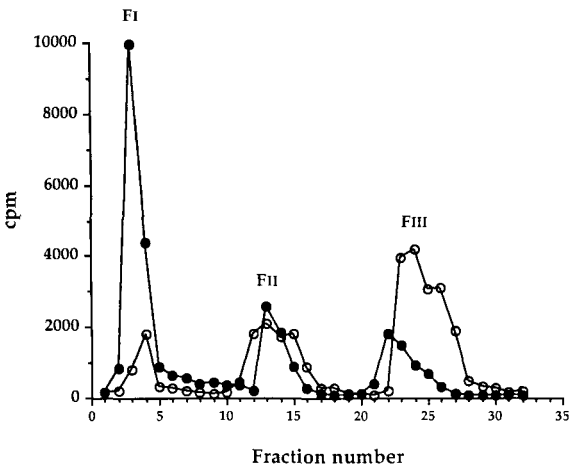

Fig. 2. Effect of dMNJ on mannose incorporation in HT29-D4 cells. HT29-D4 cells were labeled with ^{3}H-mannose in the absence (●) or in the presence (○) of 100 μg/ml of dMNJ. ^{3}H-mannose-labeled oligosaccharides were obtained and submitted to concanavalin A-agarose chromatography as described in Materials and Methods. FI, flowthrough; FII, elution with 10 mM α-methylglucoside; FIII, elution with 10 mM α-methylglucosid; FIII, elution with 0.5 M α-methylmannoside.

As depicted in Figure 3, lane 2, after dMNJ treatment the mixed αv-HC/β6 chains and the β5 subunit immunoprecipitated by the 69-6-5 mab possessed, respectively, Mr of 108,000 and 90,000. Moreover, the αv-LC appeared as a doublet of Mr 30,000 and 27,000. As shown in lane 3, no difference between the PNGase F-de-N-glycosylated integrins extracted from treated or untreated cells was detected, indicating that dMNJ did not affect the polypeptide chain synthesis. In agreement with the action of dMNJ, leading to the accumulation of high-mannose-containing proteins, αv integrins of dMNJ-treated cells were fully Endo H sensitive (Fig. 3, lane 4). Indeed, they behaved similarly during electrophoresis after either Endo H or PNGase F hydrolysis. The slight difference in the distance of migration obtained between the two treatments could be due to the presence of N-acetylglucosamine residues remaining after Endo H hydrolysis. Anti-β5 antibody immunoprecipitated immature αv (Mr 108,000) and β5 (Mr 90,000) subunits, converted into N-glycan-free proteins by Endo H or PNGase F (Fig. 3, lanes 5–7). We also precipitated immature Endo H–sensitive β6 chains with anti-β6 antibody (Fig. 3, lanes 8–10). The αv-LC doublet was observed whatever the antibody used (data not shown).

Data obtained from TM-treated cells are shown in Figure 4A. The 69-6-5 antibody immu-

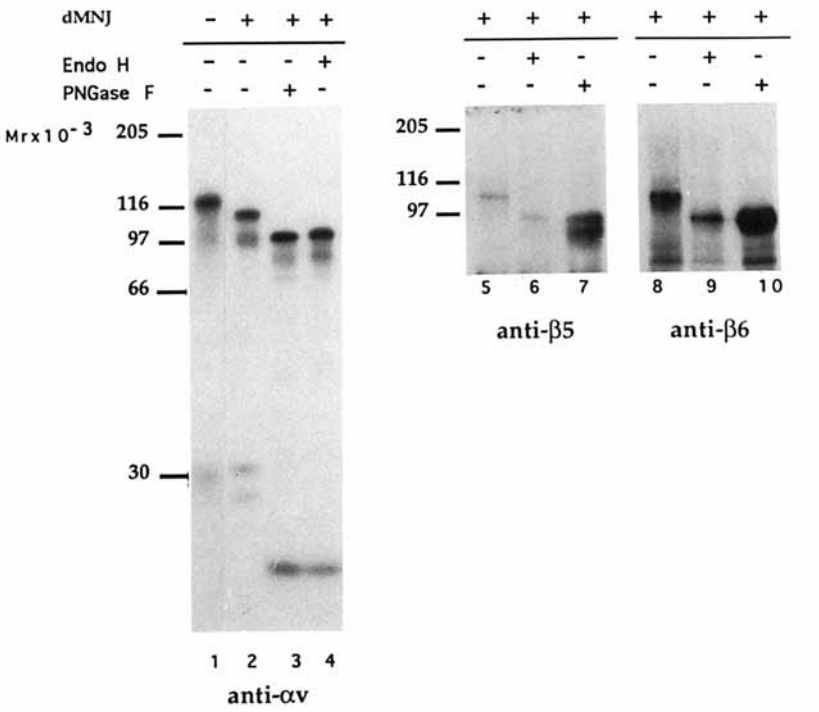

Fig. 3. Cell surface labeling of αvβ5 and αvβ6 integrins after dMNJ treatment. HT29-D4 cells were incubated for 3 days in DMEM containing dMNJ (100 μg/ml). Labeled cell surface proteins were immunoprecipitated with 69-6-5 anti-αv mAb, anti-β5, or anti-β6 antibodies. Immunopurified integrins were digested as indicated by Endo H or PNGase. Labeled integrins were then analyzed by SDS-PAGE and revealed as described in Materials and Methods. The position of the molecular weight marker proteins (Mr × 10^{-3}) is indicated on the side of each autoradiogram.

noprecipitated three polypeptides of Mr 99,000, 81,000 and 25,000 (Fig. 4, lane 1). The Mr 99,000 (αv-HC) and the Mr 81,000 (β5) polypeptides were virtually unaffected by a subsequent PNGase F treatment, indicating that TM generated N-glycan-free αv-HC and β5 subunits. On the contrary, TM treatment induced the expression of an Mr 25,000 αv-LC, which, as shown by glycohydrolase treatments, contained complex (Endo H–resistant) sugars. When an anti-β5 antibody was used instead of the 69-6-5 mAb, similar data were obtained, i.e., the cell surface expression of de-N-glycosylated αv-HC and β5 subunits (Fig. 4A, lanes 4, 5). Also on the contrary, patterns generated from the anti-β6 immunoprecipitation of the TM-treated cells were quite different (Fig. 4A, lanes 6, 7). Two polypeptides, of Mr 115,000 and 100,000, were obtained; the latter could be related to the αv integrins. To identify the second band, we performed immunoblotting experiments on control and TM-treated cells. Cell lysates were immunoprecipitated with the 69-6-5 mAb, resolved on SDS-PAGE, and proteins were revealed using an anti-β6 antibody. As shown in Figure 4B, β6 chain from control cells appeared as a single polypeptide (Mr 116,000). After TM treatment, we revealed two polypeptides, of Mr 116,000 and 104,000, suggesting that under these conditions the β6 subunit coexisted under a native (Mr 116,000) and a de-N-glycosylated form (Mr 104,000). This result was confirmed by the total conversion of the native form to an N-glycan-free β6 subunit after PNGase F treatment (Fig. 4B, lane 3).

Considering that the immunoprecipitated material derived from cell surface labeling, these results demonstrate that de-N-glycosylated αv integrins of TM-treated cells were effectively expressed at the cell surface. Interestingly, when such TM deglycosylation experiments were performed on another cell line, the human melanoma cells IGR39, a very small amount of only native αv integrins was found at the cell surface (data not shown).

Adhesion Properties of HT29-D4 Cells Treated With dMNJ or TM

The extent of cell surface expression of partially or totally de-N-glycosylated integrins is a

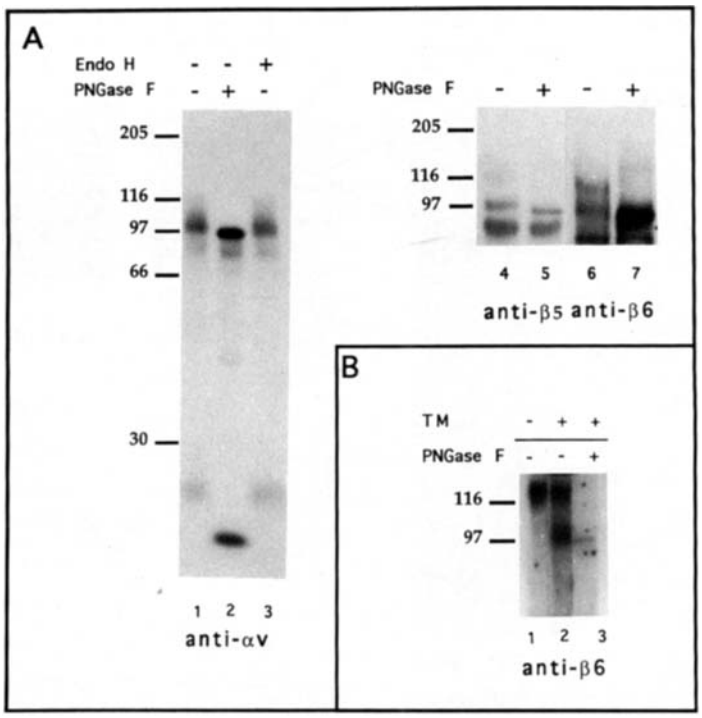

Fig. 4. Structural characterization of αvβ5 and αvβ6 integrins after TM treatment. **A:** TM-treated HT29-D4 cells were surface labeled and total protein extract was immunoprecipitated with 69-6-5 anti-αv mAb and anti-β5 or anti-β6 antibodies. Integrins were then digested by Endo H or PNGase F as indicated. Proteins were analyzed on SDS-PAGE and revealed as described in Materials and Methods. **B:** Cells were treated or not for 3 days with 1 μg/ml TM. αv integrin from crude plasma membrane was immunopurified with 69-6-5 mAb and separated on 7.5% SDS-PAGE. Integrins, digested or not by PNGase F, were blotted onto nitrocellulose sheets. β6 subunit was revealed with rabbit anti-β6 polyclonal antibody and ^{125}I-protein A. Positions of molecular size standards are shown on the left of the autoradiogram.

crucial point for the study of cell adhesion. The surface expression of αv and β1 integrin subunits of treated or untreated HT29-D4 cells was compared by flow cytometry. As shown in Figure 5, the binding level of two anti-αv antibodies, 69-6-5 and AMF7, was not affected by the drugs. Similar data were obtained with anti-β1, -α3, and α6 mAbs. These results indicate that integrins were fully expressed at the cell surface regardless of culture conditions. Moreover, it appears that alterations of αv integrin N-glycosylation did not impair their cell surface targeting.

The ligand binding properties of native and immature αvβ5 and αvβ6 integrins were preliminary compared by cell adhesion experiments. To this end, the adhesion properties of treated or untreated HT29-D4 cells were studied in detail by dose–response experiments on four different ECM proteins (vitronectin, fibronectin, laminin, and collagen I). Figure 6 shows that the efficiency of laminin and collagen I in supporting attachment was quite similar in both cell culture conditions. The situation was different in

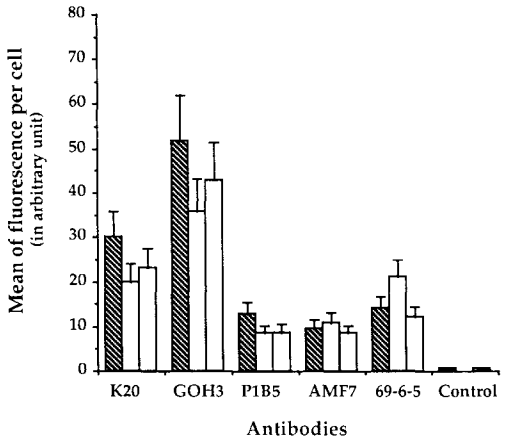

Fig. 5. Comparative studies of integrin subunits cell surface expression between control, dMNJ- and TM-treated HT29-D4 cells. HT29-D4 cells were cultured for 3 days in the absence (▨) or the presence of dMNJ (*stippled*) or TM (*white*). Integrin cell surface expressions were compared by flow cytometry analysis as described in Materials and Methods using anti-αv (AMF7 and 69-6-5), anti-β1 (K20), anti-α3 (P1B5), and anti-α6 (GOH3) mAbs, or an irrelevant antibody (control). Results are expressed as mean of fluorescence per cell in arbitrary units ±SEM.

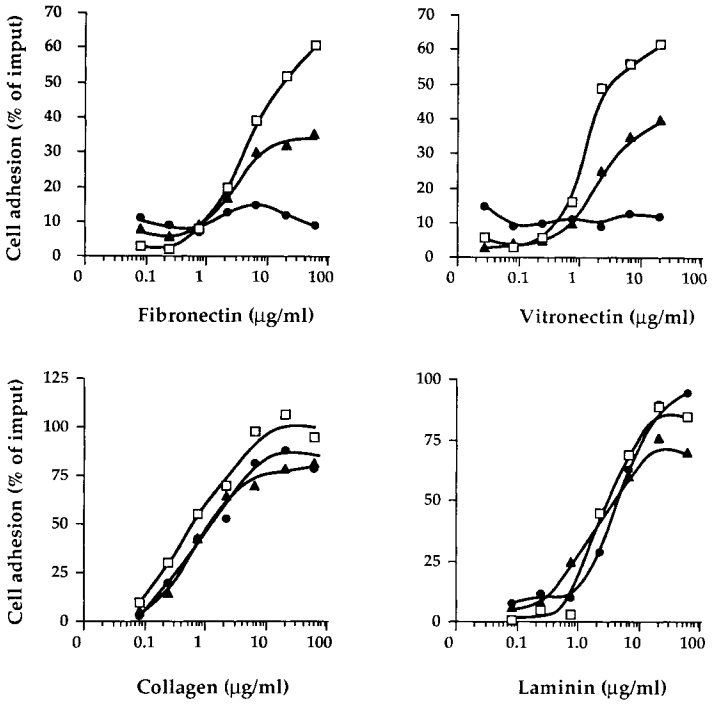

Fig. 6. Dose–response attachment assays of dMNJ- or TM-treated HT29-D4 cells to ECM proteins. Control (□), dMNJ- (▲), and TM-treated (●) cells were labeled with ^{3}H-thymidine, and seeded in plastic wells previously coated with vitronectin, fibronectin, laminin, or collagen I at indicated concentrations. After incubation (90 min, 37°C) and washing of unattached cells, the radioactivity in the lysates of attached cells was counted and is expressed as a percentage of radioactivity relative to input cells. Presented data are the more representative of three different experiments done in triplicated wells (SD below 5%).

the case of cell attachment to vitronectin and fibronectin, the two substrata of αvβ5 and αvβ6 [Lehmann et al., 1994]. Dose–response experiments showed that adhesion to these ECM proteins was weakest in TM-treated cells, and in dMNJ-treated cells than in control cells. We confirmed these results by several single-dose experiments at plateau concentration (i.e., vitronectin, 20 μg/ml, and fibronectin, 50 μg/ml). TM and dMNJ induced highly significant inhibition on vitronectin (TM, 89 ± 5%; dMNJ, 49 ± 6%) and fibronectin (TM, 91 ± 2%; dMNJ, 39 ± 4%).

Effect of Neuraminidase Treatment on HT29-D4 Cells' Adhesiveness

Given that HT29-D4 cell surface αv integrin were sialylated (Fig. 1, lane 5), we evaluated the role of αvβ5 and αvβ6 sialic acid by performing adhesion assays after neuraminidase treatment of intact cells. As shown in Figure 7A, adhesion on either collagen I, laminin, vitronectin, or fibronectin was not affected at all, indicating that terminal acid sialic residues were not involved in the adhesion of HT29-D4 cells to these extracellular matrix components. As a control, Figure 7B shows the pattern of integrins immunoprecipitated with 69-6-5 mAb from neuraminidase-treated cells. The slight shift of the Mr 120,000 and 95,000 bands indicated the efficiency of neuraminidase digestion.

DISCUSSION

We have recently described that the human colonic adenocarcinoma cell line HT29-D4 adheres on vitronectin and fibronectin in an RGD-dependent manner through the αvβ5 and αvβ6 integrins, respectively. In the present study, we reported that the N-glycosylation of these two integrins was required neither for subunit association nor for cell surface expression, but played a crucial role in their cell adhesive function.

The N-glycosylation state and the oligosaccharide processing of αvβ3 and the closely related αIIbβ3 integrins have been clearly reported. The α and β subunits are synthesized from two different mRNAs in the endoplasmic reticulum. The oligosaccharide cores are cotranslationally added to the Asn residues of nascent proteins. The association of the α and β subunits and the

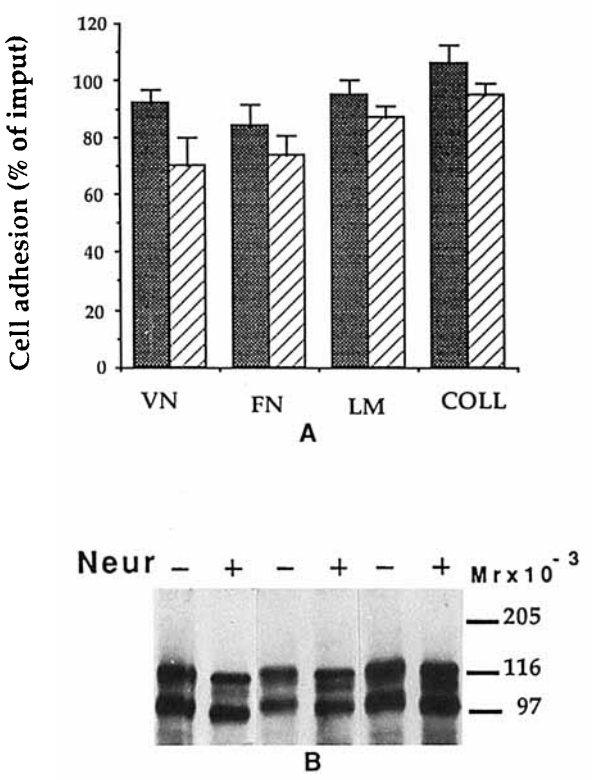

Fig. 7. Effect of cell surface neuraminidase treatment on HT29-D4 cell adhesion. **A:** Metabolically labeled HT29-D4 cells were treated (□) or not (▨) for 30 min with neuraminidase, and seeded on culture wells coated with various extracellular matrix proteins: fibronectin (FN) (50 μg/ml), vitronectin (VN) (20 μg/ml), collagen I (COLL) (100 μg/ml), and laminin (LM) (25 μg/ml) as described in Materials and Methods. The results are the average of three independent experiments ±SEM. **B:** From the batch of cells used for adhesion experiments described above, 0.5–1 × 10^7 cells were removed and cell surface labeled with NHS-biotin. Extracted proteins were immunoprecipitated with the 69-6-5 mAb, separated on 10% SDS-PAGE, blotted on nitrocellulose, and revealed as described in Materials and Methods. Molecular weight standards are shown on the right of the autoradiogram.

initial trimming of oligosaccharide chains likely occur in a pre-Golgi compartment. Integrins are then transferred to the Golgi apparatus where the α subunit is endoproteolytically cleaved and its sugar chains fully processed and sialylated, while the N-glycan chains of the β3 subunit remain Endo H sensitive [Cheresh and Spiro, 1987; Polack et al., 1989; Spiro et al., 1989; Troesch et al., 1990].

Here, we have established that the αv, β5, and β6 subunits expressed on HT29-D4 cells were also highly N-glycosylated. The Mr of PNGase F-de-N-glycosylated αv and β5 subunits were in good agreement with the molecular weight calculated from their amino acid sequences [McLean et al., 1990; Susuki et al., 1987]. In the case of β6 subunit, a discrepancy was observed [Sheppard et al., 1990], indicating that this protein could be O-glycosylated, in opposition to αv and β5 proteins, which carried exclusively N-linked oligosaccharides.

Endo H sensitivity of cell surface αvβ5 and αvβ6 integrins indicates that their oligosaccharide processing was quite different from those of the αvβ3 and αIIbβ3 integrins. On HT29-D4 cells, the mature β5 and β6 subunits carried only complex oligosaccharide chains, while the αv carried mixed carbohydrate chains of high-mannose and complex type. This discrepancy could be due to intrinsic differences between oligosaccharide processing according to the cell line, suggesting that the Endo H sensitivity of mature cell surface αv integrins might be related to a cell lineage specificity. Alternatively, the glycosylation state of αv and β subunits could be directly related to the nature of the β subunits, that differently contribute to the conformation of the heterodimers.

In order to understand the relationship between carbohydrate structure and cell adhesion properties, we have investigated the effect of dMNJ and TM, two inhibitors of the N-glycosylation pathway.

Recent works have shown that dMNJ effectively inhibits carbohydrate maturation of integrins without quantitatively affecting their cell surface expression [Akiyama et al., 1989; Spiro et al., 1989; Troesch et al., 1990; v Lampe et al., 1993]. Besides, these results indicate that neither the α/β association nor their exportation to the plasma membrane were delayed by such an altered N-glycosylation. We show here that dMNJ-treated HT29-D4 cells expressed at their cell surface the expected fully Endo H–sensitive αvβ5 and αvβ6 integrins at the same extent as untreated cells, as shown by flow cytometry analysis.

TM blocks the cotranslational addition of N-linked carbohydrates to nascent polypeptides. On many cell lines, the effect of TM results in a dramatic inhibition of cell surface expression of integrins. Indeed, Polack and coworkers [Polack et al., 1989] reported that TM prevents the association of αv and β3 subunits in endothelial cells. We also found that the human melanoma cell line IGR39 failed to express de-N-glycosylated αv integrins after TM treatment (data not shown). Interestingly, we found that on TM-treated HT29-D4 cells, αvβ5 and αvβ6 integrins were expressed at the plasma membrane

at a similar extent as in control cells, as demonstrated by flow cytometry analysis. Under these conditions, the αv and β5 subunits were virtually unglycosylated. It is noteworthy that on TM-treated cells, the β6 subunit extracted from a total cell lysate was present under both glycosylated and unglycosylated forms. We have determined that at least the glycosylated form was effectively expressed in association with the αv subunit at the cell surface. We cannot exclude that the turnover of cell surface expression of the αvβ6 was longer than the time of TM treatment. It appears, from our experiments, that a default of N-linked oligosaccharide chain processing neither impaired the association nor the cell surface expression of αvβ5 and αvβ6 integrins.

Troesch and coworkers [Troesch et al., 1990] provided evidence of one complex carbohydrate chain of ≈ Mr 5,000 on the αv-LC from endothelial cells (HUVE cells). On HT29-D4 cells, the αv-LC Mr carried ≈ Mr 10,000 of N-linked carbohydrates, 50% of which were of high-mannose type and 50% of complex type. This discrepancy reflects the presence of additional high-mannose chain(s) on the αv-LC from HT29-D4 cells. Our study gave some additional data of interest concerning the αv-LC: (1) The αv-LC subunit from dMNJ-treated cells was expressed into a doublet of Mr 30,000 and 27,000. As both forms were converted by Endo H and PNGase F to a unique Mr 20,000 polypeptide, this doublet was not a result of a proteolysis. Similar data were obtained whatever the antibody used for the immunoprecipitation experiments. The αv-LC doublet was also detected on the human melanoma cell line IGR39 (data not shown). This suggests the existence of at least two glycoforms of the αv-LC, reflecting a possible heterogeneity of the endogenous αv population. Filardo [Filardo and Cheresh, 1994] recently reported the presence of such glycoforms on αv-transfected M21-L melanoma cells. (2) Under the action of TM, the αv-LC was expressed as a peptide carrying complex-type oligosaccharides. This result cannot be explained by a longer protein turnover (as supposed for β6) and supports that an alternative glycosylation pathway might exist on HT29-D4 cells, compensating in part the inhibition of N-linked oligosaccharide synthesis by TM of the αv-LC and/or β6 subunits. It remains to be determined if the oligosaccharide side chains of the αv-LC play a direct role in the association, protein maturation, cell surface expression, or function of the αv/β heterodimer.

The modulation of the glycosylation state of αvβ5 and αvβ6 integrins, induced by the drugs, allowed us to investigate the role of their oligosaccharide moieties in their cell adhesive function. Thus, in the first set of experiments, we assayed the direct adhesion of treated or untreated cells on extracellular matrix components, such as collagen type I, laminin, fibronectin, and vitronectin. In HT29-D4 cells, we have recently identified the αvβ5 and αvβ6 integrins as the main receptors for vitronectin and fibronectin [Lehmann et al., 1994], Whereas the cell receptors for laminin and collagen are α6β1/β4, α3β1, and α2β1 [v Lampe et al., 1993] (our unpublished data). It has been described that dMNJ induces divergent effects on the α5β1 integrin cell adhesion properties [Akiyama et al., 1989; Koyama and Hughes, 1992]. We demonstrated here that dMNJ inhibited both vitronectin- and fibronectin-mediated adhesion of HT29-D4 cells (≈ 50% and ≈ 40%, respectively). These results are partially consistent with those of v Lampe [v Lampe et al., 1993], who report that the parental cell line HT29 treated with dMNJ presented a reduced adhesion activity, not only on fibronectin, but also on laminin. The discrepancy of some of our data (especially for the reduced adhesion on laminin) with those obtained with the parental HT29 cell line could be related to the cell origin, i.e., heterogeneous or clonal cell population. TM had a more dramatic effect. TM treatment induced a quasi total inhibition of the HT29-D4 cell adhesiveness on vitronectin and fibronectin (90%). The inhibition induced by dMNJ and TM was not a consequence of a lowered cell surface expression of αv integrins. Indeed, no significant difference between treated and untreated cells could be observed in the cell surface binding activities of two anti-αv mAbs. Moreover, whatever the culture condition used, collagen I and laminin remained able to mediate HT29-D4 cell attachment, indicating that the cell adhesion inhibition was specific toward vitronectin and fibronectin. Although we have not controlled the drug effect on their structure, our results suggest that the oligossacharide moieties of the laminin and collagen receptors might not play an important role on their adhesive functions. Our observations are consistent with a crucial function of N-glycan core in the binding of αv-bearing integrins to their ligand.

We cannot exclude, however, that unexamined changes of accessory molecules could partici-

pate in the alteration of the HT29-D4 cell adhesive behavior induced by drug treatment. This is particularly the case for gangliosides, which are well known to modulate cell–cell and cell–matrix interaction and to regulate integrin function [Merzak et al., 1995; Zheng et al., 1993].

The desialylation of αvβ5/β6 integrins was found to have no effect on cell adhesion properties on any extracellular matrix tested. The role of sialic acid of the adhesive receptors has been extensively studied, but remains controversial [Acheson et al., 1991; Symington et al., 1989; v Lampe et al., 1993]. Neuraminidase treatment of two human colonic adenocarcinoma cells, HT29 and HCT116, does not impair adhesion on laminin but inhibits adhesion on collagen IV [Kemmer et al., 1992; Morgenthaler et al., 1990].

Our results suggest that the presence of N-linked sugars as well as their maturation were crucial for αvβ5- and αvβ6-dependent cell adhesion. This observation reflects the complexity of the N-glycan role in integrin regulation. This is one example of the divergent results obtained in the study of the relationship between cell adhesion and integrin glycosylation.

Malignant transformation and metastatic process are known to be associated with altered glycosylation of cell surface proteins. Moreover, these modifications could contribute to a perturbation of cell adhesion properties [for review, see Humphries and Olden, 1989]. Studies of the β1 integrins, expressed on weakly or highly metastatic murine melanoma cell lines [Kawano et al., 1993], demonstrated that N-glycosylation is crucial for integrin function, not only for cell adhesion to purified ECM proteins, but also in the multistep process of cancer metastasis. The vitronectin receptor, αvβ3, involved in the proliferation of human melanoma cells *in vivo* [for review, see Juliano and Varner, 1993], illustrates the potential role of the αv-containing integrins in the control of cell proliferation and tumor progression. The presence of αvβ5 and αvβ6 at the surface of human colonic adenocarcinoma cells and the functional role of their oligosaccharide moieties might open up a new perspective for the understanding of colonic tumor growth and metastasis. Our data contribute to support the idea that carbohydrate moieties fulfill important functions in specific adhesion on particular extracellular matrix components, perhaps by their contribution to a proper folding of integrins necessary for their adhesive functions. The understanding of these mechanisms at the molecular level is of great importance for the control of the inhibition of cancer cell invasion and metastasis.

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