Growth Factor–dependent Activation of αvβ3 Integrin in Normal Epithelial Cells: Implications for Tumor Invasion

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Abstract. Integrin activation is a multifaceted phenomenon leading to increased affinity and avidity for matrix ligands. To investigate whether cytokines produced during stromal infiltration of carcinoma cells activate nonfunctional epithelial integrins, a cellular system of human thyroid clones derived from normal glands (HTU-5) and papillary carcinomas (HTU-34) was employed. In HTU-5 cells, αvβ3 integrin was diffused all over the membrane, disconnected from the cytoskeleton, and unable to mediate adhesion. Conversely, in HTU-34 cells, αvβ3 was clustered at focal contacts (FCs) and mediated firm attachment and spreading. αvβ3 recruitment at FCs and ligand-binding activity, essentially identical to those of HTU-34, occurred in HTU-5 cells upon treatment with hepatocyte growth factor/scatter factor (HGF/SF). The HTU-34 clone se-

creted HGF/SF and its receptor was constitutively tyrosine phosphorylated suggesting an autocrine loop responsible for αvβ3 activated state. Antibody-mediated inhibition of HGF/SF function in HTU-34 cells disrupted αvβ3 enrichment at FCs and impaired adhesion. Accordingly, activation of αvβ3 in normal cells was produced by HTU-34 conditioned medium on the basis of its content of HGF/SF. These results provide the first example of a growth factor–driven integrin activation mechanism in normal epithelial cells and uncover the importance of cytokine-based autocrine loops for the physiological control of integrin activation.

Key words: integrins • thyroid • hepatocyte growth factor/scatter factor • c-*Met* • tumor invasion

ADHESION to neighboring cells and the extracellular matrix (ECM)[1] plays a crucial role in different biological phenomena, including cell motility and tumor invasion (Juliano and Varner, 1993; Chapman, 1997), differentiation (Adams and Watt, 1993; Lin and Bissell, 1993; Gumbiner, 1996), and survival (Frisch and Francis, 1994). The malignant behavior of carcinoma cells is not simply characterized by alteration or loss of growth control, a feature shared with benign neoplasms, but also by the ability to weaken tissue constraints and invade foreign districts, where cancer cells may migrate, proliferate, and survive. This xenophilic tendency is fostered by coopera-

tion among ECM molecules, proteases, growth factors (GFs), and the adhesion receptors expressed on the surface of the invading cells, which together provide signals controlling the organization of the cytoskeleton (Clark and Brugge, 1995; Yamada and Miyamoto, 1995; Brooks et al., 1996; Wei et al., 1996).

Physiological interactions between normal epithelial cells and the underlying basal lamina, as well as recognition of matrix components by carcinoma cells during stromal infiltration are mediated by the integrin family of adhesion receptors, a class of transmembrane noncovalently associated glycoprotein heterodimers composed of one α and one β chain (Hynes, 1992; Sonnenberg, 1993). Conceivably, migration of epithelial neoplastic cells within stromal tissues involves changes in the expression, topography, cytoskeletal association, and signaling properties of the integrin repertoire. In fact, many in vivo and in vitro studies have reported surface modifications of integrin levels, or even neo-expression of some integrins, in carcinoma versus normal cells (Plantefaber and Hynes, 1989; Zutter et al., 1995; Serini et al., 1996; for a comprehensive review see Ben-Ze'ev, 1997). Such modifications might be

driven either by the ECM itself (Langhofer et al., 1993; Rabinovitz and Mercurio, 1996), by GFs secreted by stromal cells and stimulating the invasive neoplastic elements in a paracrine fashion (Klemke et al., 1994; Doerr and Jones, 1996), or even by cytokines synthesized de novo by carcinoma cells and acting back on the tumor mass via an autocrine circuit (Aasland et al., 1988; Bachrach et al., 1988; Mizukami et al., 1991).

Cellular attachment to immobilized ECM ligands commonly results in cytoskeletal reorganization and clustering of integrins at discrete adhesive sites known as focal contacts (FCs). In these specialized structures, an array of submembranous proteins ranging from structural molecules to regulatory enzymes forms a multimolecular complex linking the actin microfilament network with integrins and, hence, with the ECM (Burridge and Chrzanowska-Wodnicka, 1996). The formation of FCs triggers signal cascades that act in concert with GF-activated transduction pathways and can alter gene expression (Clark and Brugge, 1995). Under given conditions, intracellular signals that originate at FCs or at downstream targets result in modulation of the affinity and/or avidity of certain integrins for extracellular ligands, a process termed activation or inside-out signaling (Hynes, 1992).

Our goal was to investigate how variations in integrin composition and GF receptor activation correlate with changes in cell adhesion in carcinoma versus normal elements. We assumed that the tuning of the integrin adhesive machinery occurring when tumor cells invade surrounding tissues might include not only changes in quantity but also changes in quality such as conversion of integrins from a dormant, nonadhesive state into an active one endowed with high adhesive capabilities. Specifically, we postulated that such conversion could be induced by one or more GFs acting within the neoplastic environment. The cellular model used in our experiments was a panel of human thyroid clonal strains corresponding to normal and malignant in vivo cell populations. These clones can form epithelial colonies and, when cultured in three-dimensional gels, develop aggregates whose biological functions, differentiation parameters, and morphological architecture are strictly related to the in vivo counterpart (Curcio et al., 1994; Perrella et al., 1997; De Filippi, R., P.C. Marchisio, G. Serini, and L. Trusolino, manuscript in preparation). Such a feature allows exhaustive comparisons between normal and carcinoma cells and is usually limited or compromised in established cell lines, where relationship studies of normal versus transformed phenotypes appear to be extremely difficult.

In this report, we provide evidence that the αvβ3 integrin is expressed by normal cells in a latent state characterized by its inability to form cytoskeletal connections and to promote cell adhesion to ECM ligands. In contrast, αvβ3 is highly enriched at FCs of carcinoma cells and mediates tight adhesion. We also demonstrate that the multifunctional cytokine hepatocyte growth factor/scatter factor (HGF/SF), but not other serum GFs, is an autocrine factor for carcinoma cells and show that the signaling pathway stimulated by HGF/SF, when elicited in normal cells, can fully recapitulate the adhesive pattern of neoplastic elements. Indeed, no connections have been ever studied between a specific GF and activation of the adhesive capabilities of an integrin in normal epithelial cells under physiological conditions. Here we unravel such a functional interplay and underscore the importance of autocrine production of GFs for the integrin-dependent invasive behavior of carcinoma cells and, possibly, for the activation state of integrins in general.

Materials and Methods

Cell Cultures

Clonal strains from human normal thyroid (HTU-5) and papillary carcinoma (HTU-34) were obtained and cultured as previously described (Curcio et al., 1994; Perrella et al., 1997). In brief, tissue samples from pathological specimens of different patients were freed from adherent connective tissue, cut into small pieces, washed in Ca^{2+}- and Mg^{2+}-free HBSS, and enzymatically digested with a solution consisting of 20 U/ml collagenase (CLSPA; Worthington Biochemical Corp., Freehold, NJ), 0.75 mg/ml trypsin (1:300; GIBCO BRL, Gaithersburg, MD), and 2% heat-inactivated dialyzed chicken serum (GIBCO BRL), in Ca^{2+}- and Mg^{2+}-free HBSS. Cell suspensions were collected after a 2 h digestion and seeded onto 100-mm plastic tissue culture dishes (Falcon; Becton Dickinson, Lincoln Park, NJ). Culture medium was a modified F-12 further varied to contain 0.48 mM $MgCl_2$, 3 mM KCl, 5% Fetal Calf Serum (GIBCO BRL), 1 mg/ml Na-insulin (Elanco, Indianapolis, IN), 5 μg/ml bovine transferrin (GIBCO BRL), 0.01 mM hydrocortisone, 2 ng/ml selenous acid, 3 pg/ml triiodothyronine (all from Sigma Chemical Co., St. Louis, MO), 75 μg/ml bovine hypothalamus extracts and 5 μg/ml bovine pituitary extracts (Pel Freez Biologicals, Rogers, AR). The purity of both strains was assessed by examining the expression of thyroid-specific molecular markers (thyroglobulin, thyroperoxidase, TTF-1, and PAX-8) and by evaluating thyrotropin-dependent c-AMP production and thymidine incorporation. This in vitro profile was found to correlate with the degree of differentiation of the starting specimen and with the pathological diagnosis (Perrella et al., 1997). Throughout the experiments, only cells from the 2nd to the 5th passage were used.

Antibodies

The integrin-specific mAbs used in this study (with the investigators who provided them) were as follows: MAR4 against β1 and MAR6 against α6 (from Sylvie Ménard, Istituto Nazionale Tumori, Milano, Italy; Bottini et al., 1993); F2 against α3 (from Luciano Zardi, Istituto Scientifico per lo Studio e la Cura dei Tumori, Genova, Italy); L230 against αv (from Paola Defilippi, Dipartimento di Genetica, Biologia e Chimica Medica, University of Torino, Italy); AA3 against β4 (from Vito Quaranta, Scripps Research Institute, La Jolla, CA); VIPL-2 against β3 (from Walter Knapp, Institüt für Immunologie der Universität, Vienna, Austria); IA9 against β5 (from Martin Hemler and Renata Pasqualini, Dana-Farber Cancer Institute, Boston, MA). Other mAbs against integrin subunits were commercially obtained: Gi9 against α2 and SAM-1 against α5 (Immunotech, Marseille, France); a rabbit polyclonal antiserum against β3 and the function-blocking mAb LM609 against the integrin complex αvβ3 (Chemicon International Inc., Temecula, CA). The inhibitory mAb AIIB2 against β1 was provided by Caroline H. Damsky (Department of Stomatology, University of California at San Francisco, CA). Rabbit polyclonal antisera against β1 and αv were, respectively, from Ivan de Curtis (DIBIT, Istituto Scientifico San Raffaele, Milano, Italy) and Guido Tarone (Dipartimento di Genetica, Biologia e Chimica Medica, University of Torino, Italy). mAb VIN11.5 against vinculin was from Sigma Chemical Co. The C-28 rabbit antiserum against human HGF/SF receptor, used in immunoprecipitation experiments, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the mAb DQ-13 against human HGF/SF receptor, used in Western blotting analyses, and the 4G10 anti-phosphotyrosine mAb were from Upstate Biotechnology Inc. (Lake Placid, NY). 1W53, a neutralizing sheep antiserum directed against human HGF/SF, was produced in the laboratory of Ermanno Gherardi (Imperial Cancer Research Fund, Cambridge University Medical School, UK) and kindly supplied by Paolo Amati and Sergio Anastasi (Dipartimento di Biotecnologie Cellulari ed Ematologia, Università "La Sapienza", Rome, Italy). The neutralizing activity was titrated in scatter assays on MDCK cells after HGF/SF stimulation and found to be optimal at a 1:80 dilution.

Cytokines

HGF/SF and TGFβ1 were purchased respectively from R & D Systems Inc. (Minneapolis, MN; Van der Voort et al., 1997), and Boehringer Mannheim GmbH (Mannheim, Germany). EGF was kindly donated by Laura Beguinot (DIBIT, Milano, Italy). Insulin and insulin-like growth factor were a generous gift of Franco Folli (Divisione Universitaria di Medicina Interna, Istituto Scientifico San Raffaele, Milano, Italy).

Immunoprecipitation and Western Blotting

Immunoprecipitations were carried out on surface-biotinylated cells as previously described (Rabino et al., 1994). In brief, confluent monolayers were washed three times at 4°C with Hank's balanced salt biotinylation buffer (HBB), pH 7.4, consisting of 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 5 mM KCl, 138 mM NaCl, 5.6 mM D-glucose, and 25 mM Hepes, pH 7.4. Sulfo-succinimido biotin (Pierce Chemical Co., Rockford, IL) was made 0.5 mg/ml in HBB and applied to the cells for 20 min at 4°C. The biotin solution was then removed and replaced with fresh biotin solution for another 20 min. The reaction was stopped by incubating four times at 4°C with Minimal Essential Medium containing Hank's balanced salts, 0.6% BSA, 20 mM Hepes, pH 7.4. After three washes in cold HBB, cells were lysed for 30 min at 4°C in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium-deoxycholate, 5 mM EGTA, 50 mM NaF, and 5 mM $MgCl_2$, supplemented with various phosphatase and protease inhibitors (leupeptin, pepstatin, aprotinin, PMSF, soybean trypsin inhibitor, and sodium-orthovanadate). Extracts were centrifuged at 15,000 rpm for 30 min at 4°C, and supernatant protein content was normalized with the BCA Protein Assay Reagent Kit (Pierce Chemical Co.). Cell lysates were precleared onto protein A–Sepharose CL-4B (Pharmacia Biotech Sverige, Uppsala, Sweden) and rotated 2 h at 4°C with different mAbs. Immunocomplexes were collected with affinity-purified rabbit anti–mouse IgG (Pierce Chemical Co.) coupled to protein A–Sepharose. After several washes with lysis buffer, the final pellets were eluted in boiling Laemmli buffer and proteins were electrophoresed on 8% SDS-PAGE. Samples were transferred onto Immobilon-P™ filters (Millipore Corp., Bradford, MA), probed with peroxidase-conjugated streptavidin, and visualized on Kodak X-OMAT AR films (Rochester, NY) by the Enhanced Chemiluminescence System (Amersham Life Sciences, Little Chalfont, UK). In biotinylation experiments of β1 integrins, cells were previously treated with 100 μg/ml trypsin in PBS twice for 20 min at 4°C, followed by inactivation with complete medium (Boll et al., 1991).

Adhesion Assay

Cell adhesion was performed according to Grano et al. (1994), with minor modifications. In brief, 96-well microtiter plates (polystyrene, nontissue culture treated; Nunc Inc., Naperville, IL) were coated with increasing concentrations of vitronectin (VN; Sigma Chemical Co.), fibrinogen (Sigma Chemical Co.), and osteopontin (from Cecilia M. Giachelli and Marta Scatena, Department of Pathology, University of Washington, Seattle, WA) in PBS, pH 7.4. In other assays, standard concentrations of 10 μg/ml laminin (Sigma Chemical Co) and fibronectin (FN; from Paola Defilippi, University of Torino) were used. Proteins were allowed to bind overnight at 4°C before the wells were rinsed and blocked for 2 h at 37°C with 3% heat-denatured BSA (RIA grade; Sigma Chemical Co.) in PBS, pH 7.4. Cells were harvested and washed twice with serum-free medium (SFM). To allow surface reexposure of integrin receptors, cells were incubated on a rotating platform for 1 h at 37°C in SFM containing 0.1% BSA, and then added to the wells at a concentration of 50,000 cells/0.1 ml of the same medium. After a 3-h incubation at 37°C, wells were gently washed twice in PBS. Adherent cells were fixed in 11% glutaraldehyde in PBS, rinsed in distilled water, and stained with 0.1% crystal violet, 20% methanol for 15 min. Cell numbers were obtained by counting all cells in four grids using a phase-contrast light microscope fitted with a 32 grid eyepiece at a total magnification of 100× (Doerr and Jones, 1996). All data presented are the means ± SD of duplicate wells from three or more experiments. Nonspecific cell adhesion as measured on BSA-coated wells has been subtracted.

In adhesion inhibition experiments, cells were plated onto the substrata in the presence of serial dilutions of the function-blocking mAbs LM609 or AIIB2. Alternatively, HTU-34 cells were treated for 2 d with 250 μg/ml suramin or with 1W53 antibody against HGF/SF and then processed for the adhesion assay; suramin or the inhibitory antibody was kept in all steps of the assays. Preimmune sera were used in control experiments.

In some assays, adherent HTU-5 cells were serum-starved for 36 h, harvested, and plated onto VN in SFM-0.1% BSA in the presence of single GFs or HTU-34–conditioned medium. In the case of TGFβ1 treatment, before harvesting cells were pretreated with TGFβ1 for 24 h. Alternatively, HTU-34–conditioned medium was preincubated with the 1W53 antibody against HGF/SF or with sheep normal IgGs (Sigma Chemical Co.) for 30 min and then applied to HTU-5 cells (see Results).

Indirect Immunofluorescence Microscopy

Cells from confluent monolayers were plated onto 24-well plates (Costar Corp.) containing 1.4-cm^2 glass coverslips. After 4 d in culture, cells were fixed for 5 min at room temperature in a freshly prepared solution of 3% formaldehyde (from paraformaldehyde) in PBS, pH 7.6, containing 2% sucrose. In some cases, cells were permeabilized by soaking coverslips for 3 min at room temperature in Hepes-Triton X-100 buffer (20 mM Hepes, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X-100; Fey et al., 1983; Rabinovitz and Mercurio, 1997). Indirect immunofluorescence was performed as previously reported (De Luca et al., 1990; Marchisio et al., 1991). In brief, after a 15-min saturation with PBS-2% BSA at 37°C, the primary antibodies were layered onto cells and incubated in a moist chamber for 30 min. After rinsing in PBS-0.2% BSA, coverslips were incubated with the appropriate rhodamine-tagged secondary antibody (DAKOPATTS, Copenhagen, Denmark) for 30 min at 37°C in the presence of 2 μg/ml of fluorescein-labeled phalloidin (Sigma Chemical Co.). Coverslips were mounted in Mowiol 4-88 (Hoechst AG, Frankfurt, Germany) and observed in a photomicroscope (Axiophot; Zeiss, Jena, Germany) equipped with epifluorescence lamp and planapochromatic oil immersion lenses. Fluorescence images were recorded on Kodak T-Max 400 photographic films exposed at 1,000 ISO and developed in T-Max Developer for 10 min at 20°C.

In some experiments, coverslip-attached HTU-5 cells were serum-starved for 36 h and then treated with HGF/SF or with HTU-34–conditioned medium (see Results). In other cases, HTU-34 cells were treated for 2 d with 1W53 antibody against HGF/SF or with sheep preimmune sera (Sigma Chemical Co.) and then processed for immunofluorescence.

FACS® Analysis

FACS® analysis was performed according to Peruzzi et al. (1996), with minor modifications. In brief, HTU-5 and HTU-34 cells were harvested with 1 mM EDTA in PBS, washed in ice-cold PBS-2%BSA, 5 mM NaN_3, and incubated with mAb VIPL-2 against the β3 integrin subunit (10 μg/ml) for 40 min at 4°C. Cells were then rinsed and treated with fluorescein-tagged rabbit anti–mouse IgG (DAKOPATTS) for 30 min. All incubations were performed in PBS-0.2%BSA, 5 mM NaN_3 at 4°C. Fluorescence was measured using a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA) set to count 10,000 cells per sample. The data were collected and analyzed with a MacIntosh Power PC computer equipped with CELLQuest research software (Becton Dickinson). Positive fluorescence was determined on a four log scale and expressed as channel number mean intensity fluorescence (MIF). Background fluorescence was determined on each cell population using fluorescein-tagged rabbit anti–mouse IgGs alone.

Northern Blotting

Total RNA was isolated from confluent monolayers of HTU-5 and HTU-34 cells by the acid guanidium method (Chomczynski and Sacchi, 1987). Northern blots were performed with 30 μg total RNA per lane. Ethidium bromide at a concentration of 0.2 μg/ml was added before electrophoresis in 1% agarose gels containing formaldehyde to verify the integrity of the RNA by short-wavelength UV detection and to monitor the equivalence of loading before and after transfer to GeneScreen Plus filters (Du Pont NEN, Boston, MA). A full-length HGF/SF cDNA probe (from Gianni Gaudino, Dipartimento di Scienze e Tecnologie Avanzate, University of Alessandria, Italy) and a probe for the housekeeper gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH, from Fanny Sciacca, DIBIT, Milano, Italy) were labeled with random priming (Rediprime DNA labeling system; Amersham Life Sciences) and [^{32}P]dCTP (3,000 Ci/mmol; Amersham Life Sciences). Membranes were pretreated and hybridized in 50% formamide (Merck, Darmstad, Germany), 10% dextran sulfate (Sigma Chemical Co.), 1% SDS, and 50 μg/ml salmon sperm DNA, at 42°C. Blots were washed twice with 2× SSC at room temperature for 10 min, then twice in 2× SSC 1% SDS at 65°C for 30 min, and finally once in 2× SSC at

room temperature for 5 min, followed by exposure to autoradiography for 48 h at –80°C with intensifying screens.

Results

Thyroid Clonal Strains Express αvβ3, αvβ1, and α3β1 Integrins

To characterize the surface adhesive repertoire of normal (HTU-5) and malignant (HTU-34) thyroid cells, a battery of integrin-specific mAbs was used in immunoprecipitation experiments on membrane biotinylated cell monolayers. The αv mAb L230 immunoprecipitated three bands of 150, 130, and 90 kD in both the normal and carcinoma strains (Fig. 1 *A*). The 150/90-kD doublet was also brought down by the β3-specific mAb VIPL2 (Fig. 1 *B*) but not by the β5 mAb IA9 (not shown). Based on band intensities, the αvβ3 integrin appeared to be more expressed in the HTU-34 clone. The higher surface exposure of αvβ3 in carcinoma cells was confirmed by FACS® analysis on HTU-5 (Fig. 1 *C*) and HTU-34 (Fig. 1 *D*) cells.

The faint 130-kD band coprecipitating with the αv subunit could be interpreted as an αv-associated β1 chain. To test the nature of this band, anti-αv immunoprecipitates from equal amounts of HTU-5 and HTU-34 cell extracts were transferred onto Immobilon-P™ membranes and probed with a β1 polyclonal antiserum: in fact, a specific 130-kD band corresponding to the β1 subunit was detected in both clones (Fig. 1 *E*). Under standard conditions, surface biotinylation of β1 integrins was extremely difficult, possibly because the accessibility of this integrin to biotin was compromised by the adhesive meshwork of basement membrane components (Gottardi and Caplan, 1992). To overcome this problem we employed a mild trypsinization protocol that enhances the ability of biotin to interact with ventral proteins (Boll et al., 1991). By this procedure, immunoprecipitation of HTU-5 and HTU-34 cell lysates with the β1 mAb MAR4 yielded two bands of similar intensity at 150 and 130 kD, representing one or more β1-associated α chains and the β1 subunit (Fig. 1 *F*), respectively. To further define which α subunits, besides αv, could heterodimerize with the β1 chain, mAbs against α2, α3, α5, and α6 were used in immunoprecipitation assays on biotinylated normal and malignant cells. Only the α3-mAb F2 was able to precipitate two bands of 150 and 130 kD comigrating with the α3 and β1 integrin subunits (Fig. 1 *G*). These same mAbs were also used in immunofluorescence experiments; consistently with the immunoprecipitation analysis, among the α subunits tested only α3 and αv were immunoreactive (not shown).

Taken together, these data demonstrate that the integrins expressed at the surface of normal and malignant thyroid clones include the αvβ1, αvβ3, and α3β1 heterodimers. The β1 chain is thus shared by the α3 and αv subunits.

The Integrin αvβ3 Is Clustered at Focal Contacts and Mediates Adhesion in Malignant but Not in Normal Thyroid Cells

The only modification in integrin repertoire observed in malignant versus normal thyroid cells was the higher surface expression of the αvβ3 heterodimer in HTU-34 cells (Fig. 1, *A–D*). To evaluate the subcellular distribution of αvβ3 in normal and carcinoma clones, cells were plated onto glass coverslips, cultured for several days, and then subjected to immunofluorescence. Under these conditions, cell adhesion occurs because of endogenous production of matrix molecules. Immunofluorescence experiments on fixed, nonpermeabilized cells were in accordance with the immunoprecipitation data: a fine grainy pattern of immunoreactivity was much stronger in HTU-34 (Fig. 2 *B*) than in HTU-5 cells (Fig. 2 *A*). Interestingly, treatment of fixed cells with permeabilization buffer (0.5% Triton X-100), which extracts freely diffusing molecules yet preserves actin cytoskeletal connections (Fey et al., 1983;
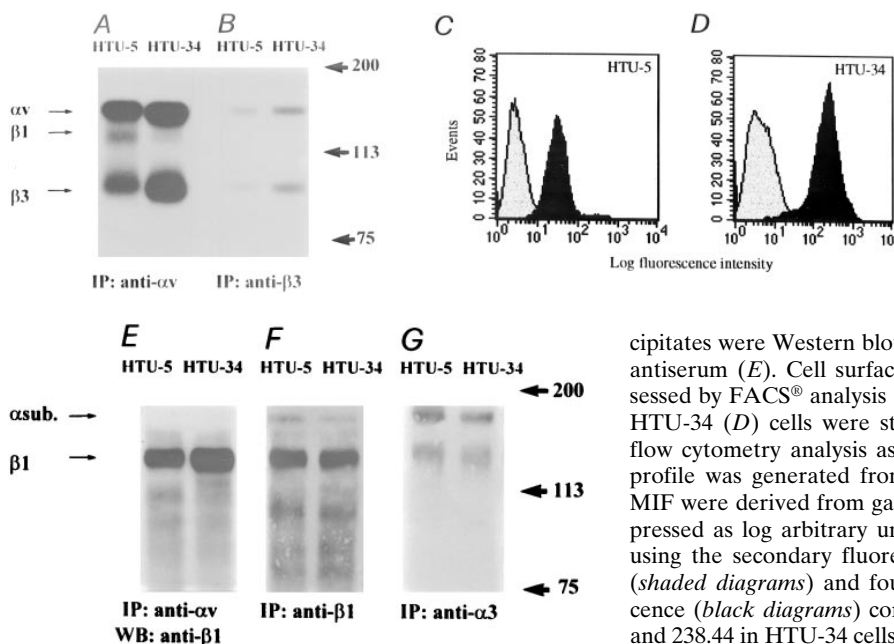

Figure 1. Characterization of the surface integrin repertoire in HTU-5 and HTU-34 thyroid clonal strains. Confluent cells were surface biotinylated and detergent lysates were immunoprecipitated with the indicated mAbs to different integrin subunits as described in Materials and Methods. The eluates were then analyzed by SDS-PAGE under nonreducing conditions. To demonstrate association of the β1 subunit with the αv chain, anti-αv immunoprecipitates were Western blotted and then decorated with a β1 polyclonal antiserum (*E*). Cell surface expression of the β3 subunit was also assessed by FACS® analysis (*C* and *D*). Washed, unfixed HTU-5 (*C*) and HTU-34 (*D*) cells were stained for indirect immunofluorescence and flow cytometry analysis as described in Materials and Methods. Each profile was generated from analyzing 10,000 cells. Relative values of MIF were derived from gated computerized histogram analysis and expressed as log arbitrary units. Nonspecific fluorescence was measured using the secondary fluorescein-tagged rabbit anti–mouse IgGs alone (*shaded diagrams*) and found to have a MIF <7. Specific β3 fluorescence (*black diagrams*) corresponded to a MIF of 38.7 in HTU-5 cells and 238.44 in HTU-34 cells.

Rabinovitz and Mercurio, 1997), completely removed β3 immunoreactivity in HTU-5 cells (Fig. 2, *C* and *D*) and selectively concentrated the β3 fluorescent signal of HTU-34 cells at the endings of microfilament bundles in sites compatible with FCs (Fig. 2, *E* and *F*). The same result was obtained when Triton X-100 permeabilization was performed before fixation (not shown). Thus, recruitment of the αvβ3 heterodimer to microfilament-associated adhesion sites occurs only in malignant but not in normal thyroid cells.

To induce ligand-mediated clustering of αvβ3 in HTU-5 normal cells, subconfluent cultures were detached and plated in serum-free conditions onto a plastic substratum coated with a concentration range (2.5 to 25 μg/ml) of VN (Fig. 3 *A*), fibrinogen (Fig. 3 *B*), and osteopontin (Fig. 3 *C*). Surprisingly, HTU-5 cells could not attach and spread. In contrast, HTU-34 cells rapidly adhered and firmly spread; indeed, cells attached proportionally to the amount of substratum and were detectable even at very low doses of matrix ligands (Fig. 3, *A–C*). HTU-34 cell attachment and spreading on VN was progressively impaired by adding increasing concentrations of the αvβ3 inhibitory mAb LM609 but not by the β1 function-blocking mAb AIIB2 (Fig. 3 *D*). Moreover, in HTU-34 cells plated on VN and processed for immunofluorescence after fixation and permeabilization, the only integrin receptor clustered at nascent FC, strictly colocalizing with vinculin (Fig. 3 *F*), was αvβ3 (Fig. 3 *E*). Conversely, β1 integrins were almost undetectable on the cell surface (Fig. 3 *G*). Thus, adhesion of HTU-34 cells to VN was specifically mediated by αvβ3.

When HTU-5 were plated onto FN, a ligand for both αvβ3 and αvβ1, cells could attach and spread. In this case as well, αvβ3 was not involved in the adhesive phenomenon: only the β1 inhibitory mAb efficiently blocked adhesion whereas mAb LM609 did not display any significant effect (Fig. 4 *A*). Conversely, β1 and β3 integrins were equally responsible for adhesion to FN in HTU-34 cells: function-blocking mAbs against either integrins could partially impair adhesion when added individually, and almost totally when added together (Fig. 4 *A*). Immunofluorescence experiments showed that HTU-5 cells, when plated on FN, organized β1 integrins at adhesive structures (Fig. 4 *B*), whereas αvβ3 was almost undetectable (Fig. 4 *C*). On the contrary, in HTU-34 plated on FN both β1 and β3 integrins were highly enriched at focal adhesions; double immunostaining for β1 and β3 revealed colocalization of the two integrin subunits within the same FCs (Fig. 4, *D* and *E*).

In summary, these results indicate that adhesion to VN, fibrinogen, and osteopontin occurs only in malignant HTU-34 cells and that, in this strain, adhesion is specifically driven by αvβ3. Adhesion to FN occurs in both strains and is governed by the selective activity of β1 integrins in HTU-5 cells and by the cooperative action of β1 and β3 integrins in HTU-34 cells.

We hypothesized that the discrepancy in adhesion efficiency between HTU-5 and HTU-34 cells was due to the different expression levels of the αvβ3 integrin receptor. To test this hypothesis, HTU-5 were treated with TGF-β1, known to induce upregulation of αvβ3 synthesis and surface exposure (Ignotz et al., 1989). Indeed, TGF-β1 increased the expression of αvβ3 but could not induce αvβ3

recruitment at cytoskeleton-associated FCs nor enhance HTU-5 cell adhesion to VN (not shown). These findings suggest that αvβ3 upregulation is not sufficient per se to trigger cluster formation and firm adhesion in HTU-5 cells. Thus, we can reasonably rule out the possibility that the different adhesive capabilities of carcinoma versus normal cells are simply related to the higher surface exposure of αvβ3 in the HTU-34 clone.

A Carcinoma-specific Autocrine Loop Sustains αvβ3-mediated Adhesion

Since the assembly of integrin adhesion complexes requires serum soluble factors in some cell types (Hotchin and Hall, 1995), and on the basis of mounting evidence that integrins and GF receptors share common signaling pathways (Clark and Brugge, 1995), we assumed that signals derived from a GF receptor could be responsible for maintaining αvβ3 in a constitutively proadhesive activated state.

In a preliminary test of this possibility we plated HTU-34 cells on VN after preincubation with suramin, a drug that
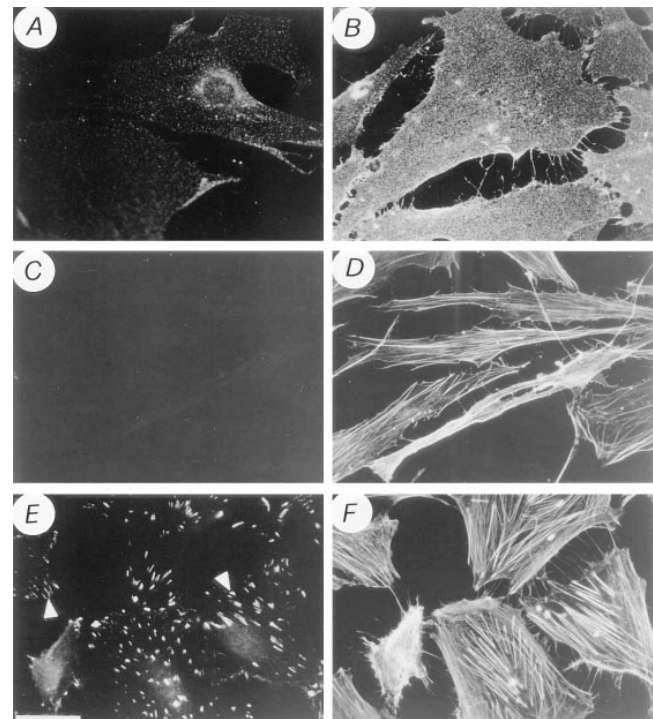

Figure 2. β3 integrin is clustered at FCs only in HTU-34 cells. In *A* and *B*, HTU-5 (*A*) and HTU-34 (*B*) cells were fixed and directly immunostained with a β3 mAb. In *C* and *F*, HTU-5 (*C* and *D*) and HTU-34 (*E* and *F*) cells were fixed, permeabilized with a Triton X-100 buffer that removes most of the soluble proteins but not cytoskeleton-associated molecules, and then stained by a double immunofluorescence protocol using the β3 mAb followed by a rhodamine-conjugated rabbit anti–mouse IgG (*C* and *E*) and by fluorescein-tagged phalloidin (*D* and *F*). In nonpermeabilized cells, β3 displayed a fine grainy pattern (*A* and *B*) and the signal was much stronger in the HTU-34 strain (*B*). After permeabilization, β3 immunoreactivity was completely abrogated in HTU-5 cells (*C* and *D*) and selectively enriched at FCs in the HTU-34 clone (*E* and *F*). Bar, 10 μm.

blocks any cytokine–receptor interactions (La Rocca et al., 1990; Adams et al., 1991; Ferracini et al., 1995; Zumkeller and Schofield, 1995). Indeed, HTU-34 cells completely lost their adhesion potential (Fig. 5 *A*) suggesting that αvβ3 adhesive properties were controlled by a soluble factor interacting with a receptor. To test this hypothesis we challenged HTU-5 cells with SFM conditioned by the HTU-34 clone and found that cells acquired de novo adhesion to VN (Fig. 5 *A*). When HTU-34–conditioned medium was applied to HTU-5 cells previously plated onto glass coverslips, thus adhering to endogenous ECM molecules, αvβ3 recruitment at FCs was observed (Fig. 5, *B* and *C*). It was deduced that a soluble factor produced by malignant cells, but not by normal cells, controlled αvβ3-mediated adhesion by acting on a receptor shared by the two cell types.

HGF/SF Promotes αvβ3-mediated Adhesion

The multifunctional cytokine HGF/SF was selected as the object of closer investigation for several reasons: (*a*) the HGF/SF receptor c-*Met* is constitutively activated in thyroid carcinomas (Di Renzo et al., 1992, 1995); (*b*) HGF/SF autocrine release has been reported to represent a selective advantage for tumor progression (Tsao et al., 1993; Ferracini et al., 1995); (*c*) the morphogenic responses to HGF/SF are critically dependent on cell adhesion (Matsumoto et al., 1995); and (*d*) finally, HGF/SF has been shown to enhance adhesion of B cells and lymphoma cells, thus suggesting its involvement in integrin activation mechanisms (Van der Voort et al., 1997; Weimar et al., 1997).

Indeed, HGF/SF clearly promoted attachment and spreading of HTU-5 cells on VN in a dose-dependent manner (Fig. 6 *A*). HGF/SF-induced adhesion was specifically inhibited by mAb LM609 against αvβ3 (Fig. 6 *B*). In agreement with these findings, HTU-34 adhesion on VN was impaired by a functional antibody to HGF/SF (Fig. 6 *B*). Moreover, this antibody, but not normal sheep serum, blocked the ability of HTU-34–conditioned medium to induce adhesion of HTU-5 cells (Fig. 6 *B*). The proadhesive effect of HGF/SF was specific insofar that TGF-β1 could not enhance HTU-5 cell adhesion to VN (not shown) nor could EGF, insulin, and insulin-like growth factor-1 (Fig. 6 *B*). It has already been demonstrated that receptors for EGF, insulin, and insulin-like growth factor-1 are present in thyroid cells (Dumont et al., 1991); however, to ascertain that also HTU-5 cells express these receptors, we performed Western blot experiments on total cell lysates after GF stimulation and verified the induction of multiple tyrosine phosphorylated bands (not shown).

When HTU-5 cells were plated on FN in the presence of HGF/SF, adhesion was markedly enhanced (Fig. 6 *C*). Blockade of the β1 integrin receptors by means of mAb AIIB2 under basal conditions abolished adhesion, that was partially restored by adding HGF/SF (Fig. 6 *C*). Adhesion levels after inhibition of αvβ3 by mAb LM609 in the presence of HGF/SF were roughly comparable to those obtained under basal conditions (Fig. 6 *C*). When both β1 and β3 integrins were blocked by their respective inhibitory mAbs, adhesion was totally abolished and stimulation with HGF/SF was ineffective (Fig. 6 *C*). Taken together, these results indicate that HGF/SF enhances adhesion effi-
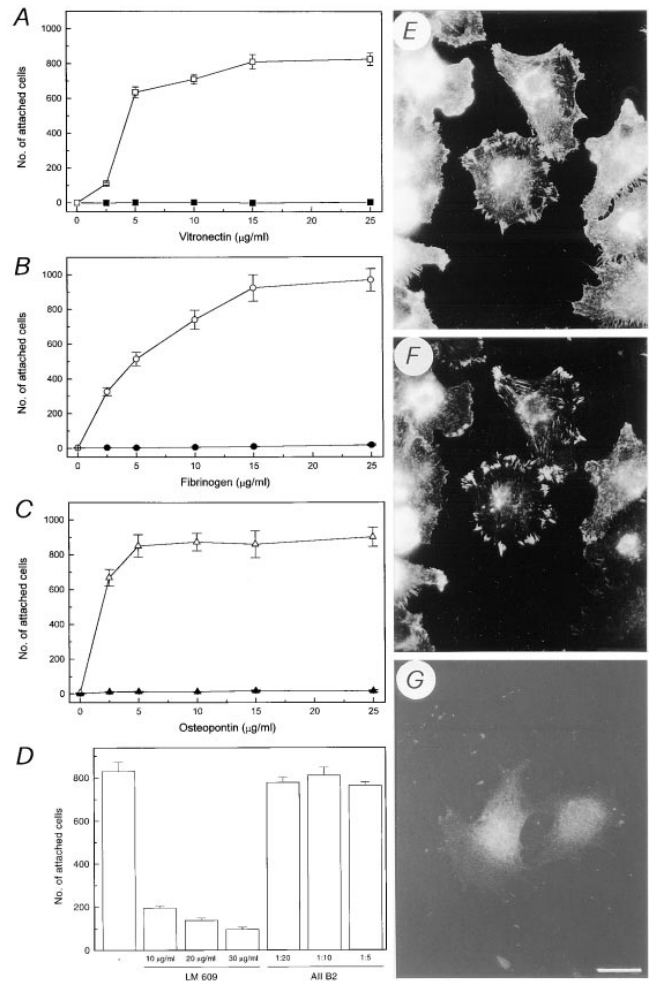

Figure 3. αvβ3 integrin displays ligand-binding activity and is recruited to newly forming adhesive structures only in HTU-34 cells. (*A–D*) Adhesion assays onto αvβ3-specific ECM molecules. (*A–C*) Cells from HTU-5 (*closed symbols*) and HTU-34 (*open symbols*) cultures were detached, resuspended in SFM, and then plated onto microtiter wells coated with increasing amounts of VN (*A*), fibrinogen (*B*), and osteopontin (*C*). After allowing cells to attach, the extent of cell adhesion was quantitated as described in Materials and Methods. (*D*) Adhesion inhibition assay. HTU-34 cells were plated onto 10 μg/ml VN alone (−) or in the presence of increasing concentrations of function-blocking mAbs to αvβ3 (*LM 609*) or to β1 (*AII B2*). (*E–G*) Immunofluorescence. HTU-34 cells were detached, resuspended in SFM, and plated for 2 h onto glass coverslips coated with VN. Cells were then fixed, permeabilized, and processed for double immunofluorescence using a rabbit polyclonal antiserum against β3 (*E*) and an mAb against vinculin (*F*). An mAb against β1 was also used (*G*) in single immunofluorescence experiments. In adhering HTU-34 cells, β3 and vinculin, but not β1, were recruited to maturing FCs. Bar, 7 μm.

ciency of HTU-5 cells on FN by promoting αvβ3-mediated attachment and spreading. Hence, αvβ3 activation significantly increases the level of basal adhesion mediated by β1 integrins.

As a control, HTU-5 and HTU-34 cells were plated on laminin (Fig. 6 *D*). Both clones adhered at comparable levels and adhesion was blocked by the β1 mAb AIIB2, act-
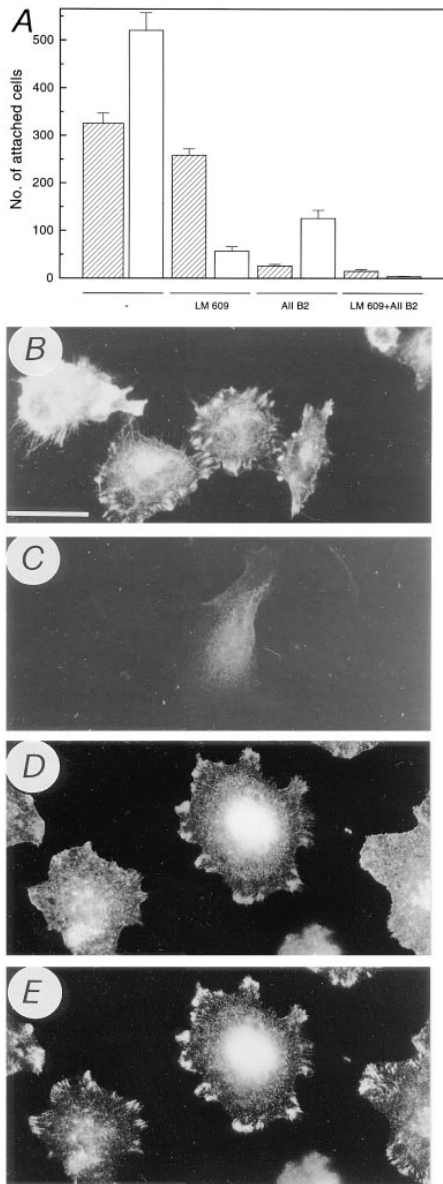
Figure 4. (A) Adhesion and adhesion inhibition assay of HTU-5 (*hatched bars*) and HTU-34 (*open bars*) cells onto FN. Cells were plated onto 10 μg/ml FN alone (−) or in the presence of mAbs LM609 (10 μg/ml) and/or AIIB2 (1:10 dilution). (*B–E*) Immunofluorescence. When HTU-5 cells were allowed to adhere to FN (*B* and *C*), the β1 chain (*B*) but not the β3 subunit (*C*) was clustered at nascent FCs. In HTU-34 cells plated on FN, both integrins were recruited to adhesion sites (*D* and *E*). Double-immunofluorescence analysis using an mAb to β1 (*D*) and a rabbit polyclonal antiserum to β3 (*E*) showed that both subunits were clustered within the same focal adhesions. Bar, 10 μm.

ing conceivably on the α3β1 heterodimer. Addition of HGF/SF did not modify cell attachment and spreading. Thus, the effect of HGF/SF on cell adhesion is specifically mediated by αvβ3 and is independent of the substratum recognized by this integrin, being exerted on both VN and FN.

In immunofluorescence experiments on HTU-5 cells adhering onto endogenous ECM molecules, HGF/SF triggered clustering of αvβ3 at FCs (Fig. 7, *A* and *B*). Consis-

tent with this observation, treatment of HTU-34 cells with the inhibitory antibody to HGF/SF resulted in a clear-cut decrease in the focal immunostaining for αvβ3 (Fig. 7, *C* and *D*), but no modifications in the topography of the β1 subunit (Fig. 7, *E* and *F*) or vinculin (Fig. 7, *G* and *H*) were observed. αvβ3 expression levels in HTU-5 clones did not increase upon stimulation with HGF/SF, nor did they decrease after antibody-mediated neutralization of HGF/SF activity in the HTU-34 cultures (not shown).

To determine whether HTU-5 and HTU-34 cells expressed the HGF/SF receptor c-*Met* and to verify whether the receptor was constitutively activated in HTU-34 cells because of a chronic autocrine loop, cell lysates were subjected to immunoprecipitation with the C-28 human *Met* polyclonal antibody. Anti-*Met* immunoprecipitates were then split into two equal fractions, Western blotted, and decorated with the anti-*Met* mAb DQ-13 (Fig. 8 *A*) or the phosphotyrosine mAb 4G10 (Fig. 8 *B*). In both cell lines, the 145-kD mature form of the c-*Met* β subunit was clearly detected (Fig. 8 *A*). The c-*Met* β subunit was phosphorylated on tyrosine residues in HTU-34 cell extracts, but not in unstimulated HTU-5 cell lysates. When HTU-5 cells were treated with conditioned medium from the HTU-34
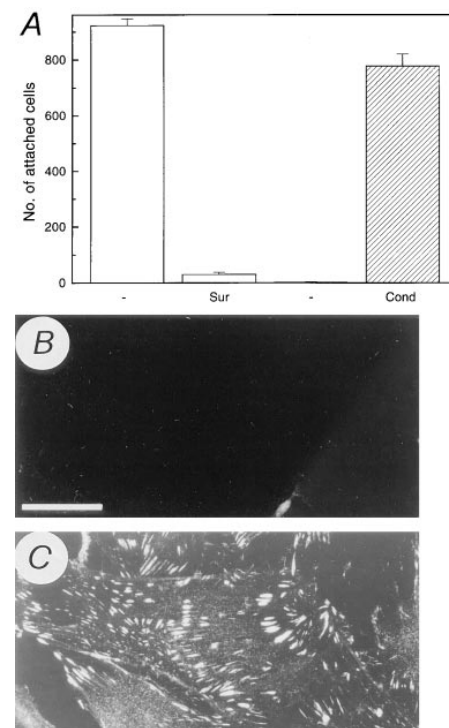

Figure 5. A soluble factor produced by HTU-34 cells is responsible for αvβ3 ligand-binding activity and recruitment to FCs. (*A*) Adhesion assay. Treatment of HTU-34 cells (*open bars*) with suramin (*Sur*) markedly reduced adhesion to VN. Conversely, conditioned medium from the HTU-34 clone (*Cond.*) triggered adhesion of HTU-5 cells (*hatched bars*). (*B* and *C*) Immunofluorescence. HTU-5 cells were either left untreated (*B*) or stimulated for 1 h with conditioned medium from the HTU-34 clone (*C*); cells were then fixed, permeabilized, and processed for immunofluorescence using an mAb against the β3 integrin subunit. Bar, 10 μm.

clone or with purified HGF/SF, specific tyrosine phosphorylation of the c-*Met* β subunit was detected (Fig. 8 *B*).

The presence of HGF/SF in the supernatant of HTU-34 cells was tested by assaying its scatter activity in MDCK epithelial cells after serial dilutions in standard medium. At a 1:10 dilution, conditioned supernatant was able to dissociate epithelial colonies, although it did not achieve the maximal effect observed with recombinant HGF/SF (not shown).

Finally, Northern blot analysis identified a specific 6-kb transcript, equivalent in size to the principal HGF/SF mRNA species (Nakamura et al., 1989), only in HTU-34 cells (Fig. 8 *C*). We believe that these results unequivocally substantiate the existence of a natural autocrine loop for HGF/SF in HTU-34 cells.

Discussion

A New Physiological Mechanism for Integrin Activation in Epithelia

Normal epithelial cells require integrin-mediated adhesion to ECM molecules of the basement membrane for GF control of the cell cycle (Assoian, 1997). In this paper we report that the opposite phenomenon also occurs, i.e., that integrin-dependent cellular adhesion requires GFs to take place. Namely, we report that the HGF/SF-dependent sig-

nal transduction pathway can induce ligand-binding activity in functionally inactive αvβ3 integrins.

The activation of integrins is characterized by conformational changes in their extracellular domain, reorganization of their cytoplasmic connections, and clustering of heterodimers within the plane of the plasma membrane, which together augment integrin affinity and avidity for ligands and stabilize adhesion (Du et al., 1991; Diamond and Springer, 1994; Li et al., 1995; Yednock et al., 1995). The molecular mechanisms responsible for physiological activation are still unclear (Lasky, 1997). Moreover, although this phenomenon has been extensively studied in platelets and leukocytes, little information is available for cells that are part of compact tissues and adhere to basement membranes, such as epithelial cells. Recently, Pelletier et al. (1996) reported that αvβ3 activation in a melanoma cell line involves a cation binding site that regulates integrin conformation. Even more recently, Fenczik et al. (1997) identified CD98, a type II membrane glycoprotein involved in early T cell activation and expressed in many adherent cell lines, as a factor responsible for β1 integrin activation. The mechanisms that regulate clustering of integrins and their recruitment at FCs also are poorly understood. Experiments with antibody- and ligand-coated beads have shown that clustering of integrins depends upon binding to multivalent matrix molecules and that FC assembly requires both integrin–ligand interaction and aggregation of integrins (Miyamoto et al., 1995).
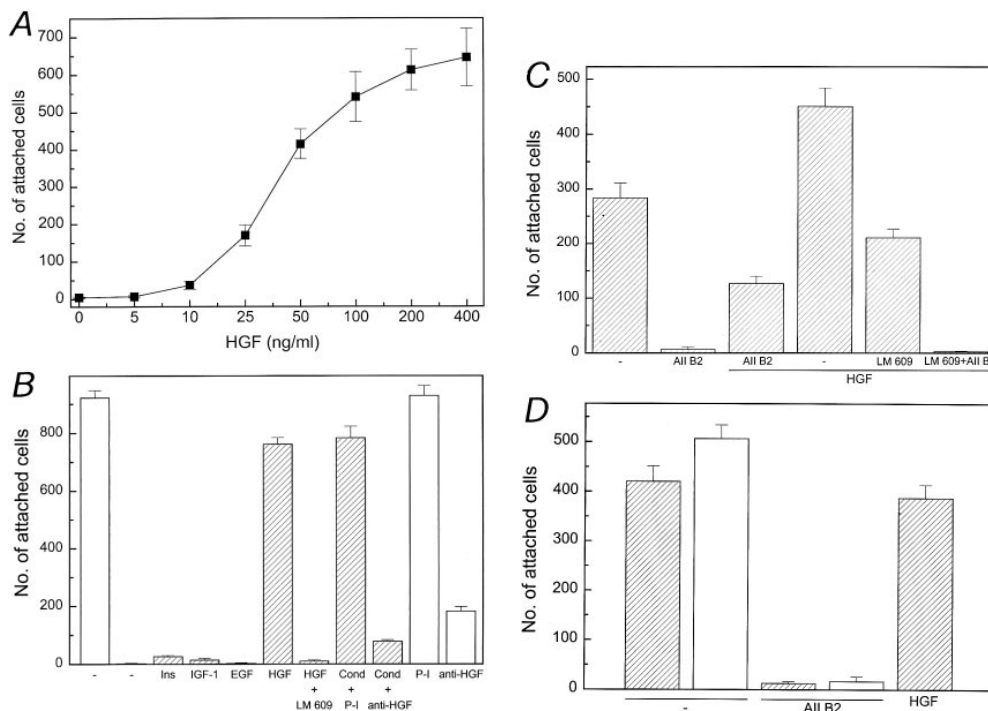

Figure 6. HGF/SF selectively activates the adhesive potential of αvβ3 integrin in HTU-5 cells. (*A*) HGF/SF promotes αvβ3-mediated adhesion to VN in a dose-dependent manner. Cells from HTU-5 cultures were detached, resuspended in SFM containing increasing amounts of HGF/SF, and then plated onto VN. (*B*) The effect of HGF/SF on αvβ3-mediated adhesion is specific and in malignant cells it results from an autocrine loop. HGF/SF was the only stimulus able to trigger adhesion of HTU-5 cells (*hatched bars*) to VN. Treatment of HTU-34 cells (*open bars*) with a blocking polyclonal antiserum to HGF/SF (*anti-HGF*) markedly reduced adhesion. HGF/SF-induced adhesion in HTU-5 cells was inhibited by mAb LM609 to the β3 subunit (*HGF+LM609*) and by treating HTU-34–conditioned medium with the inhibitory antiserum against HGF/SF (*Cond + anti-HGF*). In contrast, addition of preimmune sheep IgGs to HTU-34 cells (*P-I*) did not modify their adhesive properties. Similarly, treatment of HTU-5 cells with HTU-34–conditioned medium in the presence of preimmune IgGs (*Cond + P-I*) had no effect on the ability of conditioned medium to trigger cell adhesion. (*C*) HGF/SF increases adhesion efficiency of HTU-5 cells on FN via the enhancement of αvβ3 ligand-binding ability (see Results). (*D*) HGF/SF does not modify the adhesive function of laminin-binding integrins. Attachment of both HTU-5 (*hatched bars*) and HTU-34 (*open bars*) cells to laminin was inhibited by the β1 function-blocking mAb AIIB2, but was not modulated by HGF/SF treatment.
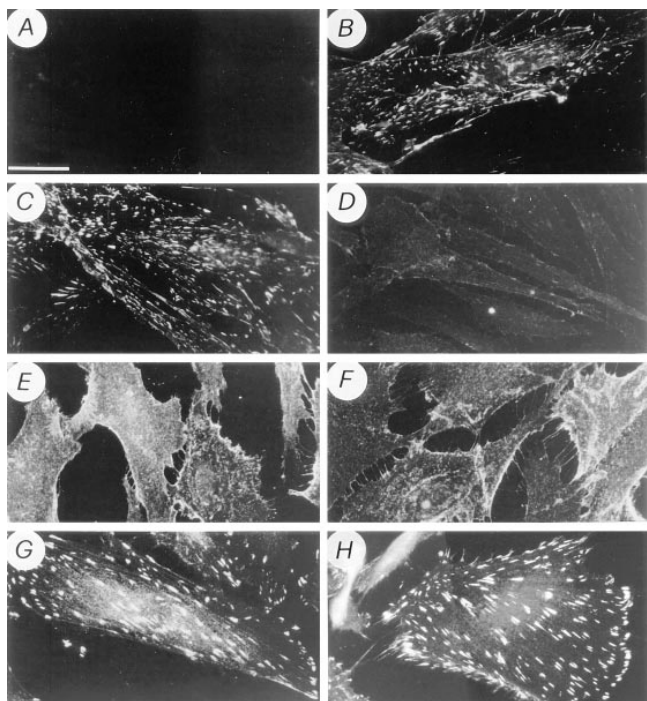
Figure 7. HGF/SF promotes clustering of β3 integrin at FCs (*A* and *B*). HTU-5 cells were either left untreated (*A*) or incubated for 1 h with 50 ng/ml purified HGF/SF (*B*); cells were then fixed, permeabilized, and processed for immunofluorescence using an mAb against β3. Antibody-mediated inhibition of HGF/SF function in HTU-34 cells selectively induces β3 disconnection from FCs (*C-H*). HTU-34 cells were incubated for 2 d with either a preimmune sheep serum (*C*, *E*, and *G*) or with a function-blocking sheep antiserum to HGF/SF (*D*, *F*, and *H*); cells were then fixed, permeabilized, and processed for immunofluorescence using mAbs against β3 (*C* and *D*), β1 (*E* and *F*) and vinculin (*G* and *H*). HGF/SF inhibition resulted in strong reduction of the β3 staining at FCs, with partial peripheral redistribution. No modifications of the immunoreactivity for β1 and vinculin could be observed upon HGF/SF blockade. Bar, 10 μm.

Data presented in this study indicate that both integrin aggregation and triggering of efficient ligand-binding capability in adherent normal cells require the presence of GFs; in particular, HGF/SF displays the unique ability to recruit αvβ3 to FCs and to stimulate αvβ3-mediated adhesion. Since no changes in αvβ3 expression levels can be observed upon HGF/SF treatment, we interpret HGF/SF-induced adhesion as a conversion of the integrin functional state from inactive to active, with consequent acquisition of ligand-binding ability. To our knowledge, these findings are the first example of a GF-driven integrin activation mechanism in adherent cells. Moreover, because we use an epithelial cell model presumably mirroring the adhesive environment of solid normal tissues and tumors, we elucidate one of the mechanisms that coordinate integrin and GF receptor function in normal and transformed epithelia under conditions that parallel several in vivo situations.

How can HGF/SF elicit clustering and activation of αvβ3? One possibility is that the activated GF receptor directly acts on the β3 cytoplasmic domain and that this, in turn, induces a conformational change in the integrin resulting in ligand binding. Integrin–ligand interaction would then trigger αvβ3 clustering at FCs. However, we could never show any obvious biochemical modification of the β3 integrin tail like HGF/SF-induced tyrosine phos-
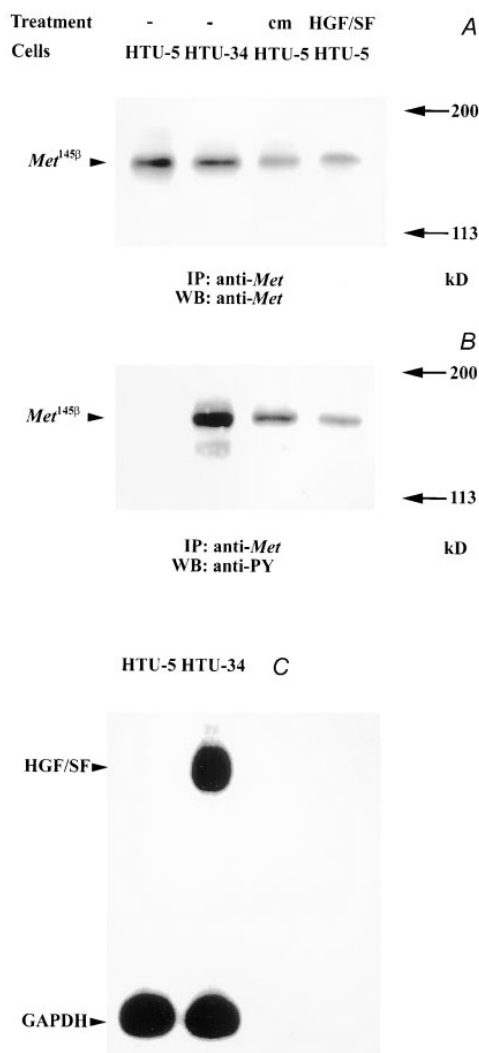

Figure 8. Biochemical evidence of HGF/SF autocrine production by the HTU-34 clone. HTU-5 cells were either left untreated or incubated for 10 min with conditioned medium from the HTU-34 clone (*cm HTU-5*) or with 50 ng purified HGF/SF (*HGF/SF HTU-5*). Unstimulated and stimulated HTU-5 cells, together with untreated HTU-34 cells, were extracted and immunoprecipitated with a human c-*Met* polyclonal antibody. The eluates were then split into two equal fractions, Western blotted, and decorated with mAbs to c-*Met* (*A*, *anti-Met*) or to phosphotyrosine (*B*, *anti-PY*). The c-*Met* receptor β chain (*Met*^{145β}) appeared to be constitutively tyrosine-phosphorylated in the HTU-34 strain. Specific tyrosine-phosphorylation of the *Met* protein could be induced in HTU-5 cells by treatment with HTU-34–conditioned medium or with HGF/SF. The HGF/SF transcript is specifically expressed in HTU-34, but not in HTU-5, cells (*C*). 30 μg of total RNA from HTU-5 and HTU-34 cells were separated by electrophoresis, transferred to nylon filters, and hybridized to ^{32}P-labeled probes specific for HGF/SF and for the housekeeper gene GAPDH.

phorylation nor formation of a complex between the activated HGF/SF receptor and the αvβ3 integrin.

A second possibility is that HGF/SF activates the αvβ3 integrin in an indirect manner, e.g., by modifying a range of intermediate effectors. Good candidates for this role would include CD98 analogues (see above) or β3-endonexin, a cytosolic protein that selectively binds the β3 cytodomain and modulates its affinity state (Shattil et al., 1995; Kashiwagi et al., 1997).

In a third scenario, HGF/SF might affect one or more of the submembranous components of the FCs. Because most of these components, as well as integrin cytoplasmic tails, have been shown to interact in vitro with each other, this could lead to αvβ3 recruitment via the formation of multiple cross-links (Turner and Burridge, 1991; Sastry and Horwitz, 1993; Gilmore and Burridge, 1996). According to this hypothesis, clustered integrins would form a high density, high valency complex with increased avidity for ligands, thus leading to stabilization of integrin–ligand interactions, firm adhesion, and spreading. Interestingly, HGF/SF is known to induce tyrosine phosphorylation of pp125^{FAK} (Matsumoto et al., 1994), a cytosolic tyrosine kinase enriched at FCs and able to phosphorylate other FC components including paxillin and tensin (Schaller and Parsons, 1994).

Implications for Tumor Invasion

All the morphological and functional features evoked by HGF/SF in normal thyroid cells spontaneously occur in carcinoma cells because of a natural HGF/SF autocrine loop. Inhibition of this loop markedly reduces αvβ3 enrichment at FCs and binding to immobilized ligands. Hence, whereas HGF/SF treatment of normal cells recapitulates the overall adhesive phenotype of carcinoma cells, neutralization of HGF/SF activity in neoplastic elements can per se revert αvβ3 from a functional to a latent state.

Although in different ways, both HGF/SF and αvβ3 integrin contribute to the malignant behavior of neoplastic cells. HGF/SF is responsible for invasive growth of tumors, a complex phenomenon resulting from the combination of proliferation, motility, ECM degradation, and cell survival. Specifically, HGF/SF impairs the compaction of polarized epithelia by disrupting the architecture of adherens junctions and inducing the appearance of a fibroblastoid phenotype endowed with motile properties (Stoker et al., 1987; Gherardi et al., 1989; Weidner et al., 1990, 1991; Igawa et al., 1991; Kan et al., 1991; Matsumoto et al., 1991; Naldini et al., 1991; Rubin et al., 1991). This scatter activity is corroborated by HGF/SF ability to promote the synthesis of ECM-degrading proteases, including urokinase plasminogen activator (uPA; Pepper et al., 1992; Boccaccio et al., 1994; Jeffers et al., 1996) and matrix metalloproteinase-2 (MMP-2; Zeigler et al., 1996), thus enhancing cell invasiveness into stromal compartments. Finally, HGF/SF can protect epithelial cells from anoikis, a form of programmed cell death occurring when adherent cells are detached from their physiological matrix substrata (Frisch and Francis, 1994; Longati et al., 1996; Amicone et al., 1997). In fact, when carcinoma cells infiltrate connective tissues and blood vessels before systemic dissemination,

they lose contact with their basal lamina and, to escape anoikis, must recognize previously unknown ECM components. It is tempting to speculate that the survival message conveyed by HGF/SF resides, at least partially, in its ability to activate the function of the αvβ3 integrin, thus supplying an adhesive information that may confer resistance to anoikis. From this viewpoint, the ability of HGF/SF to activate αvβ3 in carcinoma cells results in a double selective advantage: (*a*) it provides a functional receptor for stromal invasion and (*b*) it protects tumors from massive apoptosis.

Similarly to HGF/SF, αvβ3 is directly involved in invasive and antiapoptotic phenomena. This integrin is upregulated in melanoma clones endowed with high metastatic potential (Nip et al., 1995) and is physically associated with MMP-2 at the invasive front of infiltrating cells, in order to concentrate matrix degradation at adhesive sites and facilitate directed cellular motility (Brooks et al., 1996). Interestingly, αvβ3 is de novo expressed on the surface of endothelial cells during intratumoral formation of blood vessels (Brooks et al., 1994) and regulates the survival of newly sprouting blood vessels (Stromblad et al., 1996); likewise, HGF/SF displays powerful angiogenic activity (Bussolino et al., 1992). Altogether, data support the concept of a functional synergy between HGF/SF-dependent biological pathways and αvβ3-mediated adhesion processes in several neoplastic phenomena including matrix degradation, invasion, cell survival, and tumor neoangiogenesis.

It is worth noting that in many cell lines (the HTU-5 thyroid clone being a prominent exception) αvβ3 appears to be spontaneously clustered at FCs. In some cells, the basal activation state of the integrin may be intrinsically high, or perhaps more likely, maintained by autocrine production of HGF/SF. Accordingly, many examples of natural autocrine cells for HGF/SF have been described (Adams et al., 1991; Rong et al., 1992, 1993; Tsao et al., 1993; Grano et al., 1994; Ferracini et al., 1995; Woolf et al., 1995; Maier et al., 1996; Anastasi et al., 1997). An alternative hypothesis stems from the observation that in a variety of cell lines simple cellular adhesion is sufficient to elicit ligand-independent activation of the HGF/SF receptor (Wang et al., 1996). In this case, recruitment of αvβ3 at FCs would be the consequence of adhesion-dependent constitutive activation of the kinase receptor rather than a cellular response to chronic autocrine stimulation by the growth factor ligand. Notably, activation of the HGF/SF receptor by cell attachment occurs in many tumor cells, but not in primary or clonal cultures of normal cells (Wang et al., 1996).

In conclusion, we propose here a novel regulatory mechanism that epithelial cells use for integrin activation and in the ensuing integrin–ligand interaction phenomena. To our knowledge, this is the first report describing the specific modulation operated by a GF on the adhesive state and aggregation rate of an individual integrin in epithelial cells. Moreover, because αvβ3 activation is obtained upon stimulation with a GF that is physiologically present within the interstitial milieu of compact tissues and can be pathologically overexpressed in cancer, this mechanism can have strong in vivo implications for the adhesive behavior of parenchymal cells and for their interactions with stromal components. In addition, our results highlight the

importance of GF autocrine production in the regulation of integrin function during tumor invasion.

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