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Integrin $\alpha 3\beta 1$ Participates in the Phagocytosis of Extracellular Matrix Molecules by Human Breast Cancer Cells

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The mechanisms and receptors involved in phagocytosis by nonhematopoietic cells are not well understood. The involvement of the $\alpha 3\beta 1$ integrin in phagocytosis of the extracellular matrix by human breast cancer cells was studied. The possible role of this integrin was suggested since $\alpha 3$ and $\beta 1$ but not $\alpha 2$ subunits are concentrated at membrane sites where local degradation of fluorescently labeled gelatin occurs. Strikingly, anti-$\alpha 3$ integrin monoclonal antibodies (mAbs) stimulate the phagocytosis of fluorescently labeled gelatin films, gelatin beads, and Matrigel films in a quantitative phagocytosis assay. Stimulation of the gelatin uptake by the anti-$\alpha 3$ mAb is dose responsive, saturable, and time dependent. Antibodies against other integrin subunits have a lower stimulatory effect (anti-$\beta 1$) or no significant effect (anti-$\alpha 2$, -$\alpha 5$, -$\alpha 6$, and -αv) on gelatin phagocytosis. The synthetic HGD-6 human laminin peptide that binds specifically the $\alpha 3\beta 1$ integrin, but not the scrambled HSGD-6 control peptide, also markedly stimulates gelatin uptake in a dose-responsive way. Furthermore, the stimulatory effects of the HGD-6 peptide and the anti-$\alpha 3$ mAb are additive, suggesting that they might promote phagocytosis in different ways. Other laminin (YIGSR, IKVAV) and fibronectin (GRGDS) peptides have no effect on gelatin phagocytosis. Immunofluorescence shows that the $\alpha 3$ and the $\beta 1$, but not the $\alpha 2$ integrin subunit, concentrate into patches on the cell surface after treatment with their respective mAbs. And, both gelatin and the $\alpha 3\beta 1$ but not the $\alpha 2\beta 1$ integrin are cointernalized and routed to acidic vesicles such as lysosomes. In conclusion, we demonstrate that human breast cancer cells locally degrade and phagocytose the extracellular matrix and show for the first time that the $\alpha 3\beta 1$ integrin participates in this phagocytosis. We hypothesize that the anti-$\alpha 3$ antibodies and the laminin peptide HGD-6 activate the $\alpha 3\beta 1$ integrin, which results in a downstream signaling cascade stimulating phagocytosis.

INTRODUCTION

Integrins are a family of integral membrane glycoprotein cell surface receptors that mediate cell-to-extracellular matrix and cell-to-cell interactions and that regulate biological activities such as adhesion, migration, proliferation, development, differentiation, and tumor dissemination (reviewed by Hynes, 1992; Juliano and Varner, 1993). They consist of noncovalently bound α and β subunits that associate in various combinations to form integrin heterodimers with a variety of ligand-binding specificities. Integrins connect the extracellular matrix to the cytoskeleton and, therefore, transmit biological signals into the cell (reviewed by Yamada and Miyamoto, 1996). The $\alpha 3\beta 1$ integrin (VLA3) is a promiscuous receptor reported to bind different forms

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of laminin such as murine laminin (Wayner and Carter, 1987), human laminin (Gehlsen *et al.*, 1989), merosin (Chang *et al.*, 1995), epiligrin/kalinin/nicein (Carter *et al.*, 1991; Weitzman *et al.*, 1993; Delwel *et al.*, 1994) but also to collagen (Wayner and Carter, 1987; Elices *et al.*, 1991), fibronectin (Wayner and Carter, 1987; Elices *et al.*, 1991), and entactin (Dedhar *et al.*, 1992). Binding of the $\alpha 3\beta 1$ integrin to other integrins such as $\alpha 2\beta 1$ (Symington *et al.*, 1993) and to itself (Sriramarao *et al.*, 1993) is reported, but seems not to be a widespread phenomenon (Weitzman *et al.*, 1995). The specificity of $\alpha 3\beta 1$ for its ligands depends on the presence of other integrins that may display a higher avidity for the same ligands and on the presence of bivalent cations (Elices *et al.*, 1991). The cellular activities reported to be mediated by the $\alpha 3\beta 1$ integrin include adhesion and spreading (Weitzman *et al.*, 1993), migration (Yoshinaga *et al.*, 1993), cell-to-cell interaction (Sriramarao *et al.*, 1993; Symington *et al.*, 1993), collagenase synthesis (Larjava *et al.*, 1993), morphogenesis (Berdichevsky *et al.*, 1994), proliferation (Chang *et al.*, 1995), extracellular matrix assembly (Wu *et al.*, 1995), and differentiation (Symington and Carter, 1995). Gehlsen *et al.* (1989) determined that the region in laminin that is recognized by the $\alpha 3\beta 1$ integrin is in the carboxy-terminal region at the end of the long arm. They further localized this binding site to a 22-amino acid sequence in the globular domain of the murine and human laminin α chains (Gehlsen *et al.*, 1992). A synthetic peptide corresponding to this binding site in murine laminin (GD-6) was linked to Sepharose and specifically retained the $\alpha 3\beta 1$ integrin from cell extracts that could be eluted with exogenous GD-6 peptide. The synthetic GD-6 peptide has been reported to support cell adhesion (Wilke and Skubitz, 1991; Gehlsen *et al.*, 1992), inhibit cell attachment on laminin-coated surfaces (Gehlsen *et al.*, 1992), and stimulate cell migration (chemokinesis; Harvath *et al.*, 1994).

Phagocytosis is defined as the receptor- and actin-dependent internalization of large particles or microorganisms into large vesicles (phagosomes) that subsequently mature into phagolysosomes (terminology taken from Rabinovitch, 1995; reviewed by Brown, 1995). It is mainly associated with infection, inflammation, and wound repair where it is necessary for the destruction of microorganisms, damaged or senescent cells, and pollutant particulates. Consequently, this process is primarily studied in professional phagocytes such as polymorphonuclear granulocytes, macrophages, and monocytes. Phagocytosis also occurs in epithelial cells, fibroblasts, and other cells in vivo and in culture (Rabinovitch, 1995). Interestingly, malignant tumor cells and macrophages have several cellular activities in common such as degradation of the surrounding tissues, invasion, transport via the blood or lymph vessels, extravasation, and proliferation at distant sites in the host (reviewed by Opdenakker and Van Damme, 1992). We previously reported the endocytosis of colloidal gold-labeled laminin by mammary gland cell lines (Coopman *et al.*, 1991). Phagocytosis has been observed in virally transformed mouse and human epitheloid cervix carcinoma cells (Van Peteghem *et al.*, 1980), rat glioma cells (Bjerknes *et al.*, 1987), transformed chicken embryo fibroblasts (Mueller and Chen, 1991), and malignant human breast cancer cells (Montcourrier *et al.*, 1994), but the mechanisms and the receptors involved are not yet elucidated.

In macrophages, phagocytosis is mainly mediated by IgG-Fc and complement receptors (reviewed by Brown, 1995). Recent experiments suggest a functional role for $\beta 3$ integrins (cytoadhesins) in phagocytosis by hematopoietic cells such as the $\alpha v\beta 3$ vitronectin receptor (Blystone *et al.*, 1995; Savill *et al.*, 1990), the $\alpha IIb\beta 3$ fibrinogen receptor (Ylänne *et al.*, 1995), and the leukocyte response integrin (Gresham *et al.*, 1989), which was characterized as a novel $\beta 3$ integrin-like receptor (Carreno *et al.*, 1993). It has been proposed that the engagement of integrins with integrin ligand-opsonized prey leads to an increased ingestion via IgG-Fc receptors (Brown and Goodwin, 1988). However, the exact function of the integrins in phagocytosis is unknown (Greenberg, 1995). Interestingly, the $\alpha v\beta 5$ integrin was shown to mediate endocytosis of the conformationally altered heparin-binding form of vitronectin in human fibroblasts (Panetti and McKeown-Longo, 1993). Multiple $\beta 1$ integrins were reported to mediate the entry of bacteria into mammalian cells via the interaction of the integrin with the bacterial outer membrane protein invasin (Isberg and Leong, 1990). Endocytosis of $\beta 1$ integrins has been described also in the absence of bacteria (Raub and Kuentzel, 1989; Bretscher, 1992; Gaietta *et al.*, 1994), but its physiological role was not reported.

Cancer cell invasion depends on a repertoire of cell behaviors including reduced cell adhesion, reduced cell-to-cell contact, increased proteolysis at the cell surface, and increased migration (reviewed by Monsky and Chen, 1993; Stetler-Stevenson *et al.*, 1993). We are studying the interactions between cancer cells and extracellular matrix molecules and have developed an in vitro model to identify localized invasion using microscopy to detect holes made by invasive cells in fluorescent cross-linked gelatin films (Chen, 1989; Mueller and Chen, 1991). This technique identifies subcellular membrane protrusions that participate in matrix adhesion and degradation. We have called them invadopodia to distinguish them functionally from focal adhesion contacts, rosettes, or podosomes that are expressed when cells are cultured on glass. Among the constituents of invadopodia, we can distinguish proteolytic enzymes (Monsky and Chen, 1993; Monsky *et al.*, 1993; Kelly *et al.*, 1994), cytoskeletal molecules, and as yet undefined tyrosine-phos-

phorylated molecules suggesting the occurrence of complex signaling events in invadopodia (Mueller *et al.*, 1989; Mueller *et al.*, 1992). Using immunoelectron microscopy of transformed chicken embryo fibroblasts, we demonstrated the presence of the integrin $\beta 1$ subunit in invadopodia and in phagocytic vesicles associated with fragments of the matrix (Mueller and Chen, 1991).

In the present study, we demonstrate that malignant human breast cancer cells locally degrade and phagocytose the extracellular matrix and show that the $\alpha 3\beta 1$ integrin is involved in this phagocytosis.

MATERIALS AND METHODS

Cells

The human breast cancer cell line MDA-MB-231 was isolated from a pleural effusion of a human breast carcinoma and was obtained from the Tissue Culture Shared Resources of the Lombardi Cancer Center and originated from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in Falcon (Becton and Dickinson Labware, Plymouth, England) flasks in a 1:1 mixture of DMEM and RPMI 1640 (Mediatech, Washington, DC) supplemented with 10% bovine calf serum (Hyclone, Logan, UT), 5% NU-serum (Collaborative Biomedical Products, Becton and Dickinson Labware, Bedford, MA), 2 mM L-glutamine (Life Technologies, Grand Island, NY), 1 U/ml penicillin, and 10 μg/ml streptomycin (Life Technologies), which is referred to throughout as complete culture medium.

Antibodies

The following antihuman integrin mAbs were used: rat anti-$\beta 1$ (mAb13, kindly provided by Dr. S. Akiyama, National Institute of Dental Research, Bethesda, MD), mouse anti-$\beta 3$ (AP3; ATCC), mouse anti-$\alpha 2$ (P1E6; Becton and Dickinson Immunocytometry Systems, San Jose, CA and Telios, San Diego, CA), mouse anti-$\alpha 3$ (P1B5, Becton and Dickinson and Telios; M-KID2, Immunotech, Westbrook, ME; 11G5, Serotec, Washington, DC), mouse anti-$\alpha 4$ (P4G9; Telios), rat anti-$\alpha 5$ (mAb11 and mAb16; kindly provided by Dr. S. Akiyama), mouse anti-$\alpha 6$ (4F10; Serotec), and rat anti-$\alpha 6$ (GoH3; Serotec) and mouse anti-αv (L230; ATCC). The rat mAb C27 (kindly provided by Dr. W.T. Chen, Department of Cell Biology, Georgetown University Medical School, Washington, DC) was raised against the antigen derived from isolated shed membrane vesicles from LOX human melanoma cells. The rat C27 mAb was coupled to cyanogen bromide-activated Sepharose and used to immunoaffinity purify the antigen from the MDA-MB-231 human breast cancer cells. Immunoblotting and microsequencing of affinity-purified C27 antigen showed that this mAb recognizes a 120-kDa polypeptide that is also recognized by the mAb13 anti-$\beta 1$ antibody and therefore identifies it as the $\beta 1$ integrin subunit. The polyclonal rabbit anti-human laminin antiserum and the monoclonal mouse anti-human laminin α chain globular domain antibody were obtained from Life Technologies, and the polyclonal rabbit anti-mouse laminin antiserum was obtained from Telios. All fluorescently labeled affinity-purified antimouse and antirat IgG + IgM secondary antibodies (fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), Texas Red) were obtained from Jackson Immunoresearch Laboratories (West Grove, PA) and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Rockland (Gilbertsville, PA).

Reagents

Porcine skin gelatin, Lucifer Yellow, and the actin polymerization inhibitor cytochalasin D were purchased from Sigma (St. Louis, MO), the acidotropic probe LysoTracker red and the filamentous actin dye TRITC-phalloidin were purchased from Molecular Probes (Eugene, OR), and human placenta laminin was purchased from Life Technologies. The basement membrane matrix Matrigel was purchased from Collaborative Biomedical Products. The laminin peptides HGD-6 (KQKCLRSQTSFRGCLRKLALIK), HSGD-6 (KQCLKSQRSFTRGLCRLKAKIL), KRGD (VEKRGDREEA) (Gehlsen *et al.*, 1992) were synthesized by the La Jolla Cancer Research Foundation (La Jolla, CA) and Genosys (The Woodlands, TX). The laminin peptides YIGSR and IKVAV and the fibronectin peptides GRGDS and GRGES were kindly provided by Dr. M. Nomizu (National Institute of Dental Research).

FACS-Phagocytosis

Porcine skin gelatin and Matrigel were labeled overnight by dialysis against FITC (Research Organics, Cleveland, OH) as described by the manufacturer. The labeling was done at either 37°C (gelatin) or 4°C (Matrigel). The unbound FITC was removed by a 3-day dialysis against multiple changes of phosphate-buffered saline (PBS) followed by chromatography on a Sephadex G25 M column (Pharmacia, Uppsala, Sweden).

Precooled plastic 24-well plates (Costar, Cambridge, MA) were coated with a thin film ($\pm$100 μl/well) of warmed FITC-gelatin/sucrose (2%/2% in PBS) for 15 min at 4°C and then cross-linked with 0.5% glutaraldehyde in PBS (15 min at 4°C). The free aldehyde groups were quenched with DMEM (1 h at 37°C) as described for films on coverslips (Mueller *et al.*, 1992). Thin FITC-Matrigel films (0.2% in serum-free medium) gel at 37°C and were therefore not cross-linked with glutaraldehyde. Cross-linked FITC-gelatin beads were prepared as described earlier and were selected by differential centrifugation either for a subcellular size (diameter <10 μm) (Mueller *et al.*, 1989) or a size much larger than a cell (diameter >100 μm) (Mueller and Chen, 1991).

For the quantification of phagocytosis, 150,000 cells were seeded per coated well in 0.5 ml of serum-containing or serum-free culture medium. Cells were allowed to attach and spread for 3 h or overnight at 37°C in a humid atmosphere containing 7.5% CO_2. Then they were subsequently detached with trypsin/EDTA, washed in serum-containing medium and PBS, and fixed in 0.3% paraformaldehyde in PBS. The amount of phagocytosed FITC-matrix (mean value of the fluorescence intensity of 10,000 cells) was determined by fluorescent activated cell sorting (FACStar Plus; Becton and Dickinson) and expressed as fold over background (cells incubated on unlabeled matrix). The background settings for FACS were, however, similar in all experiments. The stimulation of FITC-matrix uptake is expressed as fold over untreated cells (phagocytosis of FITC-gelatin without treatment), and treatments with either the P1B5 anti-$\alpha 3$ mAb or the HGD-6 laminin peptide were included as positive controls and to judge the magnitude of the stimulation by other reagents. Trypan blue controls to quench extracellular fluorescence were tested but not found to be necessary since the trypsin/EDTA treatment of cells before FACS analysis completely removed the cell surface-associated fluorescence.

Immunocytochemistry

For microscopic observation of localized gelatin degradation, we developed a variant on a previously described assay (Mueller *et al.*, 1992) and used glutaraldehyde cross-linked FITC-gelatin films instead of unlabeled gelatin films coated with a thin layer of FITC-fibronectin. For this gelatin degradation assay as well for general immunostaining of cells, 50,000 cells were incubated overnight at 37°C in 2 ml of serum-containing or serum-free culture medium on 18 mm in diameter glass coverslips, plain or coated with FITC-gelatin films (see FACS-Phagocytosis). Cell surface immunostaining was done on nonpermeabilized living cells that were fixed with 3.7% formaldehyde in PBS only after incubation with the primary antibodies (15 min at 4°C). For total cell immunostaining, cells were

fixed with 3.7% formaldehyde/0.1% Triton X-100 in PBS for 15 min and further permeabilized in 0.5% Triton X-100 in PBS for 15 min at room temperature. All primary antibodies were used at a 1:100 dilution in PBS, all secondary antisera at a 1:100 (FITC and TRITC conjugates) or a 1:400 (Texas Red conjugates) dilution in PBS, and all incubations were done for 15 min at room temperature. Actin filaments were visualized in permeabilized cells using TRITC-phalloidin (1:400). For detection of acidic vesicles, living cells were incubated for 3 h at 37°C in culture medium containing 0.1 μM of the acidotropic probe LysoTracker red (Molecular Probes) and fixed without permeabilization. Coverslips were permanently mounted using the ProLong antifade kit (Molecular Probes).

Fluorescence photomicrographs of fixed cells were made using a Zeiss Photomicroscope III (Carl Zeiss, Thornwood, NY) equipped with epifluorescence and a Planapo 63 X/1.4 NA phase 3 objective. Confocal microscopy was done using a Bio-Rad 600 confocal laser scanning microscope equipped with an argon laser (Bio-Rad, Hercules, CA). Living cells in culture were photographed with a Zeiss IM35 inverted microscope equipped with a 16× phase 2 objective. Images were digitized from photographic negatives using a Zeiss IBAS 2000 image analyzer coupled to a Panasonic WV-CD50 black and white camera and lightbox.

Quantification of Cell Surface Integrin Expression by FACS

Subconfluent cell monolayers were detached with 0.02% Na_2-EDTA in PBS. After two washes in cold PBS, 300,000 cells were incubated under gentle rotation in 0.4 ml of a 1:50 dilution of anti-integrin mAbs for 15 min at 4°C. After three washes in cold PBS, cells were incubated in 0.4 ml of a 1:200 dilution of donkey anti-mouse-FITC or donkey anti-rat-FITC for 15 min at 4°C. After fixation of the cells in 3% paraformaldehyde in PBS, samples were diluted down to 0.3% paraformaldehyde and the fluorescence intensity (mean value of 10,000 cells) was quantified by FACS. The cell surface-associated fluorescence was expressed as fold over background (cells incubated with secondary antiserum alone).

Cell Surface Biotin Labeling and Immunoprecipitation

A subconfluent monolayer cell culture was washed with PBS containing calcium and magnesium and incubated for 30 min at 4°C with sulfo-N-Hydroxysulfosuccinimide-biotin (0.5 μg/ml in PBS with calcium and magnesium; Pierce, Rockford, IL) under gentle agitation. After several PBS washes, cells were lysed and scraped in an extraction buffer containing 50 mM Tris (pH 8), 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Equal protein amounts (150 μg, as determined with the bicinchoninic acid assay; Pierce) were incubated with 5 μl of anti-integrin mAb for 1.5 h at 4°C. Protein A-Sepharose beads (10 μl suspension; Boehringer Mannheim, Indianapolis, IN) were preincubated with 5 μg of unlabeled rabbit anti-rat or rabbit anti-mouse serum for 1.5 h at 4°C and then added to the cell lysate/mAb mixtures and further incubated for 1.5 h at 4°C. In negative controls, the primary mAbs were omitted from the immunoprecipitation. After six washes with 0.1 M Tris (pH 8.6)/0.5 M LiCl/1% Tween 20, proteins were removed from the Sepharose beads by boiling and separated using 8% SDS-polyacrylamide gels. After electroblotting on nitrocellulose, immunoprecipitated proteins were detected with streptavidin-HRP (1:5000; Jackson) using the Enhanced Chemiluminescence method (Amersham, Arlington Heights, IL).

For laminin immunoprecipitation, complete culture medium was conditioned overnight by MDA-MB-231 cells and concentrated to 20% of its original volume using a Centriprep 10 concentrator (Amicon, Beverly, MA). One milliliter of concentrated conditioned medium was incubated with 5 μl of polyclonal antiserum or monoclonal antihuman laminin antibodies and protein A-Sepharose beads alone (for polyclonal antilaminin) or preincubated with unlabeled rabbit anti-mouse antiserum (for monoclonal antilaminin) as described above for the integrin immunoprecipitation. In negative controls, the primary antiserum was omitted from the immunoprecipitation. Proteins were separated on 6% SDS-polyacrylamide gels under reducing conditions, and after electroblotting on nitrocellulose, laminin was detected using a polyclonal rabbit anti-mouse laminin antiserum (1:1000) and goat anti-rabbit-HRP (1:10000) and Enhanced Chemiluminescence. Purified human placenta laminin (Life Technologies) was used as a positive control (5 μg/lane).

Transmission Electron Microscopy

Five hundred thousand cells per ml complete culture medium were coincubated overnight with approximately 2×10^6 large (>100 μm) gelatin beads at 37°C under continuous end-over-end rotation to promote the even attachment and spreading of the cells to the surface of the beads. The pellet of gelatin beads coated with cells was then fixed with 2% glutaraldehyde/3% paraformaldehyde in PBS for 2 h, postfixed in 1% osmium tetroxide in distilled water for 1 h, and processed for electron microscopy as described previously (Mueller and Chen, 1991). Sections were observed with a JEOL 1200EX transmission electron microscope operated at 60 kV.

RESULTS

The α3β1 Integrin Is Present in Invadopodia and Colocalizes with Sites of Local FITC-Gelatin Degradation

Transformed cells and some human cancer cells exhibit cell surface extensions, called invadopodia, that project into the surrounding extracellular matrix and that are actively involved in localized degradation of this matrix (Monsky and Chen, 1993). Here, we show that invadopodia are also present in the malignant human breast carcinoma cells MDA-MB-231. These cells actively degraded the underlying gelatin matrix as demonstrated by the disappearance of the FITC-gelatin (Figure 1,B, D, F, and H) under the cells. Degradation spots (Figure 1B) colocalized with actin concentrations in the cell (Figure 1A), thereby confirming the involvement of active cell membrane movements of the invadopodia. They appear as dots since they project downward into the matrix. Immunofluorescent staining also showed the colocalization of the integrin subunits β1 and α3 (Figure 1, C and E), but not α2 (Figure 1G), with local degradation of gelatin films (Figure 1, D, F, and H). The β1-containing heterodimers were concentrated in invadopodia (dots) and were also diffusely distributed over the cell surface. They may also be present in focal adhesion contacts (streaks, not seen in Figure 1C but visible in Figure 6, E and G). Also the α3 integrin subunit was concentrated in invadopodia and localized diffusely on the cell membrane (Figure 1E), whereas the α2 subunit showed only diffuse staining (Figure 1G) that did not colocalize with FITC-gelatin degradation spots (Figure 1H). As seen also in Figure 2, the α2 integrin staining was less intense than β1 or α3. Because of the dynamic nature of the invadopodia (evaluated by videomicroscopy in Chen, 1989), not all α3 or β1 inte-

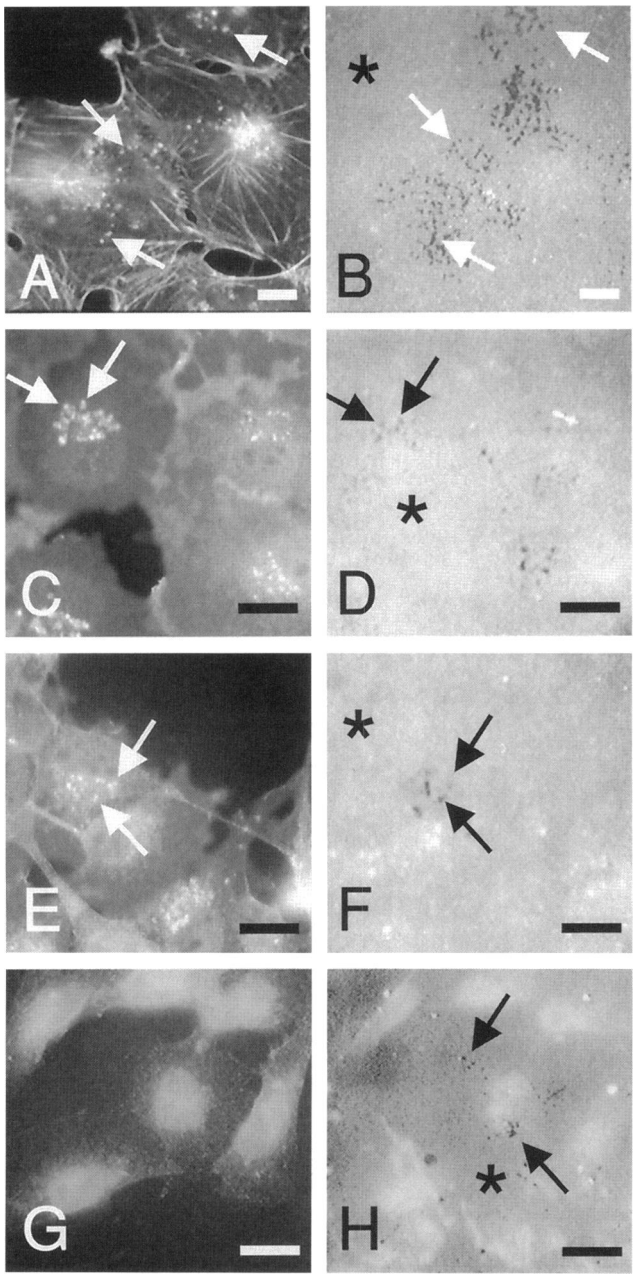

Figure 1. The α3β1 integrin is present in invadopodia and colocalizes with sites of local FITC-gelatin degradation. MDA-MB-231 cells were cultured overnight on glass coverslips coated with cross-linked FITC-gelatin films in complete culture medium as described in MATERIALS AND METHODS. After fixation and permeabilization, cells were stained with TRITC-phalloidin detecting filamentous actin in active cell membrane extensions (invadopodia, A) or immunostained for the β1 (mAb C27, C), α3 (mAb P1B5, E) and α2 (mAb P1E6, G) integrins using Texas Red-labeled anti-rat (for β1) or anti-mouse (for α2 and α3) antiserum as described in MATERIALS AND METHODS. Actin is observed as cables and dots (A, arrows) that colocalize with FITC-gelatin degradation spots (B, arrows). Both β1 (C) and α3 (E) integrin subunits were diffusely present all over the cell surface, but appeared also concentrated in invadopodia (dot-like, arrows) colocalizing with FITC-gelatin degradation spots (D and F, arrows). The α2 integrin subunit (G) was not concentrated in invadopodia but diffusely present all over the cell surface and did not colocalize with FITC-gelatin degradation spots (H, arrows). *, FITC-gelatin films. Bar, 10 μm.

grin staining in invadopodia colocalized with FITC-gelatin degradation spots and vice versa at a given time point. Gelatin under newly formed invadopodia might not yet be degraded, whereas formerly active invadopodia might have disappeared leaving zones of degraded gelatin.

Breast Cancer Cells Locally Degrade and Phagocytose Cross-linked FITC-Gelatin Matrix

Using confocal microscopical sectioning of cells incubated on thick FITC-gelatin films (~10 μm as determined by confocal scanning in the vertical dimension), the fluorescent matrix was detected in vesicles inside the cells demonstrating its internalization (Figure 2, A and B). Using transmission electron microscopy of cells attached and spread on large gelatin beads (diameter >100 μm), cell membrane extensions (invadopodia) were observed projecting into the gelatin and were associated with vesicles in the process of forming that contain fragments of the gelatin matrix (Figure 2C). The term phagocytosis is used for the uptake of relatively large particles (~0.5 μm) by cells. We therefore tested whether the MDA-MB-231 cells could also take up FITC-gelatin beads with a subcellular size (diameter <10 μm). Active uptake of beads could be followed in living cells by a phase shift using phase-contrast microscopy: beads appeared to be black when extracellular and white after uptake by the cells (Figure 2D). After washing and fixation, intracellular beads were detected with fluorescence microscopy (Figure 2, E and F). Moreover, the fluorescence could be exclusively associated with the bead or diffusely spread over the cytoplasm, suggesting that intracellular degradation of the gelatin bead led to the release of the FITC label which subsequently diffused into the cytoplasm. These results demonstrate that cells not only locally degrade but also actively phagocytose the underlying gelatin matrix.

Anti-α3 Integrin Antibodies Stimulate Phagocytosis of Gelatin Films or Beads and Matrigel Films

Based on the observation that MDA-MB-231 cells not only locally degrade the underlying gelatin matrix but also ingest it, we developed an assay to quantify the uptake of fluorescent matrix using FACS analysis. As expected for phagocytosis, treatment of MDA-MB-231 cells with the actin polymerization inhibitor cytochalasin D (1 μM, 3 h) decreased the uptake of FITC-gelatin by ~27% on average and maximally 42%. Since immunofluorescent colocalization of the α3β1 integrin

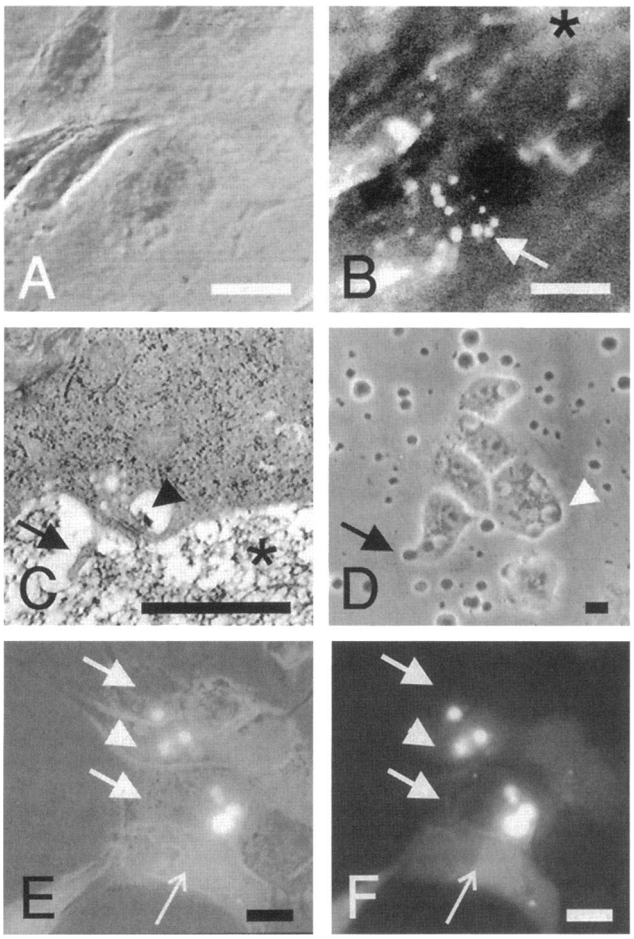

Figure 2. Phagocytosis of cross-linked FITC-gelatin matrix. MDA-MB-231 cells in complete culture medium were cultured overnight on cross-linked FITC-gelatin films or beads as described in MATERIALS AND METHODS. Phase-contrast (A) and FITC-gelatin fluorescence (B) confocal laser scanning microscopy demonstrates the active uptake of FITC-gelatin from coated films (*) and its localization in intracellular vesicles (arrow). Transmission electron microscopy of cells attached and spread on >100 μm in diameter gelatin beads (*) shows cell membrane extensions into the gelatin (arrow) and intracellular gelatin particles (arrowhead, C). Uptake of subcellular sized gelatin beads was monitored on living cells using phase-contrast microscopy (D). Because of a phase shift, extracellular beads are black (arrow) and can be distinguished from intracellular beads which are white (arrowhead). Comparing mixed phase-contrast-fluorescence (E) with exclusive fluorescence (F) microscopy images, intracellular FITC appeared to be associated with only the bead in some cells (thick arrows), diffusely distributed all over the cytoplasm in other cells (thin arrow), or a mixture of both (arrowhead). Bars, 50 μm (A and B); 10 μm (D–F); or 1 μm (C).

with sites of localized gelatin degradation suggests an involvement of this integrin in degradation and uptake of the extracellular matrix, we tested the effect of a series of anti-integrin antibodies against different integrin subunits on the phagocytosis of FITC-gelatin by MDA-MB-231 cells (Figure 3). In an initial screening, unpurified mAbs (hybridoma supernatant or ascites) were used and the assay was performed in serum-containing medium. All other experiments were done in the absence of serum, using purified antibodies to confirm the initial results. Two different clones of anti-α3 mAbs (P1B5 and M-KID2) significantly stimulated the uptake of FITC-gelatin, but had no additive effects when present together, suggesting that they recognize a similar epitope. The anti-α3 mAb (P1B5) was routinely included as a positive control in phagocytosis assays testing other anti-integrin mAbs to compare the magnitude of the responses. The two- to threefold stimulation of FITC-gelatin uptake by MDA-MB-231 cells in the presence of the P1B5 anti-α3 mAb was consistently observed in more than 50 experiments. This stimulation was not restricted to MDA-MB-231 cells, but also occurred with the BT549

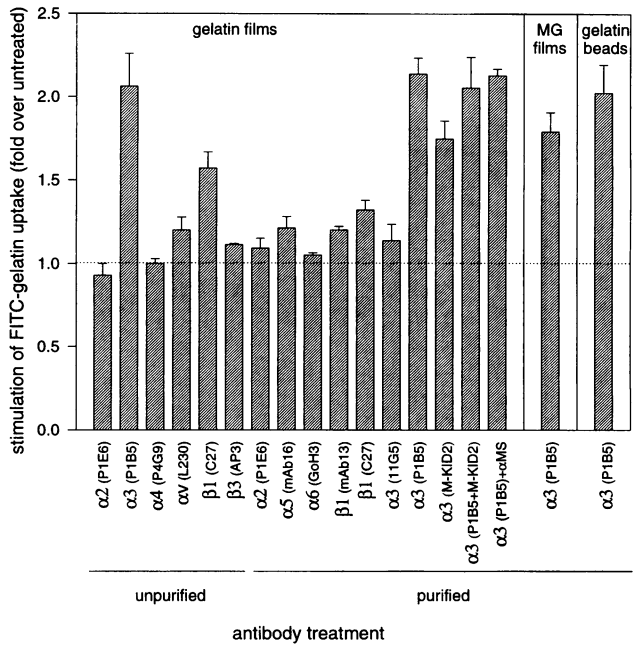

Figure 3. Anti-α3 integrin antibodies stimulate phagocytosis of FITC-gelatin films or beads and FITC-Matrigel films. The uptake of FITC-labeled extracellular matrix by cells was quantified using FACS as described in MATERIALS AND METHODS (FACS-phagocytosis assay). MDA-MB-231 cells were incubated overnight on 24-well plates coated with FITC-gelatin or FITC-Matrigel films or with subcellular sized FITC-gelatin beads in the presence of unpurified (2 μl of anti-α2, -α3, and -α4 ascites or 10 μl of anti-αv, -β1, or -β3 hybridoma supernatant) or purified monoclonal antibodies (5 μg/ml; mAb clones are indicated between parentheses). Complete culture medium was used for testing unpurified antibodies and serum-free culture medium for testing purified antibodies. In one case, cells were incubated with a combination of the anti-α3 mAb (P1B5) and unlabeled rabbit anti-mouse (αMS) antiserum (5 μg/ml). The background fluorescence was determined on cells incubated on unlabeled gelatin or Matrigel. Dotted line, amount of FITC-gelatin phagocytosis by untreated cells. The stimulation of FITC-gelatin or FITC-Matrigel (MG) phagocytosis is expressed as fold over untreated cells (phagocytosis of FITC-matrix without treatment) and represents the mean ± SEM of 4–22 experiments.

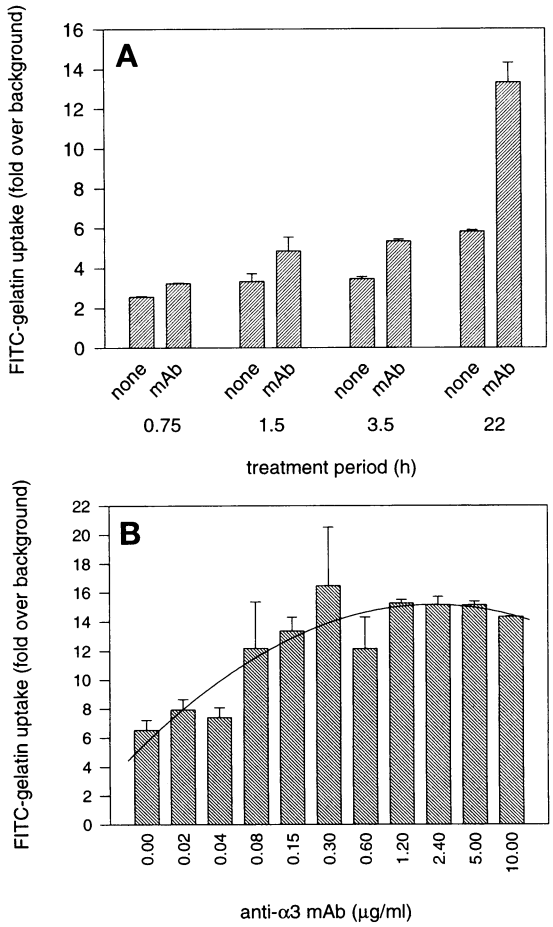

Figure 4. The stimulation of gelatin phagocytosis by anti-α3 integrin antibodies is time and dose dependent and saturable. The FACS-phagocytosis assay to quantify uptake of FITC-gelatin was performed as described in MATERIALS AND METHODS. For the time course (A), MDA-MB-231 cells were incubated on FITC-gelatin films for different periods in the presence of an anti-α3 integrin monoclonal antibody (P1B5, 5 μg/ml) in serum-free culture medium. For the dose response (B), MDA-MB-231 cells were incubated overnight on FITC-gelatin films in serum-free culture medium in the presence of different concentrations of an anti-α3 integrin monoclonal antibody (P1B5). The amount of phagocytosed FITC-gelatin is expressed as fold over background (cells incubated on unlabeled matrix) and represents the mean ± SEM of two to four experiments. A regression curve (second order) indicates that the saturation point of stimulation by the anti-α3 mAb is situated around 1.2 μg/ml.

human breast cancer cell line expressing the α3β1 integrin (our unpublished observation). The 11G5 anti-α3 mAb clone, on the contrary, had no significant effect ($p = 0.22$, t test) and is described by the manufacturer not to display any biological activity. A lower, but still significant, stimulation of phagocytosis was also observed with monoclonal antibodies recognizing the β1 integrin subunit (mAb13, $p < 0.005$ and C27, $p < 0.001$, t test). All other tested antibodies (α2, α4, α5, α6, αv, and β3) had no significant effect on phagocytosis of FITC-gelatin. This is not surprising for the anti-α4 and the anti-β3 antibodies since MDA-MB-231 cells did not show detectable levels of these integrins (Figure 5). Anti-integrin mAbs might have a stimulatory or inhibitory effect or display no biological activity at all. We can therefore not exclude an eventual role for the α2-, α5-, α6-, or αv-containing integrins in gelatin phagocytosis solely based on experiments using one mAb clone. We have chosen, however, to

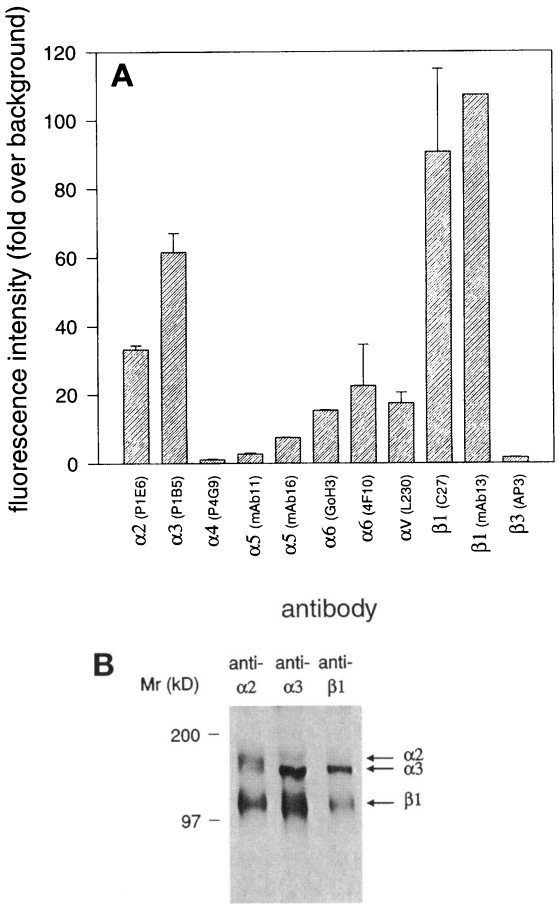

Figure 5. Integrin expression on MDA-MB-231 cells. (A) For quantification of cell surface integrin expression by FACS, MDA-MB-231 cells were incubated with different anti-integrin mAbs (antibody clones in parentheses) and FITC-labeled secondary antiserum as described in MATERIALS AND METHODS. The background staining was quantified by incubation of cells with only FITC-labeled secondary antiserum. Data represent mean ± SEM of the relative fluorescence intensity (fold over background) of two to six experiments. (B) Extracts of surface biotin-labeled MDA-MB-231 cells were immunoprecipitated with anti-α2 (P1E6), anti-α3 (P1B5), or anti-β1 (C27) integrin mAbs as described in MATERIALS AND METHODS. Immunoprecipitates were boiled and separated by 8% SDS-polyacrylamide gels. After electroblotting on nitrocellulose, immunoprecipitated proteins were detected with streptavidin-HRP and enhanced chemiluminescence. M_r, relative molecular weight. Arrows indicate the relative positions of the α2, α3, and β1 integrin subunits.

focus our study on the $\alpha3\beta1$ integrin using the stimulatory P1B5 anti-$\alpha3$ integrin mAb and the nonstimulatory P1E6 anti-$\alpha2$ integrin mAb. Both $\alpha2\beta1$ and $\alpha3\beta1$ are major integrins in the MDA-MB-231 cells (Figure 5). The stimulatory effect of the anti-$\alpha3$ (P1B5) mAb was also observed on the ingestion of FITC-gelatin beads, confirming the phagocytic nature of the uptake. For all further experiments on the quantification of uptake of the fluorescent matrix, we preferred to use fluorescent films instead of beads so that cell-to-matrix interactions would be more uniform. The use of films also better reflects the in vivo situation where cells have to locally degrade the matrix before taking it up. In addition, the anti-$\alpha3$ (P1B5) mAb also stimulated the uptake of FITC-Matrigel, a reconstituted basement membrane. The response to antibodies is therefore not restricted to gelatin. In contrast, the pinocytic uptake of Lucifer Yellow in solution (1 mg/ml) was not significantly stimulated by the anti-$\alpha3$ mAb (P1B5, 5 μg/ml; 1.2 $\pm$ 0.07-fold stimulation) or the anti-$\beta1$ mAb (C27, 10 μg/ml; 0.99 $\pm$ 0.01-fold stimulation) as determined by FACS. The additional presence of secondary antibodies did not further increase the stimulation of FITC-gelatin uptake by the anti-$\alpha3$ (P1B5) mAb.

Anti-$\alpha3$ Integrin Antibodies Stimulate Phagocytosis of FITC-Gelatin Films in a Time- and Dose-dependent Manner

To further characterize the stimulatory effects of the anti-$\alpha3$ mAb (P1B5) on phagocytosis of FITC-gelatin by MDA-MB-231 cells, we determined the time and dose responsiveness of the anti-$\alpha3$ stimulation. The time course (Figure 4A) shows that phagocytosis is a very fast event that was already measurable 45 min after seeding, even before cells were fully spread on the gelatin films. Moreover, a 1.3-fold stimulation of phagocytosis by the anti-$\alpha3$ mAb was already detectable after 45 min and reached a 1.5-fold stimulation after 3.5 h. Thereafter, it increased more slowly to its maximum level (2.3-fold) after 22 h. Maximum stimulation after overnight incubation varied generally between two- and threefold (see also Figure 3). Because of this early stimulation of phagocytosis by the anti-$\alpha3$ mAb, antibodies were added when cells were seeded and not after attachment and spreading. Except for the anti-$\beta1$ integrin mAb (C27) that retarded the spreading of MDA-MB-231 cells on gelatin, none of the other antibodies significantly affected cell attachment and spreading on gelatin films. The stimulation of phagocytosis by the anti-$\alpha3$ mAb (P1B5) was dose dependent and saturable with a maximal stimulation around 1.2 μg/ml (Figure 4B).

$\alpha3\beta1$ Is a Major Integrin in MDA-MB-231 Cells

The expression of cell surface integrins was quantified using FACS analysis. The $\alpha3$ and $\beta1$ subunits were highly expressed on MDA-MB-231 cells (Figure 5A). In addition, the anti-$\beta1$ C27 mAb was used to immunoaffinity purify $\beta1$ integrins from MDA-MB-231 cells. SDS-PAGE analysis of the immunoprecipitate showed the 120-kDa $\beta1$ integrin subunit and a copurifying polypeptide of 150 kDa that, after microsequencing (our unpublished data; Qing-Xiang Sang, Florida State University, Tallahasse, FL), demonstrated a complete identity with the human integrin $\alpha3$ subunit, suggesting that $\alpha3\beta1$ is a major $\beta1$ integrin on MDA-MB-231 cells. The $\alpha2$ integrin subunit was less abundant than $\alpha3$ as observed by FACS (Figure 5A), immunoprecipitation (Figure 5B), and immunofluorescence (Figures 1 and 6). The $\alpha4$ and $\beta3$ subunit levels were comparable to the background staining with secondary antiserum alone and therefore seem to be absent on MDA-MB-231 cells.

The association of the $\alpha3\beta1$ integrin was further confirmed by immunoprecipitation of extracts of surface biotin-labeled MDA-MB-231 cells with either anti-$\alpha3$ or anti-$\beta1$ mAbs (Figure 5B). This result also indicates that MDA-MB-231 cells express the 150-kDa $\alpha3$ subunit and not the recently reported 225-kDa hybrid of the $\alpha3$ integrin subunit heavy chain disulfide bonded to a transferrin receptor monomer (Coppolino *et al.*, 1995). No biotinylated proteins were detected after immunoprecipitation with protein A-Sepharose alone or in combination with secondary antiserum. Taken together, these results indicate that $\alpha3\beta1$ is the predominant $\beta1$ integrin in MDA-MB-231 cells.

The $\alpha3\beta1$ Integrin Forms Patches on the Cell Surface and Is Internalized after Treatment with mAbs

The FACS-phagocytosis experiments indicate that the gelatin matrix is taken up by the MDA-MB-231 cells and suggest a role for the $\alpha3\beta1$ integrin in this uptake. Since phagocytosis of gelatin is stimulated by the presence of anti-$\alpha3$ and anti-$\beta1$ antibodies, we can track the eventual translocation of these integrins coupled to antibodies after stimulation of phagocytosis. To directly demonstrate the intracellular presence of the $\alpha3$ and $\beta1$ integrin subunits, MDA-MB-231 cells were seeded on gelatin films in the presence of the stimulatory anti-$\alpha3$ (P1B5) and anti-$\beta1$ (C27) mAbs or the nonstimulatory anti-$\alpha2$ (P1E6) mAb. After overnight treatment, permeabilized and nonpermeabilized cells were incubated with secondary fluorescently labeled antiserum to trace the translocation of the treatment antibodies in and on the cells (Figure 6, B, D, F, H, and J). In addition, conventional immunofluorescence localization of the $\alpha2$, $\alpha3$, and $\beta1$ integrin subunits was done in permeabilized and nonpermeabilized control

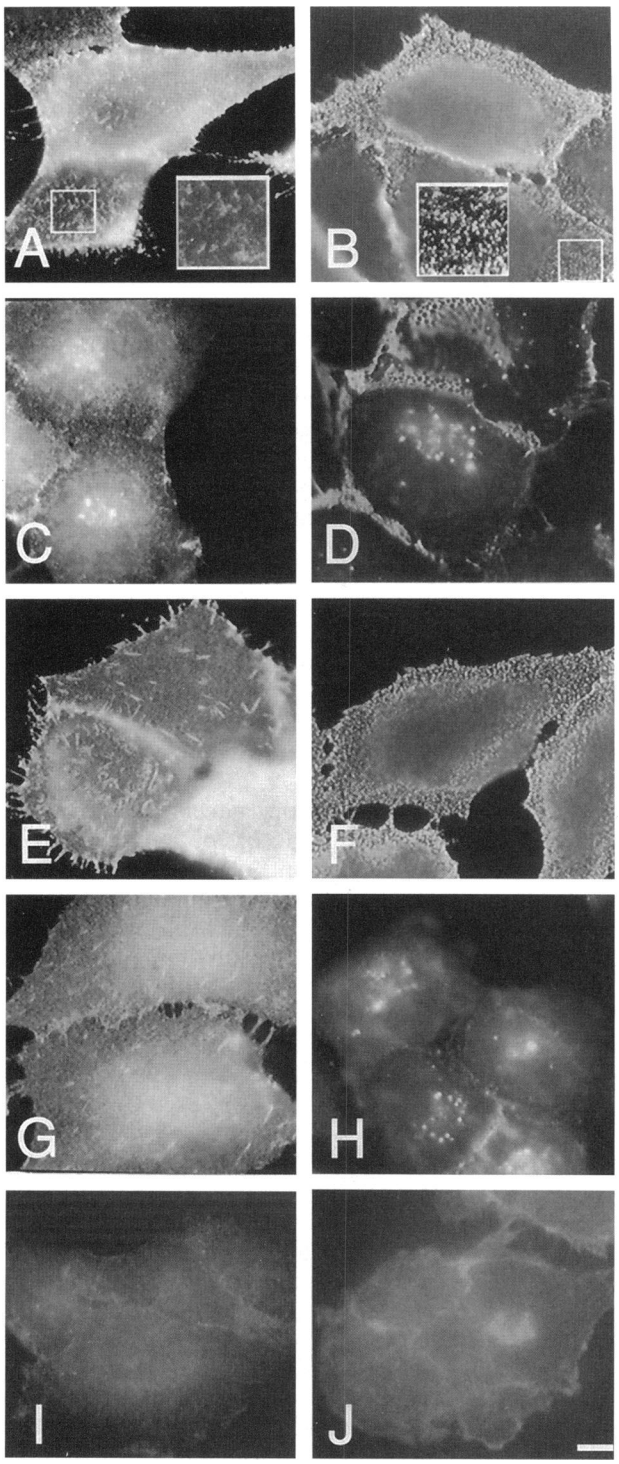

Figure 6. The α3β1 integrin forms patches on the cell surface and is internalized after treatment with monoclonal antibodies. MDA-MB-231 cells in complete culture medium were seeded on glass coverslips coated with cross-linked gelatin films as described in MATERIALS AND METHODS in the absence (left panels) or presence (right panels) of 5 μg/ml of anti-α3 (P1B5, B and D), anti-β1(C27, F and H), or anti-α2 (P1E6, J) anti-integrin monoclonal antibodies. After overnight attachment and spreading, the localization of the α3 (A and C), β1 (E and G), and α2 (I) integrins was detected on untreated cells (left panels) by conventional immunostaining of nonpermeabilized (cell surface, A and E) or Triton X-100 permeabilized (C, G, and I) cells using Texas Red-labeled anti-mouse (for α3 and α2) or TRITC-labeled anti-rat antiserum (for β1). After overnight incubation with anti-integrin antibodies (right panels), the localization of these treatment antibodies was traced by staining with secondary antiserum alone on nonpermeabilized (B and F) or in Triton X-100 permeabilized (D, H, and J) cells. Insets in A and B, 4.5-fold magnification of the zone indicated. Bar, 10 μm.

(untreated) cells to determine the localization of the entire population of each subunit (Figure 6, A, C, E, G, and I). The α3 subunit localized to the plasma membrane and was particularly abundant in cell surface protrusions (Figure 6A). The β1 subunit similarly localized to the plasma membrane but was more prominent in focal adhesions (Figure 6, E and G). Thus, the α3 localization identified the α3β1 integrin in a select plasma membrane domain, membrane protrusions, and invadopodia (Figures 1 and 6). The α2 subunit staining (Figures 6I and 1G) was diffuse and much less intense, corresponding to lower expression as quantified by FACS (Figure 5A). After overnight treatment with their respective antibodies, the α3 and β1, but not the α2 subunits, were found concentrated in patches on the cell surface (Figure 6, B and F) and were also present in intracellular vesicles (Figure 6, D and H). Some vesicular staining was observed in the β1 or α3 integrin staining (Figure 6C), but the abundance in antibody-treated compared with untreated cells suggests that this result reflects internalized rather than de novo synthesized integrin. Staining with only secondary antibodies did not reveal any significant signal. The relative absence of extracellular staining in permeabilized cells (Figure 6, D and H) was probably due to the extraction of the cell surface-bound treatment antibody during permeabilization and provided the opportunity to distinguish the extracellular from the intracellular signal. The presence of the lace-like meshwork at the cell-to-cell contacts in permeabilized anti-α3 mAb treated cells (Figure 6D) has been observed repeatedly, but its biological significance is not clear at this time. It may represent pockets of staining that are resistant to extraction due to cell-to-cell junctions.

In conclusion, these results indicate that treatment of MDA-MB-231 cells with anti-integrin mAbs induces an overall cell surface clustering and internalization of a subpopulation of the α3β1, but not the α2β1 integrin.

The α3β1 Integrin and Gelatin Are Cointernalized and Routed to Acidic Vesicles

Using FACS-phagocytosis and immunocytochemistry, we demonstrated that anti-α3 and to a lesser extent the anti-β1 integrin antibodies stimulate the uptake of

gelatin by MDA-MB-231 cells and are internalized by these cells. We next examined whether the gelatin and the α3 integrin subunits are present in the same intracellular vesicles. We also checked whether these vesicles are acidic in nature. If this was so, it would suggest intracellular routing of the phagocytosed material to lysosomes.

Cells were incubated for 3 h on FITC-labeled or unlabeled gelatin films in the presence of LysoTracker, an acidotropic probe or an anti-α3 integrin antibody (P1B5) primed with a fluorescently labeled secondary antibody (see Materials and Methods). Most gelatin was found in vesicles that colocalized with the LysoTracker (Figure 7,A and B). Staining with LysoSensor, an acidotropic probe with a pH-sensitive spectral property, showed that these acidic vesicles had a pKa ~5.2. Alternatively, vesicles containing gelatin were observed that were not acidic, and conversely vesicles were present that were acidic but lacked gelatin. These gelatin-containing vesicles that were not acidic were probably phagosomes before fusion with lysosomes. Almost all gelatin colocalized with the α3β1 integrin in vesicles, but α3β1 was also found in vesicles not containing gelatin (Figure 7, C and D), possibly indicating internalization of molecules other than gelatin via the α3β1 integrin or constitutive α3β1 internalization. However, most α3β1 integrin was present in acidic vesicles (Figure 7, E and F), suggesting it was targeted to lysosomes. The α2β1 integrin was not detected in acidic vesicles, suggesting that it was not internalized by the cells (Figure 7, G and H). Immunofluorescent staining (Figure 7, I and J) and FACS analysis (our unpublished observation) demonstrated that cells did not internalize fluorescently labeled secondary antiserum alone, indicating that antibodies were not randomly internalized by pinocytosis. Treatment of MDA-MB-231 cells with cytochalasin D (0.5 μM, 2 h) inhibited the internalization of the α3β1 integrin, similar to the results on the inhibition of gelatin uptake. Thus, these observations of colocalization of α3β1 integrin, FITC-gelatin, and acidic vesicles indicate that gelatin and the α3β1 integrin are cointernalized in phagosomes and that both are subsequently routed to lysosomes.

The Synthetic HGD-6 Laminin Peptide Stimulates Phagocytosis Independent of and Additive to the Effect of Anti-α3 Antibodies

The α3β1 integrin has been reported to be primarily a receptor for molecules of the laminin family. Gehlsen *et al.* (1992) characterized a homologous amino acid sequence in the globular domains of the murine and human laminin α chains as a binding site for the α3β1 integrin. A synthetic peptide corresponding to this sequence in human laminin (HGD-6) stimulated the uptake of FITC-gelatin by MDA-MB-231 cells and was more potent than the anti-α3 antibodies (Figures 8 and 9). Rapid stimulation was observed after addition of the peptide (2–3 h) and was dose dependent and saturable around 80 μM (200 μg/ml) peptide concentration (Figure 8). Equimolar concentrations of the scrambled HSGD-6 control peptide, as well as other laminin peptides (YIGSR, IKVAV, and KRGD) or fibronectin peptides (GRGDS) reported to have biological activity, did not affect FITC-gelatin uptake. The HGD-6 peptide also stimulated phagocytosis of FITC-gelatin by other human breast cancer cell lines expressing the α3β1 integrin (e.g., BT549, our unpublished observation). The HGD-6 peptide was routinely included as a positive control in phagocytosis assays testing other peptides to compare the magnitude of the responses. The stimulation of FITC-gelatin uptake by MDA-MB-231 cells in the presence of the HGD-6 laminin peptide was consistently observed in more than 30 experiments. A similar treatment with intact human laminin in solution (50 and 100 μg/ml) also stimulated the uptake of FITC-gelatin (1.27 ± 0.03 and 1.57 ± 0.03-fold, respectively). Intact laminin increased phagocytosis to a much lesser extent than the HGD-6 peptide, but it was used in a lower molar concentration as compared with the peptide (50 μg/ml represents 50 nM laminin or 20 μM HGD-6 peptide).

Interestingly, the stimulatory effects of the anti-α3 mAb and the HGD-6 peptide on phagocytosis were additive. When increasing concentrations (20–200 μg/ml) of the HGD-6 peptide were applied in the presence of the 5 μg/ml anti-α3 mAb (P1B5), the stimulation of phagocytosis was increased between ~1.2- and ~1.7-fold (Figure 9A). In contrast, the anti-α2 mAb did not significantly increase the HGD-6 stimulation ($p = 0.45$, *t* test) and the HSGD-6 control peptide did not significantly increase the anti-α3 mAb stimulation ($p = 0.58$, *t* test; Figure 9B).

MDA-MB-231 Cells Produce and Deposit Laminin

Since the α3β1 integrin is primarily known as a laminin receptor and since phagocytosis of gelatin is stimulated by the HGD-6 laminin peptide and intact laminin, we speculated that laminin secreted by the cells could mediate the phagocytosis of gelatin. Therefore, we tested whether MDA-MB-231 cells synthesize and deposit laminin using immunocytochemistry and immunoprecipitation followed by Western blotting. Cells incubated overnight on glass coverslips in serum-containing medium were stained with a polyclonal antiserum recognizing human laminin. The positive signal can be described as an overall punctate staining with, to a minor extent, some fibril-like structures on the cell surface (Figure 10A). Remarkably, the glass substratum surrounding the cells showed a strong punctate staining pattern suggesting that lami-

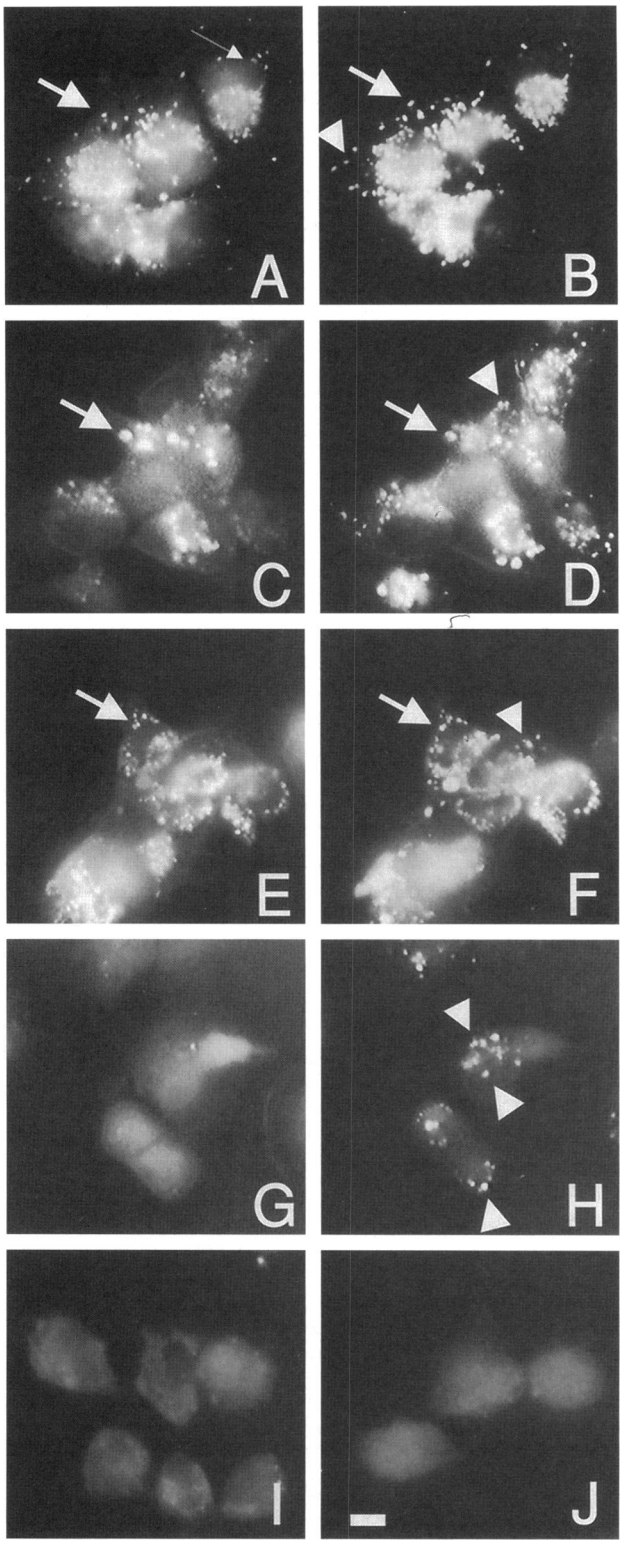

Figure 7. The α3β1 integrin and gelatin are cointernalized and routed to acidic vesicles. For the colocalization of internalized FITC-labeled gelatin with acidic vesicles or the α3 integrin subunit, MDA-MB-231 cells were incubated in serum-free culture medium for 3 h in 24-well plates coated with FITC-gelatin films in the presence of

nin was deposited on the substratum. Incubation with only secondary antiserum (negative control) did not show any significant staining of the cells or the glass substratum (Figure 10B). Complete culture medium, conditioned overnight by MDA-MB-231 cells, was used for immunoprecipitation with polyclonal antiserum against human laminin or an antihuman laminin mAb recognizing an epitope in the globular domain of the laminin α chain. This is the same domain in which the HGD-6 peptide sequence is localized. Immunoprecipitated laminin was separated by electrophoresis under reducing conditions and was detected by Western blotting using a polyclonal antimouse laminin antiserum. Both immunoprecipitations specifically pulled down two bands with apparent molecular weights of ~200 and ~400 kDa (Figure 10C, lanes 1 and 3) that corresponded to the positions of purified human laminin (Figure 10C, lane 5). Control immunoprecipitations in which the primary antiserum was omitted did not show any detectable signal after Western blotting (Figure 10C, lanes 2 and 4). These results indicate that MDA-MB-231 cells actively synthesize laminin that is secreted and deposited on the underlying substratum.

DISCUSSION

Phagocytosis has been reported in various tumor cell lines and may play a role in the spreading of malignant tumor cells. Its mechanism and the receptors involved, however, remain to be determined. Although phagocytosis is thought to be a ubiquitous

(Figure 7 cont.) 0.1 μM of the acidotropic probe LysoTracker red (A and B) or an anti-α3 integrin mAb (P1B5, 5 μg/ml) that was preincubated with a Texas Red-labeled antimouse secondary antiserum (5 μg/ml, C and D). For the colocalization of the α3 or the α2 integrin subunit with acidic vesicles, cells were incubated on unlabeled gelatin films in the presence of LysoTracker red and the anti-α3 integrin antibody (E and F) or anti-α2 integrin mAb (P1E6, 5 μg/ml, G and H) that were preincubated with a FITC-labeled antimouse secondary antiserum. As a negative control, cells were incubated only with FITC-labeled antimouse (I) or Texas Red-labeled antimouse (J) antiserum. After detachment from the gelatin films with trypsin/EDTA, cells were reseeded in complete culture medium on glass coverslips and allowed to attach and spread for 4 h. For microscopic observation of the colocalization, cells were fixed and mounted without permeabilization. (A and B) Most FITC-gelatin (A) colocalized with acidic vesicles (B, thick arrow), although FITC-gelatin was also present in nonacidic vesicles (thin arrow) and some acidic vesicles did not contain FITC-gelatin (arrowhead). (C and D) Most FITC-gelatin (C) colocalized with the α3 integrin subunit (D, thick arrow), whereas α3 integrin also occurred in vesicles not containing gelatin (arrowhead). (E and F) Most α3 integrin (E) colocalized with acidic vesicles (F, thick arrow), whereas not all acidic vesicles contained α3 integrin (arrowhead). (G and H) The α2 integrin was not detected in acidic vesicles (arrowhead). (I and J) Cells incubated with only antimouse antiserum labeled with FITC (I) or Texas Red (J) did not show any detectable fluorescence in vesicles. Bar, 10 μm.

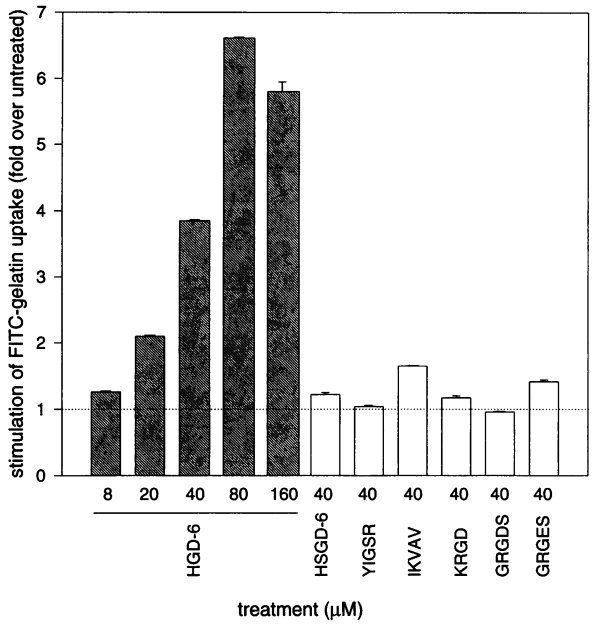

Figure 8. The synthetic HGD-6 laminin peptide, but not other peptides or the scrambled HSGD-6 control peptide, stimulates gelatin phagocytosis in a dose-dependent manner. FACS-phagocytosis was evaluated as described in Figure 3. Cells were incubated in the absence or presence of different amounts of the HGD-6 peptide from the globular domain of the human laminin α-chain, its scrambled version HSGD-6 (negative control), or other laminin (YIGSR, IKVAV, and KRGD) or fibronectin (GRGDS) peptides. The background fluorescence was determined on cells incubated on unlabeled gelatin films. Dotted line, amount of FITC-gelatin phagocytosis by untreated cells. The stimulation of FITC-gelatin phagocytosis is expressed as fold over untreated cells (phagocytosis of FITC-gelatin without treatment) and represents the mean ± SEM of two experiments.

biological activity occurring in most cells, the majority of the studies of this phenomenon have been limited to polymorphonuclear granulocytes, macrophages, and monocytes, in which phagocytosis is primarily mediated by IgG-Fc receptors. Macrophages and malignant tumor cells share common features such as local invasion into host tissues and spreading to distant sites in the host. We studied the phagocytosis of the extracellular matrix by the MDA-MB-231 human breast carcinoma cell line and provide the first evidence that α3β1, the major β1 integrin on the MDA-MB-231 cells, participates in this uptake. Different anti-α3 and anti-β1 integrin monoclonal antibodies as well as the HGD-6 laminin peptide, reported to interact specifically with the α3β1 integrin, significantly stimulated the phagocytosis of gelatin. The α3 and β1 integrin subunits were concentrated in invadopodia at sites under the cells where gelatin was locally degraded. In addition, the gelatin and α3β1 integrin were cointernalized and colocalized intracellularly in acidic vesicles. These observations were not limited to the MDA-MB-231 cell line, but were also observed with other breast cancer cell lines expressing the α3β1 integrin. Since not all monoclonal antibodies display biological activity, we cannot exclude the eventual involvement of integrins other than α3β1 in phagocytosis based on the inefficacy of only one anti-integrin monoclonal antibody clone to affect gelatin uptake. However, not only did the anti-α2 mAb not affect gelatin phagocytosis, the α2β1 integrin which is expressed in appreciable amounts on MDA-MB-231 cells was neither concentrated at sites of local gelatin degradation nor was it internalized and routed to acidic vesicles. This strongly suggests a specific role for certain integrins, such as α3β1, in the internalization of matrix molecules.

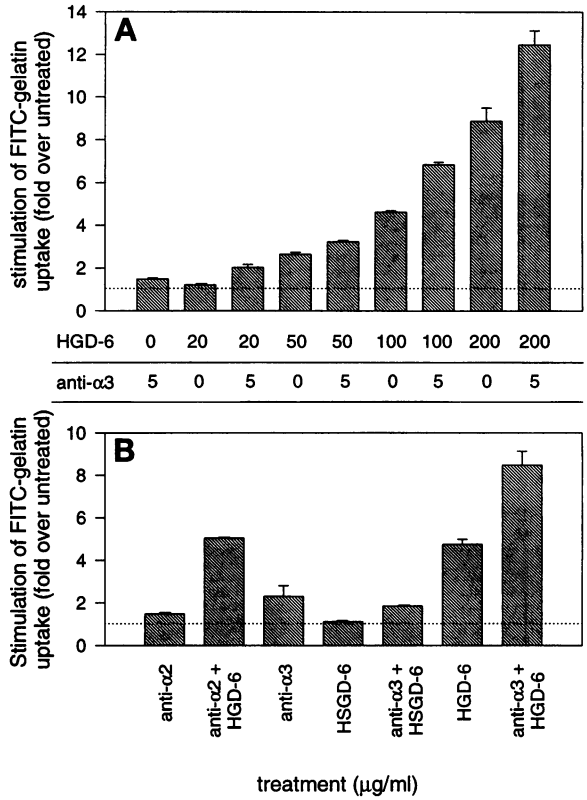

Figure 9. The HGD-6 peptide and the anti-α3 antibodies have an additive stimulatory effect on the phagocytosis of gelatin. FACS-phagocytosis was evaluated as described in Figure 3. Cells were incubated in the absence or presence of peptides or monoclonal antibody, alone or in combination. The background fluorescence was determined on cells incubated on unlabeled gelatin films. Dotted line, amount of FITC-gelatin phagocytosis by untreated cells. The stimulation of FITC-gelatin phagocytosis is expressed as fold over untreated cells (phagocytosis of FITC-gelatin without treatment) and represents the mean ± SEM of two to four experiments. (A) Cells were incubated in the presence of different amounts (20–200 μg/ml) of the HGD-6 peptide with or without anti-α3 mAb (P1B5, 5 μg/ml). (B) Cells were incubated in the presence of the HGD-6 or the scrambled HSGD-6 (negative control) peptide (100 μg/ml) with or without anti-α2 (P1E6) or anti-α3 antibodies (P1B5, 5 μg/ml).

Although phagocytosis is mediated by IgG-Fc receptors in macrophages and monocytes, the engagement of certain integrin receptors with integrin ligand-opsonized prey leads to an increased ingestion via IgG-Fc receptors (Brown and Goodwin, 1988). It is believed that binding by integrin receptors merely enhances particle uptake via other receptors, rather than mediating phagocytosis directly (Greenberg, 1995). The fate of the cooperating integrin molecules has not been reported however. Here, we demonstrate that in breast cancer cells lacking IgG-Fc receptors, the $\alpha 3\beta 1$ integrin itself is internalized along with the ingested extracellular matrix and that both are routed to acidic vesicles, thereby joining the lysosomal degradation pathway. Lysosomal targeting of the IgG-Fc receptor following phagocytosis has been reported previously, although to our knowledge the fate of the integrins has not been described (Mellman and Plutner, 1984).

This newly developed assay for quantification of the internalized extracellular matrix can be defined as measuring phagocytosis for the following reasons. 1) The cells actively ingest gelatin beads (diameter >1 μm) and large fragments of gelatin films. 2) This uptake is actin dependent because it was inhibited by cytochalasin D. 3) The uptake of gelatin is receptor mediated by the $\alpha 3\beta 1$ integrin. Unlike the latex, polystyrene, or dextran beads that are frequently used in phagocytosis studies, the gelatin and Matrigel used in our assays are naturally occurring and endogenous substrates that can interact with cell surface receptors. In contrast to most existing phagocytosis tests, the FACS-phagocytosis assay described in this article uses coated films instead of beads. This reflects the in vivo situation during matrix remodeling. This assay can also be used to measure phagocytosis in three-dimensional collagen or Matrigel gels, for example.

Cells first have to partially degrade the matrix before phagocytosing it and degrading it in the lysosomes. Partial digestion of gelatin before phagocytosis is necessary because the gelatin films are covalently cross-linked using glutaraldehyde. In addition, various inhibitors of gelatinases decrease the phagocytosis of gelatin (our unpublished results). Colocalization of the internalized gelatin with the $\alpha 3\beta 1$ integrin in acidic vesicles indicates the intracellular degradation in lysosomes. This intracellular degradation and subsequent release of free FITC label was evidenced by the presence of diffuse FITC signal in the cytoplasm after incubation with FITC-gelatin beads (see Figure 2, E and F). Although probably no extracellular degradation is needed for the phagocytosis of beads as compared with films, their uptake seems to be mediated by the same mechanism since the phagocytosis of both FITC-gelatin beads and films is stimulated by anti-$\alpha 3$ integrin mAbs. The role of the $\alpha 3\beta 1$ integrin in promoting other cell surface motility events is currently under study.

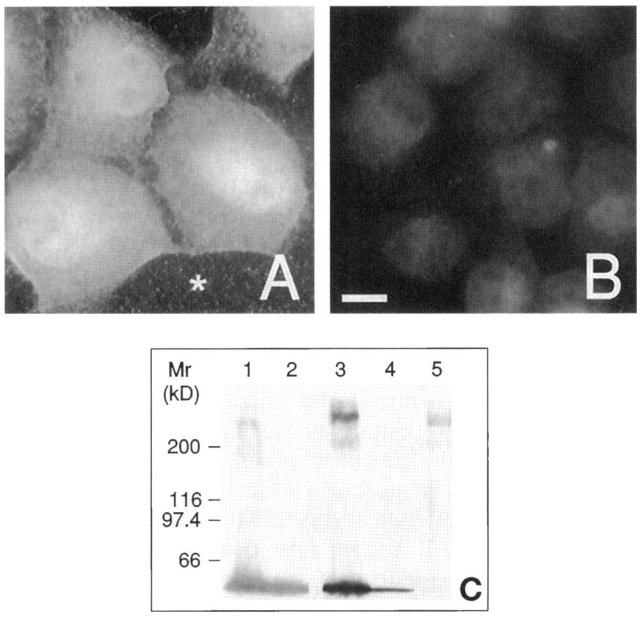

Figure 10. MDA-MB-231 cells produce and deposit laminin. (A and B) MDA-MB-231 cells were cultured overnight on glass coverslips in complete culture medium. Formaldehyde-fixed and nonpermeabilized cells were immunostained with polyclonal rabbit anti-human laminin (A) followed by Texas Red-labeled antirabbit antiserum as described in MATERIALS AND METHODS. The primary antiserum was omitted in a control experiment (B). *, laminin deposited on the coverslip. Bar, 10 μm. (C) Complete culture medium that was conditioned overnight by MDA-MB-231 cells was concentrated to 20% of its original volume. Laminin was immunoprecipitated with a polyclonal rabbit anti-human laminin antiserum and protein A-Sepharose beads (lane 1) or a monoclonal mouse anti-human laminin α chain globular domain antibody followed by protein A-Sepharose beads that had been preincubated with unlabeled rabbit anti-mouse antiserum (lane 3). Control immunoprecipitations were incubated with protein A-Sepharose beads alone (lane 2) or with protein A-Sepharose beads that had been preincubated with unlabeled rabbit anti-mouse antiserum alone (lane 4). Purified human placenta laminin was used as a positive control (5 μg/lane, lane 5). After SDS-PAGE under reducing conditions and electroblotting on nitrocellulose, laminin was visualized using a polyclonal rabbit anti-mouse laminin antiserum and HRP-labeled goat anti-rabbit antiserum followed by enhanced chemiluminescence.

The $\alpha 3\beta 1$ integrin mediates phagocytosis of different types of extracellular matrices such as gelatin (collagenous) and Matrigel (basement membrane-like). This is not surprising since the $\alpha 3\beta 1$ integrin is reported to be a promiscuous receptor recognizing a variety of extracellular matrix molecules such as collagen and laminin (the major constituent of Matrigel). The uptake of the underlying matrix might therefore be directly mediated by the $\alpha 3\beta 1$ integrin. Alternatively, since the $\alpha 3\beta 1$ is reported to be primarily a laminin receptor, the underlying matrix could be coated by laminin which is produced and deposited by the cells. The main arguments in support of lami-

nin-promoted uptake of gelatin are as follows: 1) MDA-MB-231 cells actively synthesize and deposit laminin on the underlying substratum. 2) The phagocytosis of gelatin is stimulated when intact human laminin or the HGD-6 laminin peptide are added to the culture medium. 3) The inhibition of protein synthesis with cycloheximide significantly inhibited gelatin phagocytosis (our unpublished results). The priming or opsonization of particles by extracellular matrix molecules is a common mechanism in phagocytosis (reviewed by Brown, 1986). Laminin has also been reported to enhance complement and IgG-Fc receptor-mediated phagocytosis by macrophages (Bohnsack *et al.*, 1985) and to stimulate IgG-mediated phagocytosis by neutrophils (Gresham *et al.*, 1989). Fibronectin is deposited by transformed chicken embryo fibroblasts at sites of localized gelatin degradation (Mueller and Chen, 1991). However, although fibronectin is a well-known opsonin, it seems not to be involved in the phagocytosis of gelatin in our model since neither anti-α5 integrin fibronectin receptor antibodies nor RGD-containing synthetic peptides influenced gelatin uptake. Moreover, the presence of fibronectin-containing serum in the culture medium did not affect the phagocytosis of gelatin.

We hypothesize that anti-α3 antibodies stimulate phagocytosis by activating the $\alpha3\beta1$ integrin. In support of this, we have shown that the $\alpha3\beta1$ integrin and the extracellular matrix are cointernalized and co-translocated to the lysosomal compartment. Alternatively, anti-α3 mAbs may indirectly promote phagocytosis by blocking the $\alpha3\beta1$ integrin and thereby allowing phagocytosis via another receptor. However, the concentration of the α3 integrin subunit with gelatin degradation spots and the intracellular colocalization of the α3 subunit with internalized gelatin strongly suggest a direct involvement of the $\alpha3\beta1$ integrin in gelatin phagocytosis. In addition to stimulating phagocytosis, the P1B5 anti-α3 mAb has also been reported to positively affect other downstream biological activities such as epidermal differentiation (Symington and Carter, 1995), intercellular adhesion of keratinocytes (Symington *et al.*, 1993), mammary morphogenesis (Berdichevsky *et al.*, 1994), and collagenase expression in keratinocytes (Larjava *et al.*, 1993). The anti-α3 mAb might mimic the ligand that interacts with and activates the $\alpha3\beta1$ integrin. Alternatively, this antibody might alter the conformation of the $\alpha3\beta1$ integrin, thereby augmenting binding of the ligand. The stimulation by anti-α3 antibodies is time and dose dependent and saturable, confirming its specificity. In addition, the fact that different clones of anti-α3 mAbs (P1B5 and M-KID2) stimulate gelatin phagocytosis demonstrates that this observation is not artifactual. The two anti-α3 mAbs had no additive stimulatory effect on phagocytosis when present together and thus may recognize a similar epitope. The stimulation of gelatin phagocytosis by the anti-α3 antibodies (Figure 3) and the internalization of the $\alpha3\beta1$ integrin (Figure 6, D and H) do not depend on the presence of secondary antibodies.

Stimulation of phagocytosis by the anti-α3 integrin antibodies appears to be via its effect on phagocytosis directly rather than increasing gelatinolytic activity since uptake of small beads was also stimulated. However, integrin antibodies have been reported to up-regulate the expression of matrix-degrading metalloproteases (Werb *et al.*, 1989). In particular, Larjava *et al.* (1993) reported that the P1B5 anti-α3 mAb stimulated the expression of the 92-kDa matrix metalloprotease (MMP9) in keratinocytes. However, this gelatinase seems not to account for the increased phagocytosis in our model since no detectable MMP9 protein or gelatinolytic activity was found in serum-free medium conditioned by MDA-MB-231 cells that had been stimulated with the P1B5 anti-α3 mAb (our unpublished data). Although it is possible that other proteases may be up-regulated, treatment with the anti-α3 mAb (P1B5) did not induce any other detectable gelatinolytic activities as assayed by zymography (our unpublished data).

The $\alpha3\beta1$ integrin binds the HGD-6 synthetic laminin peptide in vitro (Gehlsen *et al.*, 1992). Here, we have demonstrated that the HGD-6 peptide, but not its scrambled homologue HSGD-6, strongly stimulates phagocytosis of gelatin in a dose-dependent and saturable manner. We hypothesize that the HGD-6 peptide also directly activates the $\alpha3\beta1$ integrin, thereby stimulating the phagocytosis of extracellular matrix molecules. This peptide might elicit an effect that is similar to the ligand from which it is derived since intact human laminin also stimulated the uptake of gelatin. As in the case of anti-integrin mAbs, the HGD-6 peptide has been shown to promote other processes including cell adhesion (Wilke and Skubitz, 1991; Gehlsen *et al.*, 1992) and neutrophil motility (Harvath *et al.*, 1994).

In our experiments, RGD peptides did not affect the phagocytosis of gelatin. This is consistent with our results demonstrating that $\alpha3\beta1$ is the major $\beta1$ integrin involved in phagocytosis and with previous articles reporting that the $\alpha3\beta1$ integrin does not depend on the RGD sequence (Elices *et al.*, 1991; Dedhar *et al.*, 1992). Moreover, biologically active antibodies against the RGD-responsive integrin $\alpha5\beta1$ had no effect on phagocytosis by these cells. Other laminin peptides reported to have biological activities (YIGSR and IKVAV) had no influence on gelatin uptake.

We propose that both the anti-α3 mAb and the HGD-6 peptide bind and activate the $\alpha3\beta1$ integrin and thereby stimulate phagocytosis of the extracellular matrix since the anti-α3 mAb (P1B5) and the HGD-6 peptide had an additive stimulatory effect on gelatin phagocytosis. The additive effects of these re-

agents suggest that they do not operate via the same mechanism during stimulation of phagocytosis. Anti-$\alpha 3$ integrin mAbs and HGD-6 might bind to different epitopes on the $\alpha 3\beta 1$ integrin and activate different but functionally overlapping signal cascades. These results are also reminiscent of the extensive study performed by Miyamoto *et al.* (1995), who demonstrated that anti-integrin mAbs and monovalent ligand (peptides) each produce specific biological effects and that their actions may synergize to mimic the effect of the multivalent ligand. This results in a sensitive and complex control of downstream effects on the actin cytoskeleton and its interaction with the plasma membrane. The mechanism of $\alpha 3\beta 1$ integrin activation by the mAbs and peptide and which downstream molecules and signaling events are involved in $\alpha 3\beta 1$-mediated phagocytosis are currently under study.

We provide the first evidence that the $\alpha 3\beta 1$ integrin participates in the phagocytosis of the extracellular matrix by malignant breast cancer cells. This integrin-mediated clearing of partially degraded extracellular matrix may be of significant importance in pathological situations (e.g., malignant spreading of cancer cells) as well as in normal physiological functions (e.g., tissue reorganization) by facilitating cell migration.

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