Phagocytosis of Cross-Linked Gelatin Matrix by Human Breast Carcinoma Cells Correlates with Their Invasive Capacity

Peter J. Coopman, Michael T. H. Do, Erik W. Thompson, and Susette C. Mueller
Department of Cell Biology and Lombardi Cancer Center, Georgetown University Medical School, Washington, DC 20007

ABSTRACT

During invasion and metastasis, cancer cells interact closely with the extracellular matrix molecules by attachment, degradation, and migration. We demonstrated previously the local degradation of fluorescently labeled gelatin matrix by cancer cells at invasive membrane protrusions, called invadopodia. Using the newly developed quantitative fluorescence-activated cell sorting-phagocytosis assay and image analysis of localized degradation of fluorescently labeled matrix, we document here that degradation and site-specific removal of cross-linked gelatin matrix is correlated with the extent of phagocytosis in human breast cancer cells. A higher phagocytic capacity is generally associated with increasing invasiveness, documented in other invasion and motility assays as well. Gelatin phagocytosis is time and cell density dependent, and it is mediated by the actin cytoskeleton. Most of the intracellular gelatin is routed to actively acidified vesicles, as demonstrated by the fluorescent colocalization of gelatin with acidic vesicles, indicating the intracellular degradation of the phagocytosed matrix in lysosomes. We show here that normal intracellular routing is blocked after treatment with acidification inhibitors. In addition, the need for partial proteolytic degradation of the matrix prior to phagocytosis is demonstrated by the inhibition of gelatin phagocytosis with different serine and metalloprotease inhibitors and its stimulation by conditioned medium containing the matrix metalloproteinases MMP-2 and MMP-9. Our results demonstrate that phagocytosis of extracellular matrix is an inherent feature of breast tumor cells that correlates with and may even directly contribute to their invasive capacity. This assay is useful for screening and evaluating potential anti-invasive agents because it is fast, reproducible, and versatile.

INTRODUCTION

The extracellular matrix undergoes gross alterations during breast tumor progression and metastasis, and its constituents and their receptors are actively involved in these processes (1). The success of malignant tumor cells in the invasion and metastasis of host tissues depends on a series of closely concerted cellular activities including reduced cell-matrix adhesion, reduced cell-cell contact, increased migration, and increased matrix proteolysis (2, 3). Although less well studied, tumor cells also manifest a phagocytic capacity. This was observed in vitro in human breast carcinoma cells (4, 5), human epithelial cervix carcinoma cells (6), and rat glioma cells (7). The phagocytosis of leukocytes by metastatic tumor cells in vivo has been described in oat cell carcinoma of the lung (8), medulloblastoma (9), and endometrial adenocarcinoma (10). Phagocytosis is a cellular activity involved in the recognition and engulfment of microorganisms or tissue debris (11). It is performed mostly by polymorphonuclear granulocytes, monocytes, and macrophages, and is therefore mainly associated with infection, inflammation, and wound repair. Interestingly, macrophages and malignant tumor cells are both invasive cells and have adopted comparable strategies such as the use of proteolytic enzymes to dissolve the extracellular matrix (12). The biological significance of phagocytosis in tumor cells has, however, not yet been unveiled. We are studying the close interactions between tumor cells and the extracellular matrix that occur at well-defined plasma membrane protrusions, called invadopodia, that project into the matrix and locally degrade it (13). A multitude of molecules are shown to be concentrated in invadopodia and include integrins (5, 14), cytoskeletal molecules (15), tyrosine-phosphorylated molecules (15), proteolytic enzymes such as MMP-2 (16) and seprase (17). This suggests the occurrence of complex signaling events in invadopodia. Our current understanding is that invadopodia are primarily involved in the localized degradation of extracellular matrix molecules. In this paper, we demonstrate that breast cancer cells actively phagocytose extracellular matrix after localized degradation. Using a new in vitro assay for quantifying phagocytosis of fluorescently labeled extracellular matrix molecules and image analysis of localized degradation of fluorescently labeled extracellular matrix molecules, we show a correlation between the phagocytic and invasive capacities of different human breast cancer cell lines.

Received 6/26/97; revised 11/14/97; accepted 11/21/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by NIH Grant 5R01-CA-61273 (to S. C. M.) and by the Lombardi Cancer Center Microscopy/Imaging, Flow Cytometry/Cell Sorting, and Biostatistics shared resources supported by UPHS Grant 1P30-CA-51008.

2 To whom requests for reprints should be addressed, at Department of Cell Biology and Lombardi Cancer Center, Georgetown University Medical School, Research Building W316, 3970 Reservoir Road, NW, Washington, DC 20007. Phone: (202) 687-8484; Fax: (202) 687-7505; E-mail: muellers@gunet.georgetown.edu.

3 The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; MESF, molecules of equivalent soluble fluorescence; PBS, phosphate-buffered saline; TRITC, tetramethyl rhodamine isothiocyanate.
Phagocytosis and Invasion by Breast Carcinoma Cells

MATERIALS AND METHODS

Cell Lines and Cell Culture. All human breast cancer cell lines (MDA-MB-231, MDA-MB-436, MDA-MB-468, HS578T, MCF7, SKBr3, and BT549), the mouse embryonic cell line NIH/3T3, and the human fibrosarcoma cell line HT1080 were obtained from the Tissue Culture Shared Resource of the Lombardi Cancer Center and originated from the American Type Culture Collection (Rockville, MD). MCF7 ADR was obtained from Dr. Ken Cowan (National Cancer Institute, NIH, Bethesda, 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; for MDA-MB-231 cells) or 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY; all other cell lines) and 2 mM l-glutamine (Life Technologies, Inc.), 1 unit/ml penicillin, and 10 μg/ml streptomycin (Life Technologies, Inc.), which is referred to throughout as complete culture medium.

Reagents. Porcine skin gelatin, the actin polymerization inhibitor cytochalasin D, the lysosomotropic agent NH4Cl, the chelator 1,10-phenanthroline, and the serine proteinase inhibitors benzamide and aprotonin (4.2 trypsin inhibitor units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO); rat tail collagen type I, and the basement membrane matrix Matrigel from Collaborative Biomedical Products (Bedford, MA); the ATPase proton pump inhibitor bafilomycin A1 from LC Laboratories (Woburn, MA); the acidotropic probe LysoTracker red and the filamentous actin dye TRITC-phalloidin from Molecular Probes, Inc. (Eugene, OR); and the recombinant human TIMP-1 and TIMP-2, and the mouse monoclonal antibodies against MMP-2 (clone 42-5D11) and MMP-9 (clone 6-6B) from Oncogene Research Products (Calbiochem, Cambridge, MA). The chemically designed gelatinase inhibitors CT-1399 and CT-1847 (18) were generously provided by Dr. Andrew Docherty (Celltech Biologicals, Slough, United Kingdom), and SC-44463 was provided by Dr. George Fuller (Ref. 19; G. D. Searle & Co., Skokie, IL).

Localized Gelatin Degradation and Fluorescence Microscopy. For the microscopic evaluation of localized gelatin degradation and for filamentous actin staining, 10 μm diameter glass coverslips were coated with FITC labeled-gelatin films and cross-linked with glutaraldehyde as described (5). Then 50,000 cells were seeded in 2 ml of complete culture medium on the coated coverslips and incubated overnight at 37°C. After fixation with 3.7% formaldehyde/0.1% Triton X-100 in PBS for 15 min, cells were further permeabilized in 0.5% Triton X-100 in PBS for 15 min. Actin was visualized by incubation with TRITC labeled-phalloidin (Molecular Probes, Inc.) at 1:400 dilution in PBS for 15 min. Cell nuclei were stained using 4',6-diamino-2-phenylindole-2HCl at 0.4 μg/ml in PBS for 15 min. All incubations and PBS washes were performed at room temperature. Coverslips were mounted with the ProLong antifade kit (Molecular Probes, Inc.).

To colocalize internalized FITC-gelatin with acidic vesicles, cells were incubated for 3 h on FITC-gelatin films as described above but in the presence of 0.1 μM acidotrophic probe LysoTracker red (Molecular Probes, Inc.). Following detachment with trypsin/EDTA, cells were reseeded in complete culture medium on plain glass coverslips and allowed to attach and spread for 4 h. Cells were then fixed and mounted as described above but without permeabilization. Fluorescence photomicrographs of fixed cells were made using a Zeiss Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence and a Zeiss Planapo ×63/1.4 NA phase 3 objective.

For the quantification of localized FITC-gelatin degradation, microscopy was performed on an Olympus Vanox AH-2 fluorescence microscope (Olympus, Melville, NY) equipped with a Zeiss Plan ×40/0.65 NA objective. Images (TIFF format) were captured using an integrating Toshiba 3 chip color CCD camera (Toshiba, New York, NY) with an RGB color framegrabber (Flashpoint; I-Cube, Crofton, MD). The area of degraded FITC-gelatin zones and the number of cells (visualized by 4',6-diamino-2-phenylindole-2HCl staining of nuclei) were extracted and exported for further analysis using the Optimas 5.2 image analysis program (Optimas Corp., Bothell, WA).

Each experiment was carried out on three coverslips on which 25 fields (≡100–200 cells) were analyzed. For each field (0.01 mm²), the area of the degraded zones (μm²) and the number of cells were summed, and the area of the degraded zones per cell was calculated.

FACS-Phagocytosis Assay. The phagocytosis of extracellular matrix by cells in vitro was quantified as reported (5). Briefly, 150,000 cells were seeded in 0.5 ml of serum-containing (complete) or serum-free culture medium in plastic 24-well plates (Costar, Cambridge, MA) coated previously with a fluorescently labeled extracellular matrix film (100 μl/well). Gelatin and Matrigel were labeled with FITC as described (5). Collagen type I was first polymerized at neutral pH, labeled with FITC as a gel, and then resolubilized by acidification with 0.02 N acetic acid. Glutaraldehyde cross-linked gelatin films (20 mg/ml in PBS), Matrigel films (4 mg/ml in serum-free medium), and collagen type I films (2 mg/ml in PBS) were made as described (5, 20). After different incubation times, cells were detached with trypsin/EDTA, washed in PBS, and fixed in 0.3% paraformaldehyde in PBS. The amount of phagocytosed matrix was determined in 10,000 cells by FACS (FACStar Plus; Becton and Dickinson). The fluorescence levels measured by the FACStar Plus instrument were calibrated using fluorescence reference standards (Quantum 24 FITC-labeled microbeads; Flow Cytometry Standards Corp., San Juan, PR), and QuickCal software (Flow Cytometry Standards Corp.) was used to determine the MESF. Phagocytosis was expressed either as mean MESF units (absolute calibrated fluorescence intensity), fold over background (relative phagocytosis of FITC-labeled matrix as compared to unlabeled matrix), or percentage of inhibition or stimulation as compared with FITC-gelatin in the absence of treatment. In all experiments, controls with unlabeled matrix were included to correct for background fluorescence associated with the cell type and the unlabeled matrix. In addition, in experiments comparing matrix phagocytosis by different cell lines, MDA-MB-231 cells were always run as an internal con-
Localized degradation and phagocytosis of gelatin films by breast cancer cells. MDA-MB-231 breast carcinoma cells were cultured overnight in complete culture medium on glass coverslips coated with FITC-gelatin films as described in "Materials and Methods." After fixation and permeabilization, cells were stained with TRITC-phalloidin (TRITC-phall) to detect invadopodia containing filamentous actin (A and C). Actin is observed in stress fibers and invadopodia (dots; membrane extensions into the matrix vertical to the plane of the substrate). Degraded FITC-gelatin (FITC-gel) zones are observed as black spots (B and D). The selected areas in A and B are shown enlarged in panels C and D, respectively. The invadopodia stained by actin colocalize with FITC-gelatin degradation spots (arrows in C and D) as outlined using Optimas image analysis. For colocalization of the internalized FITC-gelatin (F) with acidic vesicles (F), cells were incubated for 3 h on FITC-gelatin films in the presence of 0.1 μM of the acidotropic probe LysoTracker Red (LysoT) as described in "Materials and Methods." Most FITC-gelatin (F) colocalized with acidic vesicles (F; thick arrows), whereas some acidic vesicles did not contain FITC-gelatin (thin arrows). Bar, 10 μm.

Control. Trypan blue treatments to quench extracellular fluorescence were tested but not found to be necessary because the trypsin/EDTA treatment of cells before FACS analysis completely removed the cell surface-associated fluorescence. In some experiments gelatin beads were used instead of films. Cross-linked FITC-gelatin beads were prepared and selected for a subcellular size (diameter <10 μm) by differential centrifugation as described earlier (21). Reagents tested for their effect on phagocytosis (cytochalasin D, NH4Cl, bafilomycin A1, proteinase inhibitors, and conditioned medium) were added simultaneously with the cells when seeded on gelatin-coated wells and left for the entire length of the assay.

Conditioned Media and Gelatin Zymography. Conditioned media were prepared by washing and incubating 80% confluent cell cultures overnight in serum-free medium (20 ml/175 cm²) in the presence of concanavalin A (25 μg/ml; Sigma) to activate MMP-2 (22). After removal of floating cells by centrifugation at 2000 rpm for 15 min, the conditioned media were concentrated 2.5–10-fold the original volume using Amicon Centrifuplus 10 concentrators (Amicon, Beverly, MA) according to the manufacturer’s instructions. Conditioned media proteins were separated on 8% SDS-PAGE gels and electroblotted onto nitrocellulose. Both the anti-MMP-2 and anti-MMP-9 monoclonal antibodies were used at 2 μg/ml in PBS. Immunodetection of proteins was visualized after incubation with horseradish peroxidase-labeled goat-anti-mouse antiserum (1:10,000; Jackson Immunoresearch Laboratories, West Grove, PA) using the Enhanced Chemiluminescence method (ECL; Amersham Corp., Arlington Heights, IL).

Chemotaxis and Chemoinvasion. The chemotactic and chemoinvasive capacity of the cells was tested in Boyden chambers as described previously (23). Briefly, polycarbonate filters (12-μm pore size and polyvinylpyrrolidone free; Nucleopore, Pleasanton, CA) were coated with 5 μg of collagen type IV (for chemotaxis) or 25 μg of Matrigel (for chemoinvasion). Cells (300,000/chamber) were diluted in serum-free improved minimal essential medium supplemented with 1 mg/ml BSA and seeded in the upper compartment of the Boyden chamber. The
Phagocytosis and Invasion by Breast Carcinoma Cells

RESULTS AND DISCUSSION

During invasion and metastasis, tumor cells produce and/or activate proteolytic enzymes necessary for the degradation of the extracellular matrix and their subsequent invasion into the surrounding host tissues. Hence, a series of fine-tuned intimate interactions between the tumor cells and the extracellular matrix are established at the cell plasma membrane. To study these interactions in situ, we used immunocytochemistry, we show that the MDA-MB-231 malignant human breast cancer cells are able to locally degrade the cross-linked FITC-labeled gelatin matrix in vitro (Fig. 1, A–D, arrows). The degradation spots (observed as black holes in Fig. 1, B and D) colocalize with invadopodia, which are active cell membrane extensions that project vertically into the matrix and which are visualized as bright, positively staining structures (dots) by actin staining (Fig. 1, A and C, arrows). Because of the dynamic nature of the invadopodia, not all degraded FITC-gelatin zones colocalize with invadopodia at a given moment and may have been created by formerly active invadopodia. In addition to the local degradation of the matrix, we demonstrate that MDA-MB-231 cells also actively phagocytose the partially digested gelatin matrix (Fig. 1E), which is then further broken down intracellularly in acidic vesicles, such as lysosomes (Fig. 1F).

We next quantified the phagocytosis of various fluorescently labeled extracellular matrices using the newly developed in vitro FACS-phagocytosis assay (5). To rule out the substrate specificity of gelatin phagocytosis, we performed phagocytosis assays also using collagen type I and Matrigel (reconstituted basement membrane) films (Fig. 2). These native physiological matrices were phagocytosed to the same extent as gelatin by MDA-MB-231 breast cancer cells, illustrating the flexibility of the substrate choice for the FACS-phagocytosis assay. For several reasons, however, we preferred to use gelatin matrices in subsequent phagocytosis experiments. Unlike native collagen type I or Matrigel, gelatin forms a dense and covalently linked structure that is not easily remodeled by cells. Also, direct comparisons can be made between the local degradation (Fig. 1) and the phagocytosis of gelatin (Fig. 2A). Unlike collagen I and Matrigel, gelatin is a cheap and easy-to-handle matrix, allowing the analysis of multiple samples in routine assays. Finally, the majority of the studies on the involvement of collagenases in tumor invasion are using gelatin as a model for collagen degradation (e.g., gelatin zymography).

Phagocytosis and intracellular digestion of collagenous matrices were described to occur in fibroblasts during connective tissue remodeling and turnover (24), but we show that this is also a customary activity in breast cancer cells. Its biological role in these cells remains, however, unclear. Phagocytosis could be responsible for the physical clearing of extracellular matrix degraded previously by invasive tumor cells and may thereby promote the malignant spreading of tumor cells. To verify this hypothesis, we tested the ability of various human
The phagocytic activity of breast cancer cells correlates with their invasive and migratory capacity. The capacity of different human breast cancer cell lines to phagocytose FITC-gelatin was determined by FACS as described in “Materials and Methods.” The amount of phagocytosed FITC-gelatin is expressed as fold over background and calculated as: (mean fluorescence intensity of 10,000 cells incubated on FITC-gelatin)/ (mean fluorescence intensity of 10,000 cells incubated on unlabeled gelatin). The amount of phagocytosed FITC-gelatin is compared with other parameters for invasiveness such as chemoinvasion (A), chemotaxis (B), and localized degradation of FITC-gelatin films (C). Phagocytosis of FITC-gelatin was tested overnight in complete culture medium, and data represent the means of 2–23 experiments; bars, SE. Chemoinvasion and chemotaxis were performed as described in “Materials and Methods,” and data are expressed as number of cells per field and represent the means of nine representative fields from two to three replicate filters as published previously (23); bars, SE. Localized degradation of FITC-gelatin films was quantified using the Optimas image analysis program as described in “Materials and Methods” and is expressed as area (µm²) degraded per cell. Localized degradation data represent the means of 71–112 fields; bars, SE. The following cell lines were tested: MDA-MB-231 (●), MDA-MB-436 ( ), MDA-MB-468 (▼), BT549 (▲), MCF7 (■), MCF7 ADR (●), SKBr3 (●), and Hs578T ( ○). First-order regression curves and Spearman rank correlations (corr.) between the different assays are shown and are considered significant when P ≤ 0.05.

Fig. 3 The phagocytic activity of breast cancer cells correlates with their invasive and migratory capacity. The capacity of different human breast cancer cell lines to phagocytose FITC-gelatin and compared it with their invasive capacity as evaluated using existing in vitro invasion assays (Fig. 3). To be able to show the combined results of different phagocytosis experiments and to compare the FACS-phagocytosis test with other assays, phagocytosis results are displayed as the mean fluorescence of 10,000 cells instead of a histogram containing individual cell fluorescence. Also, in Fig. 3 phagocytosis is expressed as fold over background, because the background levels (mean fluorescence associated with cells incubated on unlabeled gelatin) are slightly different for the various cell lines tested. This internal calibration allows the direct comparison between the phagocytic capacity of different cell lines. Phagocytosis of FITC-gelatin by the different cell lines showed a statistically significant correlation (Spearman analysis) with their chemotactic (migratory) response to medium conditioned by NIH/3T3 cells (Fig. 3B) and with the localized degradation of FITC-gelatin films expressed as area of degraded zones per cell (Fig. 3C). These results demonstrate that a higher phagocytic capacity is generally associated with increasing invasiveness documented in other assays. For most tested cell lines, with the exception of Hs578T, increased phagocytosis also corresponded with a higher invasive capacity through Matrigel, but the correlation was not statistically significant (Fig. 3A). However, when the Hs578T data are not included in the Spearman analysis, the correlation between phagocytosis and chemoinvasion becomes statistically significant (P = 0.038) with a better rank correlation (correlation, 0.75). This indicates that phagocytosis is a cellular activity that, although generally associated with an increasing invasiveness, is independent of other features of invasive cells such as chemoinvasion. Also, the correlation between the FACS-phagocytosis test and the chemotaxis or localized degradation assay was not always apparent for several of the tested cell lines, although the Spearman correlation was statistically significant for the group as a whole. In particular, Hs578T cells were relatively more phagocytic than chemotactic (Fig. 3B), and BT549 cells showed relatively less phagocytosis than degradation of the gelatin matrix (Fig. 3C). These data taken together suggest that phagocytosis is a cellular activity independent of other features of invasive cells such as cell migration and matrix degradation. All of these activities contribute, however, to the invasive process and might act in concert to accomplish invasion.

Using the FACS-phagocytosis assay, we subsequently tried to better define the phagocytic process in terms of kinetics, dependence on the actin cytoskeleton, and the involvement of proteolytic enzymes before and after phagocytosis. We show that the uptake of FITC-gelatin by MDA-MB-231 cells is a very rapid event that is already measurable 30 min after seeding, even before cells are fully spread on the gelatin film (Fig. 4A). It then increases exponentially, reaching a plateau (steady-state level) that is maintained at least between 24 and 48 h. According to our observations, phagocytosis is also dependent on the state of confluence of the cells (Fig. 4B). It is highest at low cell densities (9,400–37,500 cells/24-well or 2.55 cm²) and then decreases exponentially with increasing cell densities. Phagocytosis is, however, never fully blocked, even when the cells become confluent. Taken together, these observations suggest that phagocytosis is a continuous event that is up-regulated in breast cancer cell lines to phagocytose FITC-gelatin and compared it with their invasive capacity as evaluated using existing in vitro invasion assays (Fig. 3). To be able to show the combined results of different phagocytosis experiments and to compare the FACS-phagocytosis test with other assays, phagocytosis results are displayed as the mean fluorescence of 10,000 cells instead of a histogram containing individual cell fluorescence. Also, in Fig. 3 phagocytosis is expressed as fold over background, because the background levels (mean fluorescence associated with cells incubated on unlabeled gelatin) are slightly different for the various cell lines tested. This internal calibration allows the direct comparison between the phagocytic capacity of different cell lines. Phagocytosis of FITC-gelatin by the different cell lines showed a statistically significant correlation (Spearman analysis) with their chemotactic (migratory) response to medium conditioned by NIH/3T3 cells (Fig. 3B) and with the localized degradation of FITC-gelatin films expressed as area of degraded zones per cell (Fig. 3C). These results demonstrate that a higher phagocytic capacity is generally associated with increasing invasiveness documented in other assays. For most tested cell lines, with the exception of Hs578T, increased phagocytosis also corresponded with a higher invasive capacity through Matrigel, but the correlation was not statistically significant (Fig. 3A). However, when the Hs578T data are not included in the Spearman analysis, the correlation between phagocytosis and chemoinvasion becomes statistically significant (P = 0.038) with a better rank correlation (correlation, 0.75). This indicates that phagocytosis is a cellular activity that, although generally associated with an increasing invasiveness, is independent of other features of invasive cells such as chemoinvasion. Also, the correlation between the FACS-phagocytosis test and the chemotaxis or localized degradation assay was not always apparent for several of the tested cell lines, although the Spearman correlation was statistically significant for the group as a whole. In particular, Hs578T cells were relatively more phagocytic than chemotactic (Fig. 3B), and BT549 cells showed relatively less phagocytosis than degradation of the gelatin matrix (Fig. 3C). These data taken together suggest that phagocytosis is a cellular activity independent of other features of invasive cells such as cell migration and matrix degradation. All of these activities contribute, however, to the invasive process and might act in concert to accomplish invasion.

Using the FACS-phagocytosis assay, we subsequently tried to better define the phagocytic process in terms of kinetics, dependence on the actin cytoskeleton, and the involvement of proteolytic enzymes before and after phagocytosis. We show that the uptake of FITC-gelatin by MDA-MB-231 cells is a very rapid event that is already measurable 30 min after seeding, even before cells are fully spread on the gelatin film (Fig. 4A). It then increases exponentially, reaching a plateau (steady-state level) that is maintained at least between 24 and 48 h. According to our observations, phagocytosis is also dependent on the state of confluence of the cells (Fig. 4B). It is highest at low cell densities (9,400–37,500 cells/24-well or 2.55 cm²) and then decreases exponentially with increasing cell densities. Phagocytosis is, however, never fully blocked, even when the cells become confluent. Taken together, these observations suggest that phagocytosis is a continuous event that is up-regulated in
Phagocytosis of gelatin is time and cell density dependent and is decreased by inhibiting actin polymerization or intracellular acidification. MDA-MB-231 breast carcinoma cells were incubated in complete culture medium at a fixed cell concentration (150,000 cells/well) for different periods (0.5–48 h; A), at varying cell concentrations (9,400–600,000 cells/well) for a fixed time period (overnight; B) or at 150,000 cells/well overnight (C) on 24-well plates coated with FITC-gelatin as described in “Materials and Methods.” Regression curves of the third order are shown (A and B). The amount of phagocytosed FITC-gelatin was measured by FACS as described in “Materials and Methods” and is expressed as MESF (A and B). In C, cells were treated immediately after seeding on the FITC-gelatin films with the lysosomotropic agent NH4Cl, the inhibitor of vacuolar H+/ATPases bafilomycin A1 (Bafilom.), or the actin polymerization inhibitor cytochalasin D (Cytoch. D). The percentage of inhibition of FITC-gelatin phagocytosis by the treatments is calculated as: 100 − [100 (fold over background for treated cells)/(fold over background for untreated cells)]. All inhibitors were added when the cells were attached and spread after overnight incubation in the conditions where the architectural integrity of the tissues is interrupted, such as in tumor invasion.

By definition, phagocytosis is an actin-dependent process. We therefore tested the effect of the actin polymerization inhibitor cytochalasin D on the phagocytosis of gelatin (Fig. 4C). Treatment of MDA-MB-231 cells with 1 μM cytochalasin D, immediately after seeding on the FITC-gelatin films, inhibited phagocytosis of gelatin by ~34%, confirming the need of an intact actin cytoskeleton for phagocytosis. Furthermore, the actin cytoskeleton is also requisite for the formation of invadopodia, which are intimately involved in the local degradation and phagocytosis of gelatin (Fig. 1). Bafilomycin A1 is an inhibitor of vacuolar H+/ATPases, similar to the proton pumps that are involved in the active acidification of lysosomes and large acidic vesicles (4). MDA-MB-231 cells treated with bafilomycin A1 (1 μM) or with the lysosomotropic agent NH4Cl (100 mM) did not contain detectable acidic vesicles as verified with the acidotropic probe LysoTracker Red (data not shown). Moreover, cells treated with bafilomycin A1 or NH4Cl showed a significant decrease (Student’s t test) in FITC-gelatin phagocytosis (~27% inhibition each; Fig. 4C). These observations confirm that most of the intracellular gelatin is retained in actively acidified vesicles. Thus, inhibitors of acidification may decrease the intracellular residence time for gelatin. Alternatively, collapse of the intracellular pH gradient might directly reduce the ability of the cells to phagocytose extracellular matrix by a still unknown mechanism. Taken together, these results confirm the phagocytic nature of the gelatin uptake by breast cancer cells and clearly demonstrate its intracellular trafficking to actively acidified vesicles, where it is further degraded. The importance of phagocytosis and intracellular degradation for invasion is supported by the observation that the bafilomycin A1-sensitive V-ATPase is overexpressed in invasive human pancreatic carcinomas as compared with benign cystic neoplasms and noninvasive ductal cancers (25) and that the presence of bafilomycin A1-sensitive large acidic vesicles is correlated with increased breast cancer cell invasion through Matrigel (4).

In the FACS-phagocytosis assay, cells are incubated on cross-linked FITC-gelatin films. This implies that cells first have to partially degrade the covalently linked matrix before they are able to internalize it. We therefore tested whether proteinase inhibitors could inhibit phagocytosis of FITC-gelatin by MDA-MB-231 cells. We used inhibitors of matrix metalloproteinases, to which belong the well-documented gelatinases MMP-2 and MMP-9, as well as inhibitors of serine proteinases. Serine proteinases can either directly degrade gelatin (17) or indirectly activate a proteolytic cascade, resulting in gelatin degradation (26, 27). All inhibitors were added when the cells were seeded. Cytotoxic effects were excluded because cells were attached and spread after overnight incubation in the...
Fig. 5  Proteinase inhibitors decrease and gelatinases increase the phagocytosis of gelatin. The phagocytosis of FITC-gelatin films and subcellular-sized FITC-gelatin beads was measured by FACS as described in “Materials and Methods.” MDA-MB-231 cells were incubated overnight in complete (A) or serum-free (B) culture medium in the presence of different concentrations of proteinase inhibitors (A) or 2.5–10-fold concentrated HT1080 serum-free conditioned medium (B). The effects of these treatments on gelatin phagocytosis are expressed as percentage of inhibition (A) or percentage of stimulation (B). The percentage of inhibition of FITC-gelatin uptake is calculated as described in Fig. 4C. The percentage of stimulation of FITC-gelatin uptake is calculated as: 100 \times \text{(fold over background for treated cells)}/\text{(fold over background for untreated cells)}. *, P ≤ 0.001; **, P ≤ 0.05 (Student’s t test). Twenty-fold concentrated HT1080 conditioned medium (serum-free, concanavalin A treated) contained both latent (open arrows) and active (closed arrows) MMP-2 and MMP-9 as shown by Western blotting (WB; C) and gelatin zymography in the absence of EDTA (D). Zymography of 28-fold concentrated MDA-MB-231 conditioned medium (serum-free) showed a Mr ~130,000 gelatinase resistant to the presence of EDTA. Phen., 1–10-phenanthroline; Aprot., aprotinin; Benz., benzamidine; TIMP, tissue inhibitor of metalloproteinases; CM, conditioned medium; TIU, trypsin inhibitor unit.
The gelatinolytic activity of this yet undefined enzyme was inhibited by various serine proteinase inhibitors but was not affected by cysteine and metalloproteinase inhibitors. Although they do not synthesize MMP-2, MDA-MB-231 cells are able to activate exogenously provided pro-MMP-2 that is present in the serum of the cell culture medium (22, 29). The involvement of MMP-2 in the degradation of gelatin in our experiments is unlikely, however, because MDA-MB-231 cells need additional factors to activate pro-MMP-2, such as concanavalin A (22) or collagen, but not gelatin (29). Phagocytosis was never completely blocked by proteinase inhibitors; the maximal inhibition was 50%. This could be due to the cooperation of both serine and metalloproteinases in the degradation of gelatin, as was reported for the degradation of basement membrane Matrigel (27). Alternatively, the local concentration of the proteinase inhibitors might be low because of their limited access to the target sites. The destruction of gelatin occurs at well-defined zones under the cells (Fig. 1B), where the gelatinases are concentrated at invadopodia (16, 17).

In addition to inhibiting the phagocytosis of FITC-gelatin by proteinase inhibitors, we also tested the effect of high concentrations of exogenously added gelatinases on the uptake of FITC-gelatin by MDA-MB-231 cells. In these experiments, we used concentrated serum-free medium that was conditioned by the HT1080 human fibrosarcoma cells. This medium contains high levels of both the MMP-2 and MMP-9 gelatinases, as shown by Western blotting (Fig. 5C) and gelatin zymography (Fig. 5D). Different concentrations of HT1080 conditioned medium significantly (Student’s t test) stimulated the FITC-gelatin phagocytosis in a dose-dependent manner (Fig. 5B). Taken together, these experiments demonstrate the need for at least partial local proteolytic degradation of the cross-linked FITC-gelatin films before phagocytosis occurs. The internalized matrix is then routed to acidic vesicles (Fig. 1F), where it is further digested by lysosomal enzymes. The lysosomal aspartyl protease cathepsin D has been shown to digest extracellular matrix in breast cancer cells (31), and its levels were increased in MDA-MB-231 cells that had migrated through Matrigel (4).

The FACS-phagocytosis test for quantifying uptake of fluorescent extracellular matrix by cells in vitro is a fast, reliable, and versatile assay that can be used with different cell lines and diverse matrices. Unlike most existing phagocytosis assays, this test uses naturally occurring and endogenous substrates (extracellular matrix) as compared with latex, polystyrene, or dextran and in a conformation that simulates better the in vivo situation, i.e., extended matrices that are larger than cells. In addition to the above, the phagocytosis results correlate with the measurement of invasion obtained using other assays. Therefore, this assay is an alternative tool for assessing the invasive capacity of tumor cell lines specifically evaluating phagocytosis. Phagocytosis has been observed in metastatic tumor cells in vivo (8–10), but its mechanisms remain largely unknown and difficult to study in vivo. This in vitro assay will also help in identifying the multiple parameters involved in phagocytosis, such as receptors for extracellular matrix molecules (5) and proteolytic enzymes (this report), that may participate in the metastatic phenotype.

In conclusion, we have shown in this report that the gelatin matrix is not only locally degraded at invadopodia under breast cancer cells, but that it is also actively phagocytosed. The matrix is first partially degraded by gelatinolytic enzymes, then internalized into phagosomes and routed to lysosomes for further digestion. Our results also demonstrate that phagocytosis of extracellular matrix is an inherent feature of breast tumor cells that correlates with and may even directly contribute to their invasive capacity. This new in vitro assay for quantifying phagocytosis might be useful for screening potent antiinvasive agents specifically targeting phagocytosis.

ACKNOWLEDGMENTS

We gratefully acknowledge the following people for their gift of reagents. The gelatinase inhibitors CT-1399 and CT-1847 were generously donated by Dr. Andrew Docherty (Celltech Biologicals, Slough, England), SC-44463 by Dr. George Fuller (G. D. Searle & Co., Skokie, IL), and FITC-labeled collagen type I by Drs. G. Ghersi and W. T. Chen (Lombardi Cancer Center, Georgetown University Medical School, Washington, DC). We also thank Dr. Emma Bowden for critical reading of the manuscript; Rebecca Slack for the statistical analyses; Dr. Owen Blair and Karen Creswell for the flow cytometry; and Joseph Catanzano, Nisha Husain, and Dianne Thomas for technical assistance.

REFERENCES


Clinical Cancer Research

Phagocytosis of cross-linked gelatin matrix by human breast carcinoma cells correlates with their invasive capacity.

P J Coopman, M T Do, E W Thompson, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/4/2/507

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/4/2/507. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.