Human Platelet-Derived Mitogens. II. Subcellular Localization of Insulinlike Growth Factor I to the α-Granule and Release in Response to Thrombin

By Kenneth P. Karey and David A. Sirbasku

Platelets contain mitogenic activities for MCF-7 human breast cancer cells when assayed under serum-free chemically defined conditions. Purification from outdated human platelets identified insulinlike growth factor I (IGF-I) as the most potent breast cancer cell mitogen in lysates (Karey KP, Sirbasku DA: see accompanying article, this issue). In this study the release and subcellular localization of IGF-I was investigated. Degranulation of platelets by thrombin treatment caused release of lysosomal enzymes (β-glucuronidase and N-acetyl-D-glucosaminidase), α-granule proteins (β-thromboglobulin and fibrinogen) as well as mitogenic activity for MCF-7 cells and IGF-I as measured by radioimmunoassay (RIA) and radioreceptor assay. Release of mitogenic activity and immunologically identified IGF-I was induced tenfold over controls by thrombin and was nearly complete as compared to platelets disrupted by repeated freezing and thawing. Disruption of platelets by nitrogen cavitation followed by separation of the organelles by sucrose density gradient sedimentation showed that IGF-I and mitogenic activity localized predominantly to fractions containing α-granules rather than soluble cellular components, lysosomes, or dense granules. The morphology of MCF-7 cells in serum-free medium supplemented with supernatants from thrombin-treated platelets also indicated the release of important cell-adhesion factors for human breast cancer cells.

PREVIOUS STUDIES showed that platelet extracts contained growth factor activities for the MCF-7 human breast cancer cells maintained in serum-free chemically defined medium.12 In an accompanying report1 we purified and identified these growth factors from lysates of outdated human platelets. The mitogens identified were insulinlike growth factors I (IGF-I) and II (IGF-II) as well as a possible truncated form of DES I → 3 IGF-I. Those data and others from this laboratory4 confirmed that IGF-I was the most potent, human, breast-cancer cell mitogen yet identified from platelets or any other tissue source.

Although our study1 indicated the presence of significant amounts of insulinlike factors in lysates, the nature of their association with platelets remained to be resolved. This issue was especially clear in view of the data confirming α-granule localization of other mitogens such as platelet-derived growth factor (PDGF)4 and transforming growth factor β (TGF/β).1 These growth factors were shown to be released specifically following degranulation of fresh platelets. Of particular interest were the roles of these growth factors in wound healing2 or vascular injury sites,6 where platelet aggregation occurs. Other roles of platelet origin factors in tumor cell growth and metastasis are the subjects of much current investigation.

In this report we have addressed the issues of the subcellular localization of IGF-I in the platelet structure and specific release following thrombin-induced degranulation. Although both IGF-I and IGF-II were identified in platelet lysates,2 we elected to study only IGF-I because the evidence from our group4 as well as from others10-13 indicated that at higher concentrations IGF-II acts to mimic the mitogenic effects of IGF-I by binding the same type 1 (IGF-I) α/β2 receptor. In addition, the application of serum-free defined culture methods has revealed the presence of other important breast cancer cell factors in thrombin-released supernatants of platelets. These activities appeared to directly effect adhesion of the MCF-7 cells to plastic dish surfaces. A preliminary report of these data has been presented.14

MATERIALS AND METHODS

Platelet aggregation. Platelets from one unit of blood, concentrated into 45 to 65 mL of plasma, were obtained from the Gulf Coast Regional Blood Center (Houston) within 36 hours of collection. Platelets were harvested by centrifugation at 1000 g for 15 minutes at 4°C, suspended in a 1:1 (vol/vol) mixture of Ham's F12 and Dulbecco's Modified Eagle's media (DMEM; high-glucose formulation) supplemented with 2.2 g/L sodium bicarbonate and 15 mmol/L 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (pH 7.2; HEPES; F12/DMEM, GIBCO, Grand Island, NY) and collected again by centrifugation. All platelets were carried through three cycles of this wash process before use. The final pellets were suspended in F12/DMEM at approximately 10^6 to 10^8 platelets/mL based upon a protein estimation of the suspension (10^6 platelets = 16 mg protein).15

Portions (2.0 mL) of this suspension were incubated separately at 37°C for ten minutes with either 0.2 mL F12/DMEM or 0.2 mL of F12/DMEM containing 50 NIH units thrombin/mL (bovine thrombin, 1,000 U/mg protein, ICN Immunobiologicals, Lisle, IL). Controls consisted of 2.0 mL F12/DMEM plus 0.2 mL thrombin stock solution or 2.0 mL of the platelet suspension (with 0.2 mL F12/DMEM) that had been frozen in a dry ice-ethanol bath and thawed at 37°C for three cycles. Following the above incubations, all mixtures were cooled on ice for ten minutes and centrifuged at 18,500 g for five minutes at 4°C. The supernatants were collected, appropriately diluted, and assayed for released activities. For measurement of mitogenic activity, the samples were filter sterilized by passage through 0.22-μm pore diameter membranes. The incubations described above were performed on several occasions for independent analysis by bioassay, radioimmunoassay (RIA), and radioreceptor assay (RRA).

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Subcellular fractionation. Platelets from one unit of blood were collected, washed, and suspended as described above with the exception that 75 mmol/L Tris-HCl, 100 mmol/L potassium chloride, 12 mmol/L citrate, pH 6.4, was used in place of F12/DMEM. In a single experiment, uptake of 14C-serotonin (140 µCi/mg, Amersham, Arlington Heights, IL) into dense granules was done as described by Valtord-Hansen and Zuck. An aliquot of the crude homogenate was saved for estimate of marker recoveries. Aliquots (1.3 mL) of the homogenate were layered onto 10.0 mL linear sucrose density gradients (30% to 60%, wt/vol, containing 5.0 mmol/L EDTA, pH 7.4) and centrifuged at 134,000 g in a Beckman SW41 rotor for 105 minutes at 4°C. The gradient was collected from the bottom through a capillary tube using a peristaltic pump. Fractions were pooled based upon their light-scattering properties to provide adequate material for all subsequent assays. Pools containing subcellular particles were diluted twofold to fivefold with phosphate-buffered saline (PBS) and centrifuged at 134,000 g in a Beckman Ti50 rotor for 90 minutes at 4°C. The supernatant was discarded, and the pellet was suspended in 1.0 mL of PBS. The nonparticulate pool from the top of the gradient, was dialyzed against PBS using a Spectra/por 6 membrane (2,000 MWCO, Spectrum Medical Industries, Los Angeles). All gradient pools were frozen and thawed at least three times prior to assays. Marker distributions were calculated as "relative specific activities" as described.19,20

Assay of N-acetyl-β-glucosaminidase, β-glucuronidase, β-thromboglobulin, fibrinogen, and serotonin. The lysosomal enzymes N-acetyl-β-glucosaminidase and β-glucuronidase were assayed with p-nitrophenyl-N-acetyl-β-glucosaminidase and phenolphthalein glucuronic acid substrates (Sigma, St Louis), respectively. The assay mixture (0.2 mL; final volume) contained 0.05% (vol/vol) Tween-20 (PBS-Tween). Goat antihuman fibrinogen (Sigma), at a 1:4000 dilution in PBS-BSA, was added to each well. Plates were incubated for 0.5 to two hours at room temperature. Optical density was measured at 410 nm by using a MR600 Microplate Reader, Immunosoft-Version 1.5 computer software program (Dynatech) and an Apple IIE computer (Apple Computer, Cupertino, CA). All readings were taken within the linear range of color development and nonspecific background, as determined in the absence of antigen, was subtracted from all measurements.

Serotonin was quantified by measurement of the radioactivity in an aliquot of the test sample by liquid scintillation counting following the incubation and subsequent fractionation described above. All samples were corrected for nonspecific background and counted to a minimum of 10,000 counts for statistical reliability.

Measurement of IGF-I by RIA and RRA. Insulinlike growth factor I was quantified using the RIA described by Furlanetto and Marino. Human recombinant 71 amino acid N-Met-IGF-I,21 provided either by Dr B. Daniel Burleigh (IMC Pitman-Moore, Terra Haute, IN) or supplied courtesy of Charles E. Seeley (IMCERA Bioproducts, St Louis) was iodinated as described.22 The specific activity was approximately 100 to 150 µCi/µg. The rabit antihuman IGF-I/Smc (provided by Drs L.E. Underwood and J.J. Van Wyk of the University of North Carolina through the National Hormone and Pituitary Program, Baltimore) was used at a final dilution of 1:10,000. This antisierum showed <5% cross-reactivity with recombinant IGF-II.

Alternatively, IGF-I was quantified using the RRA described by Furlanetto and DiCarlo with the MCF-7 human breast-cancer cells in culture. Platelet-derived and control samples to be assayed were brought to 1 mg/mL BSA (RIA grade) and dialyzed against RRA-binding buffer (F12/DMEM supplemented with 1.2 g/L sodium bicarbonate and 1 mg/mL RIA grade BSA, pH 7.2) using a Spectra/per 6 dialysis membrane (2,000 MWCO). Following dialysis and prior to competition studies, samples were clarified by centrifugation at 4,500 g for ten minutes.

Cell culture and assay of mitogenic activity. The MCF-7 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cell cultures were maintained as serum-supplemented stocks and used for the measurement of growth factor activities in serum-free medium as described.5,23 One unit of mitogenic activity was defined as the concentration of factor required to achieve ED50 in a serum-free defined assay measuring cell number increase. The growth assays were done in a serum-free Ham's F12 and DMEM (1:1, vol/vol) containing 2.2 g/L sodium bicarbonate, 15 mmol/L HEPES (pH 7.2), 10 µg/mL transferrin (TF), and 200 µg/mL BSA (hereafter referred to as TF/BSA). Growth in TF/BSA without added mitogens was designated C0 and was usually 1.0 to 1.5 cell population doublings (cpd) in eight days. Maximum growth in TF/BSA was stimulated by a single addition of 100 ng/mL insulin (C90).

Miscellaneous. Protein concentrations were estimated by the method of Bradford5 using a protein assay dye kit (Bio Rad, Richmond, CA) and BSA as standard.

Photographs of cell cultures were taken with an Olympus INVBRTED Microscope (model IMT) and Photomicrographic System Camera, model PM-10-M (Olympus, New Hyde Park, NY) using Polaroid type 665 black and white film (Polaroid, Cambridge, MA).
RESULTS

Thrombin-induced release of human platelet constituents. Platelets were incubated in F12/DMEM alone or in this medium containing thrombin. The release of lysosomal enzymes (N-acetyl-β-D-glucosaminidase and β-glucuronidase), α-granule components (β-TG and fibrinogen), and IG-F-I as measured by RIA and mitogenic activity for MCF-7 cells was compared with controls of F12/DMEM containing only thrombin or platelets in F12/DMEM that had been disrupted completely by several cycles of freezing and thawing (Table 1).

Degranulation in response to thrombin was essentially complete because the amounts (concentrations/2.2 mL) of lysosomal and α-granule components released were the same as those measured after complete disruption of platelets by repeated cycles of freezing and thawing. Since ≤ 8% of the total protein was released from thrombin-treated platelets compared with freeze/thaw samples (Table 1), the protease treatment could not have disrupted platelet structure completely.

One α-granule marker showed divergent behavior. The concentration of fibrinogen in thrombin-treated samples decreased compared with controls (Table 1). This was expected because release of this protein in the presence of thrombin led to fibrin clot formation. The soft clots were removed by centrifugation before measurement of release products.

Release of RIA measured IGF-I was the same with both thrombin-incubated and freeze/thaw-treated platelets (Table 1). An 8.0-fold increase was found after degranulation. Incubations in F12/DMEM without thrombin caused ≤ 15% of the release of IGF-I seen in enzyme-treated or freeze/thaw-treated samples. Growth factor release in control samples was comparable with other α-granule markers (β-TG and fibrinogen) identified in these same incubations. The small amount of α-granule release in the control incubations may have been due to mechanical disruption and/or a low level of degranulation in response to normal components of F12/DMEM.

Potency of the breast-cancer cell mitogenic activity released from platelets. The specific activities of the mitogen(s) released from platelets under the conditions described in Table 1 were compared with the MCF-7 cells using the Tf/BSA assay.

The supernatants from thrombin-treated platelets showed a high specific activity (Fig 1). Based on the total protein in this fraction (Table 1) and the volume of supernatant required to achieve one half maximum growth (2.2 μL), an ED50 value of 1.4 ng/mL was calculated. Direct RIA measurement of the IGF-I content of this fraction showed an ED50 of 29 pg/mL (data not shown). At saturating concentrations, growth was 4.5 cdp in eight days above that seen in Tf/BSA alone (C0). Also, maximum platelet-mitogen–stimulated growth was equivalent to that promoted by a saturating concentration of insulin (CINS).

Growth in response to platelet lysates generated by freezing and thawing was compared to that of thrombin-released supernatants (Fig 1). Equivalent volumes of both supernatants caused nearly identical growth responses. Likewise, saturating concentrations of supernatants from freeze/thawed platelets promoted the same cdp/eight days as a saturating concentration of insulin. However, as expected

Table 1. Thrombin-Induced Release of Human Platelet Constituents

<table>
<thead>
<tr>
<th>Incubation Composition</th>
<th>Protein (mg)</th>
<th>N-acetyl-β-D-glucosaminidase (Units) (%)</th>
<th>β-Glucuronidase (Units) (%)</th>
<th>β-Thromboglobulin (μg) (%)</th>
<th>Fibrinogen (μg) (%)</th>
<th>IGF-I (ng) (%)</th>
<th>Mitogenic Activity (units) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets in F12/DMEM</td>
<td>0.400</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
<td>26.3</td>
<td>7.4</td>
<td>326.0</td>
</tr>
<tr>
<td>Platelets in F12/DMEM + thrombin</td>
<td>1.080</td>
<td>204.0</td>
<td>102.0</td>
<td>3.0</td>
<td>91.7</td>
<td>282.8</td>
<td>79.0</td>
</tr>
<tr>
<td>F12/DMEM only† + thrombin</td>
<td>0.0057</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Platelets in F12/DMEM-3X freeze/thaw</td>
<td>14.060</td>
<td>200.0</td>
<td>3.3</td>
<td>358.1</td>
<td>869.0</td>
<td>57.2</td>
<td>1000.0</td>
</tr>
</tbody>
</table>

*Percent release, relative to freeze/thaw control.
†Vehicle control (no platelets).
‡Figure corrected for added thrombin (4.55 μg/mL).
from the protein measurement data presented in Table 1, the ED$_{50}$ concentration of freeze/thawed samples was more than tenfold higher than that of thrombin-released samples.

Control studies shown in Fig 1 demonstrated that release of mitogenic activity into F12/DMEM without thrombin did occur but was at least tenfold lower in total units than that released by the protease. At any concentration tested, thrombin alone had no mitogenic activity (Fig 1).

The data presented in Fig 1 confirmed thrombin release of mitogenic activity from platelets but did not define specifically which of several possible mitogens or combinations of mitogens were responsible. Clearly the RIA data presented in Table 1 implicated IGF-I. Other factors may well have been present. However, the data in Fig 1 were consistent with release of members of the insulinlike family because these factors promoted more than 4.0 cpd/eight days in the T1/BSA assay, whereas epidermal growth factor (EGF) and the basic fibroblast growth factor (bFGF) were markedly less effective even at saturating concentrations.

Morphology of MCF-7 cells growing in response to serum and platelet factor samples. We have shown previously that MCF-7 cells in mitogen-supplemented T1/BSA did not attach to the plastic culture dishes but grew exponentially in suspension as large aggregates. This proved to be the case whether the cells were responding to insulin, insulinlike growth factors, EGF, bFGF, or to any combination of these mitogens. The results in the presence of thrombin-released platelet supernatants was markedly different.

Addition of supernatants from thrombin-treated platelets to T1/BSA promoted both attachment and growth of the MCF-7 cells (Fig 2A). Despite this attachment, the morphology of these cultures was very different than those grown in whole serum (Fig 2B). The cells growing in fetal calf serum (FCS) containing medium were larger and displayed more surface spreading than those grown in platelet-release-supplemented medium. The morphology of the cells in T1/BSA containing thrombin-released supernatants was nearly identical to the pattern we observed previously with MCF-7 cells grown in T1/BSA supplemented with IGF-I (20 ng/mL) and fibronectin (10 µg/mL).

Even when supernatants were derived from platelets undergoing slow degranulation in F12/DMEM without thrombin, the cells attached firmly to the plates (Fig 2C). The cultures grew more slowly than in thrombin-released supernatants (see Fig 1) but eventually showed morphology identical to that presented in Fig 2A. Again, these data were consistent with those presented in Table 1 and Fig 1 showing only limited release of mitogenic and other activities from platelets incubated in T12/DMEM alone.

The morphology effects of platelet lysates formed by repeated freeze and thaw was different than those seen with cultures containing thrombin-released components. In the presence of freeze/thaw extracts, the cells attached to the plates as aggregates, maintained a high growth rate, but failed to show spreading morphology (Fig 2D). Because adhesion proteins such as fibronectin and vitronectin are known to aggregate and precipitate during repeated cycles of freezing and thawing, it was possible that the attachment activity in platelet supernatants also was lost in this way. Also, the culture medium was viscous and contained fibrous material that probably was due to the formation of fibrin.

Data presented in Table 1 had confirmed that freeze/
thaw-prepared lysates contained a higher concentration of fibrinogen than other supernatants.

**Immunologic identification of IGF-I.** The presence of immunologically identifiable IGF-I in thrombin-released supernatants was confirmed by RIA (Fig 3). These samples showed nearly complete competition for binding of $^{125}$-labeled recombinant IGF-I to antibody. Competition by thrombin-released samples was parallel to that shown by unlabeled recombinant IGF-I. Samples of 100 μL of supernatant contained >100 ng of RIA measurable IGF-I.

In control studies, thrombin in F12/DMEM without platelets showed no competition (Fig 3). Platelets incubated in F12/DMEM without thrombin showed essentially the same competition curve as F12/DMEM without platelets. In a series of RIA determinations, the amount of IGF-I released in control incubations was <1 ng per 100 μL of supernatant (data not shown).

*Functional identification of IGF-I. Using a specific RRA,* the presence of IGF-I was examined with thrombin-released platelet supernatants (Fig 4). Specific binding of $^{125}$-labeled recombinant IGF-I to MCF-7 cell type I receptors was competed 80% by 500 ng/well unlabeled recombinant IGF-I. Unlabeled samples of thrombin-released supernatants were assayed at up to 1,000 μL/well. The curves generated through 40% to 50% competition of $^{125}$-IGF-I binding were parallel to those shown by unlabeled recombinant IGF-I. Assays of volumes of thrombin-released supernatant greater than 1,000 μL were not possible, since this was complete replacement of the binding medium. Also, the assay was limited by the concentration of IGF-I in the supernatants, which was estimated at ~20 ng/mL. Attempts to concentrate the supernatants before RRA led to highly variable results. Control experiments with F12/DMEM containing only thrombin showed no competition (Fig 4), while incubations of platelets in F12/DMEM alone showed small but variable amounts of IGF-I–like material at concentrations of <1 ng/mL (data not shown). These concentrations were in agreement with the low levels in similar supernatants as determined by RIA.

*Subcellular localization of IGF-I.** Platelets were disrupted by nitrogen cavitation and the subcellular organelles separated by sucrose density-gradient sedimentation. Bioassays of the pooled collected fractions with MCF-7 cells showed a broad area of mitogenic activity with two peaks. The first bioassay peak (pools 2 and 3, Fig 5A) corresponded to the same positions in which IGF-I was identified by RIA (Fig 5B). The sedimentation of IGF-I was faster (denser) than that of the lysosomal markers β-glucuronidase and N-acetyl-β-D-glucosaminidase (Figs 5C and 5D, respectively). The sedimentation positions of the α-granule markers β-TG and fibrinogen (Figs 5E and 5F, respectively) more closely approximated those of IGF-I. It should be noted that the peak of the α-granule markers was at a slightly slower sedimentation position than the IGF-I peak. This discrepancy was due to the methods of assaying and expressing the data. The data in Fig 5A were obtained using a constant volume of pooled fractions. The expression of “relative specific activity” in Fig 5 indicates percent total activity divided by the percent of the total protein recovered in each pool. These data were based on analysis of uniform volumes of each pool. Since pool 2 contained less protein than pools 3 and 4, the estimation of relative specific activity of the
small and 4. We conclude that the bioassay and RIA-measured growth factor was subject to this variable. Although this pool.

Likewise, when activity was evaluated, the peak of mitogenic activity had shifted to pools 3 and 4. It was concluded that the bioassay and RIA-measured IGF-I localization correlated with the elution position of the α-granule markers and that the small peak of mitogenic activity in pools 6 and 7 (Fig 5A) was not significant.

Nitrogen cavitation disrupted platelets preincubated with 14C serotonin showed radioactivity only in the densest pool at the bottom of the centrifuge tube corresponding to the expected sedimentation position of the dense granules (Fig 5G). Average recovery of all markers was 99.1% ± 16% based on the homogenate applied to the gradients.

DISCUSSION

The identification of IGF-I in platelets and released during degranulation suggests this mitogen may participate with PDGF, TGFβ, and EGF/β-urogastrone in wound healing. Release of this group of mitogens at injury sites would be expected to cause mesenchymal cell proliferation. This possibility was supported further by our data showing that thrombin-released IGF-I from platelets was in a highly active form. This was an important issue, since insulinlike mitogens become inactivated by association with plasma or cell-secreted binding proteins. Strong support for platelet release without binding protein(s) came from the 29 pg/mL ED50 value of thrombin-treated supernatants calculated from RIA data. This mitogenic potency approached that determined before for recombinant human IGF-I under the same completely serum-free conditions.

If platelet IGF-I had been released in association with carrier protein, the growth factor concentration expected for ED50 would have been greater. The studies presented here also allowed us to address the issue of the amounts of IGF-I in platelets. As discussed in the accompanying report, isolation and RIA quantification of IGF-I released from fresh platelets by thrombin or freeze/thaw proved this amount was 100 to 150 μg IGF-I per 200 units. Although significant, it was clear that platelet-derived IGF-I was only a small (ie, <0.1%) contribution to the total circulating concentration of 170 to 200 ng/mL.

Another new observation made during these studies was the apparent thrombin-induced release of an attachment factor for human breast cancer cells (Fig 2A). Previous work by Ogasawara and Sirbasku demonstrated that MCF-7 cells growing in Tf/BSA supplemented only with growth factors resulted in proliferation as large, floating colonies. In this study, the thrombin-released platelet supernatants caused MCF-7 cell adhesion to the dishes. The implication of these observations was clear. Materials different than growth factors were released from the platelets by thrombin and caused cell attachment. While the biochemical properties of this activity have not been defined, several possibilities were apparent. We have reported that fibronectin promoted adhesion of MCF-7 cells to plastic dishes in Tf/BSA. In addition, laminin and vitronectin (S protein/serum-spreading factor) have been found to have the same effect on MCF-7 attachment to plastic dishes (K.P. KAREY AND SIRBASKU)

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that IGF-I has been shown to stimulate collagen and glycosaminoglycan biosynthesis by articular chondrocytes in culture. Although direct evidence is still required, a role of IGF-I in wound sites might be to stimulate production of extracellular matrix components required for migration and/or growth of fibroblasts into these areas.

Others have shown that IGF-I/somatomedin-C was required for the completion of PDGF initiated entry into DNA synthesis by Balb/c 3T3 fibroblast cells. Likewise, PDGF and IGF-I were synergistic inducers of growth of human dermal fibroblasts in culture. Release of both IGF-I and PDGF into wound sites would be expected to cause mesenchymal cell proliferation. This possibility was supported further by our data showing that thrombin-released IGF-I from platelets was in a highly active form. This was an important issue, since insulinlike mitogens become inactivated by association with plasma or cell-secreted binding proteins. Strong support for platelet release without binding protein(s) came from the 29 pg/mL ED50 value of thrombin-treated supernatants calculated from RIA data. This mitogenic potency approached that determined before for recombinant human IGF-I under the same completely serum-free conditions.

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derived adhesion factor(s) may be another extracellular matrix component such as glycosaminoglycans with special significance for breast cancer cells.

The observation of this effect lends support to the concept that platelets may be directly involved in the process of anchorage of tumor cells at metastatic sites. Studies by a number of investigators indicated an important relationship between platelets, tumor growth, and metastasis.34-40 Earlier studies implicated platelet aggregation in the process of adhesion of circulating cancer cells,37 while more recent work showed that thrombi formed by platelet-tumor cell aggregates in capillaries were part of a "protection" permitting anchorage of tumor cells at metastatic sites. Studies that platelets time platelet aggregation. The data of Camez et al41 were important in this regard. They showed that both MCF-7 and T47D human breast-cancer cells induced adenosine-diphosphate-mediated platelet aggregation. Also, others have demonstrated both estrogen-stimulated and hormone-independent protease secretion by MCF-7 cells.42 These proteases, and others secreted by mammary tumor cells or activities shed as membrane-associated vesicles,43 might serve to initiate platelet aggregation and attachment at metastatic sites. In view of the fact the MCF-7 line was derived from a metastatic pleural effusion,44 these cells offer a very useful model for exploring further the roles of growth factors, adhesion factors, and platelet aggregation in the process of dissemination of human breast cancer.

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