Upregulation of MMP-9 expression in MDA-MB231 tumor cells by platelet granular membrane

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Abstract The interaction between tumor cells and platelets facilitates the formation of metastasis in a way depending on the platelet aggregating ability of the tumor cell, but the mechanism remains to be elucidated. We have shown, by zymography and Western blot, that platelets greatly increased the secretion to the culture medium of MMP-9 by human mammary tumor cells MDA-MB231. This increase, which was dependent on protein synthesis, was caused by the platelet aggregates interacting with the tumor cells and not by the soluble factors released during platelet activation. Platelet subcellular fractionation allowed the localization of the inducing factor to the membrane fraction of the platelet granules, thus requiring platelet aggregation in order to become accessible on the platelet surface.

Key words: Matrix metalloproteinase-9; Gelatinase; Platelet; Tumor cell; Metastasis

1. Introduction

The importance of the interaction of tumor cells with platelets was first recognized by Gasic and colleagues who showed that the number of experimentally induced pulmonary metastases was reduced in thrombocytopenic mice [1]. Drugs that interfere with platelet function were later shown to limit metastasis [2]. Morphological observations of tumor cells arrested in capillaries have shown the cancer cells surrounded with aggregated platelets with close contact between the platelet and the tumor cell [3]. These observations show that in most instances platelets surround the tumor cells until invasion of the basement membrane occurs [3].

Tumor cells can induce platelet aggregation in vitro and this ability of tumor cells to aggregate platelets was correlated with their ability to cause metastasis in vivo [4,5], suggesting that the activation and aggregation of platelets is a prerequisite for their metastatic effect. Upon activation platelets release their granular content such as growth factors and cytokines and express new adhesive glycoproteins on their cell surface. Some of these factors are involved in the metastatic process. The effect of activated platelets may result from the released soluble factors or from a direct platelet binding to tumor cells following platelet aggregation. Platelet binding to tumor cells via the integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) has been demonstrated in some cells and was suggested to contribute to tumor metastasis [6]. P-selectin (GMP-140), a transmembrane adhesion receptor translocated from the α-granules to the platelet membrane on activation, was also shown to mediate binding of platelets to small lung cancer cells [7], In a recent study, a 160-kDa platelet surface membrane component was implicated in the lung colonization of a murine colon adenocarcinoma cell line NL-17 [8]. However, the mechanism by which tumor cell-platelet aggregates increase the metastatic phenotype of the tumor cells remains unknown. Several authors have suggested that, by forming a platelet-tumor aggregate, platelets may contribute to metastasis by retaining tumor cells within the vasculature, increasing tumor cell adhesion to the endothelium, enhancing extravasation [6] or by creating distal subendothelial necrosis with consequent exposure of subendothelial matrix on which tumor cells can adhere [9].

Many studies have shown that metastasis formation is partly associated with the expression and activity of a specialized group of extracellular matrix-degrading proteinases, the matrix metalloproteinases (MMPs). Among the members of the MMP family shown to play a role in tumor cell invasion and metastasis are MMP-2 (gelatinase A or 72-kDa type IV collagenase) and MMP-9 (gelatinase B or 92-kDa type IV collagenase). Increased expression of gelatinases by tumor cells has been associated with an increased metastatic potential [10,11]. We have previously demonstrated that platelets greatly stimulated the secretion of MMP-9 by the human mammary tumor cells MDA-MB231, leading to an increased invasiveness of these cells in an in vitro invasion model [12]. We proposed this increased invasiveness as a novel mechanism by which platelets facilitate metastasis. However, it was not known whether this mechanism depends on platelet activation and aggregation. Therefore, in this study, we investigated the effect of platelet activation and aggregation on MMP-9 secretion by MDA-MB231 cells and looked for the platelet factor responsible for this effect.

2. Materials and methods

2.1. Reagents

All electrophoresis products were from Bio-Rad (Richmond, CA, USA). Bovine serum albumin (BSA), prostaglandin E_1 (PGE_1), leupeptin, benzamidine, thrombin (Th), hirudin, cycloheximide, Triton X-114, gelatin were from Sigma Chemical Co. (St. Louis, MO, USA), The rabbit polyclonal IgG (R6) directed against the type II repeats of thrombospondin (TSP) was from Dr. J. Lawler (Harvard Medical School, Boston, MA, USA); the rabbit antiserum to the IIIa chain of the glycoprotein IIb-IIIa and the monoclonal antibody against the complex GP IIb-IIIa (AP-2) were from Dr. D. Pidard (Institut Pasteur, Paris, France). The LYP-20 monoclonal antibody to P-selectin (GMP-140) was from Dr. J. McGregor (U331 INSERM, Lyon, France). Human recombinant MMP-9 (rMMP-9) and the CA-209 monoclonal antibody directed against MMP-9 were kind gifts from Dr. R. Fridman (Wayne State University, Detroit, MI, USA).

2.2. Cell culture and cell lysis

The human mammary adenocarcinoma cell line MDA-MB231 was a gift from Prof. F. Calvo (Paris, France). The cells were cultured in

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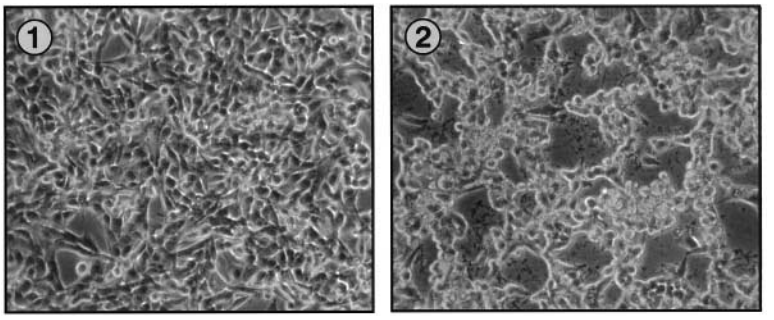

Fig. 1. Effect of platelets on the morphology of human mammary tumor cells MDA-MB231. Confluent cells were incubated without (1) or with (2) 4×10^8 platelets/ml for 24 h in a serum-free medium.

DMEM supplemented with 10% fetal calf serum (FCS). At confluence, cells were washed twice in DMEM without FCS and incubated with platelets or platelet fractions in serum-free medium for 24 h. The conditioned medium (CM) was collected and centrifuged before analysis. Cells were lysed with 2% ice cold Triton X-114 in Tris buffered saline pH 7.4 [13]. Lysates were centrifuged ($13\,500\times g$ for 15 min at 4°C) to remove Triton X-114 insoluble material, incubated for 3 min at 37°C and centrifuged for 3 min at $3000\times g$ to separate the hydrophobic and aqueous phases. The protein content of the aqueous phase was determined by a BCA test (Pierce, Rockford, USA).

2.3. Isolation and subcellular fractionation of platelets

Fresh human platelets were isolated from acid-citrate-dextrose anticoagulant, as described by Legrand et al. [14]. Washed platelets were resuspended in DMEM at 4×10^8 platelets/ml and added to MDA-MB231 cells either intact or after sonication (see below). For subcellular fractionation, washed platelets were resuspended at 3×10^9 platelets/ml in DMEM supplemented with 1 mM benzamidine, 0.2 mM leupeptin, 1 mM EDTA and 100 ng/ml PGE_1. Platelets were disrupted on ice by controlled sonication: 3 cycles of 10 s at 40 W (Vibra cell Sonifier, Bioblock Scientific, Illkirch, France), and centrifuged at $1200\times g$ for 15 min at 4°C. The resulting pellet was resuspended and sonicated again. After centrifugation, the two supernatants were combined to give the homogenate fraction (H) and centrifuged at $19\,000\times g$ for 30 min at 4°C to isolate the granular fraction (G). The resulting supernatant was further centrifuged at $100\,000\times g$ for 1 h at 4°C to separate the membrane (Mb) from the cytosolic fraction (Cyt). The granules were then further fractionated by sonication as above and centrifuged at $100\,000\times g$ for 1 h in order to separate the granular membrane fraction (Mb G) from the granular content (Cnt G). The granular, membrane and granular membrane fractions were washed once by centrifugation. Platelet subcellular fractions were characterized by measuring 5-hydroxytryptamine [15], β-thromboglobulin (radioimmunoassay kit, Amersham), β-glucuronidase [16], lactate dehydrogenase [17] and adenylate cyclase activities [18], the respective markers of platelet dense bodies, α-granules, lysosomes, cytosol and plasma membrane. Granular subfractions were further characterized by immunoblotting for TSP, P-selectin and GP IIb-IIIa (respectively markers for granular content, granular membrane and for both plasma and granule membranes).

2.4. Platelet activation

Platelets were activated by 5 min incubation at 37°C with thrombin (0.25 U/ml) while stirring. Thrombin was then inhibited by addition of 5 IU/ml hirudin and the aggregated platelets were pelleted by centrifugation at $1200\times g$ for 10 min. The supernatant containing the released factors (R) was removed and the pellet of degranulated platelets (P) was washed with DMEM and resuspended in the same volume as the initial platelet suspension. Before use, washed degranulated platelets were disrupted on ice by sonication. In some experiments, thrombin-induced activation was performed in the presence of 1 mM EDTA, to avoid the divalent-cation dependent-fixation of the released factors on the membrane of degranulated platelets [19,20].

2.5. Gelatin zymography and Western blotting

10% SDS-PAGE gels copolymerized with 1 mg/ml of gelatin were used to detect both latent and activated gelatinases [12]. Western blotting was performed as described [12].

3. Results and discussion

3.1. Effect of platelets on the morphology of MDA-MB231 cells

The morphology of MDA-MB231 cells was altered after incubation with platelets (Fig. 1). The cells seemed to retract, forming islets of more rounded cells. However, the cells were still adherent to the culture dish and did not detach during the incubation or washes, as no change in the cell count was observed. MDA-MB231 is an invasive cell line which was shown to cause intense platelet aggregation [12]. Fig. 1 shows aggregated platelets adherent to the tumor cells and to the extracellular space exposed after retraction of the tumor cells.

3.2. Effect of platelets on gelatinase production by MDA-MB231 cells

Confluent MDA-MB231 cells incubated in serum-free DMEM constitutively secreted MMP-9 into the culture me-

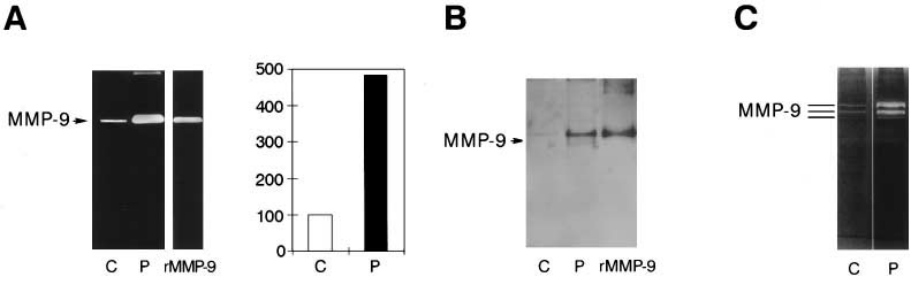

Fig. 2. Effect of platelets on MMP-9 production by MDA-MB231. Cells were treated for 24 h without or with platelets (4×10^8/ml) in serum-free medium. A: Gelatin zymography of CM and scanning of the corresponding lysis bands in arbitrary units (Visiolab 1000, BIOCOM, Les Ulis, France) (C, control; P, platelets; rMMP-9, 5 ng recombinant MMP-9). B: Identity of MMP-9 was confirmed by immunoblot of CM (third lane: 40 ng of rMMP-9). C: Gelatin zymography of the aqueous phase of the cell lysate (12.5 μg of protein by lane).

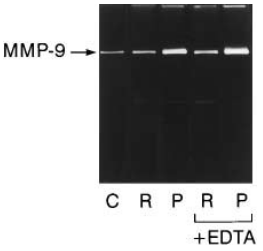

Fig. 3. Effect of thrombin-activated platelets on MMP-9 production by MDA-MB231. Platelet activation by 0.25 U/ml thrombin was performed on platelet suspension at 4×10^8 platelets/ml without or with 1 mM of EDTA. MDA-MB231 cells were incubated with serum-free medium alone (C, control), with the released soluble fraction (R) or with the pellet of degranulated platelets (P) obtained after platelet activation. The CM were analyzed by gelatin zymography.

dium. After incubation of the cells with platelets (P), the gelatinase secretion was greatly increased as demonstrated by gelatin zymography (Fig. 2A) and immunoblot using a monoclonal antibody directed against MMP-9 (Fig. 2B). It has recently been shown that platelets secrete low levels of MMP-2 [21]. However, we were unable to demonstrate either MMP-2 or MMP-9 activity in platelets alone at the concentration of platelets used. When cell lysates were analyzed, two major bands of 92 and 84 kDa and a minor band of 79 kDa were observed, all of which were greatly increased by platelets (Fig. 2C). The 84- and 79-kDa bands are consistent with those of active and non-glycosylated MMP-9, respectively [22,23].

When the protein synthesis inhibitor cycloheximide was added at 10 μg/ml, both the constitutive secretion and that stimulated by platelets were strongly inhibited (80–90% inhibition) suggesting that the increased secretion of MMP-9 by platelets corresponds to increased synthesis.

3.3. *Thrombin-activated degranulated platelets: effect on gelatinase secretion*

In order to evaluate whether the effect of platelets was due to a soluble factor released during platelet activation or to the activated platelets themselves, we compared the gelatinase secretion by MDA-MB231 cells incubated with either the platelet soluble releasate or with the remaining degranulated platelets. A platelet suspension at 4×10^8 platelets/ml was activated by thrombin and then centrifuged to separate the soluble factors from the degranulated platelets. Fig. 3 shows a much greater increase in MMP-9 secretion by the degranulated platelets (P) than by the soluble released factors (R). Thrombin or hirudin alone did not have any effect on the MMP-9 production by MDA-MB231 cells (not shown).

It is known that some of the α-granule constituents released by platelets on activation, such as TSP and fibrinogen, can then bind to the plasma membrane of activated platelets in a calcium-dependent manner [19,20] and are therefore associated with the particulate fraction of activated platelets. When platelet activation was repeated in the presence of 1 mM EDTA, which inhibits this binding, it was again the particulate fraction which caused the greatest increase in gelatinase production (Fig. 3).

3.4. *Effect of platelet subcellular fractions on gelatinase secretion*

In order to investigate the cellular localization of the factor responsible for the stimulation of gelatinase secretion, a subcellular fractionation of platelets was performed to separate the granular (containing α-granules, dense bodies and lysosomes), cytosolic and membrane fractions. The granular fraction was enriched 4-fold in 5-hydroxytryptamine, 2-fold in β-thromboglobulin and 8-fold in β-glucuronidase, the markers for α-granules, dense bodies and lysosomes, respectively; the cytosolic fraction and the membrane fraction were enriched 2.5- and 8-fold, respectively, in lactic dehydrogenase and adenylate cyclase. Fractions corresponding to the granular membrane and granular content were also prepared and characterized by immunoblotting using antibodies to specific markers of the α-granules. As is shown in Fig. 4A, P-selectin and GP IIb-IIIa were entirely found in the membrane fraction preparation (Mb G) and the TSP was found in the soluble granular fraction (Cnt G).

The various platelet subfractions were incubated with the MDA-MB231 cells at concentrations corresponding to that derived from 4×10^8 platelets/ml and the secretion of gelatinase, studied by zymography, is shown in Fig. 4B. The greatest increase in gelatinase was observed with the granular fraction (G), comparable to that obtained with whole platelets (H). After further subfractionation of platelet granules, it was the granule membranes which caused the highest increase in gelatinase secretion in MDA-MB231 cells. Thus, we suggest that the platelet factor responsible for stimulating gelatinase production is a membrane component of platelet storage granules. The fact that whole intact platelets can induce an increase in gelatinase production suggests that after activation of platelets by the MDA-MB231 cells, the inducing factor in

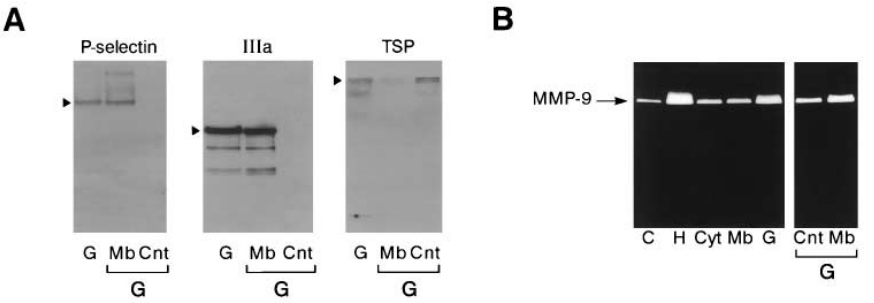

Fig. 4. Effect of platelet subcellular fractions on MMP-9 production by MDA-MB231. A: Characterization of platelet granule subfractions (G, granules; Mb, membrane; Cnt, content) by immunoblot analysis, under reducing conditions for thrombospondin (TSP), without reduction for P-selectin and the IIIa chain of GP IIb-IIIa. B: Gelatin zymography of the CM of cells incubated with platelet subfractions equivalent at 4×10^8 platelets/ml (C, control; H, homogenate; Cyt, cytosol; Mb, membrane; G, granules; Cnt G, granular content; Mb G, granular membrane).

the granular membrane is translocated to the platelet surface, as was shown for P-selectin [24].

The integrin receptor GP IIb-IIIa ($\alpha_{IIb}\beta_3$), located on both the plasma membrane and the α-granule membranes, and the P-selectin present in the α-granules, have both been shown to mediate interaction of platelets with some cancer cells [6,7]. In order to assess the role of these glycoproteins in our system, inhibition studies were performed using two antibodies, AP-2 and LYP-20, which were shown respectively to block GP IIb-IIIa and P-selectin functions in platelet aggregation studies [24,25]. However, no inhibition was observed when these antibodies were added (20 μg/ml of AP-2, 7 μg/ml of LYP-20) together with platelets or with the granular membrane fraction, suggesting that neither of these proteins is implicated in the stimulation of gelatinase by platelets. Purified TSP, fibrinogen and fibronectin, soluble α-granule adhesive glycoprotein that can be found on the surface of activated platelets, did not increase gelatinase secretion, nor did the RGDS peptide (1 mM) have any effect (data not shown).

It seems therefore that a so far unidentified platelet factor not previously implicated in tumor cell-platelet interaction and localized in the granular membrane of the platelets is responsible for stimulating the secretion of MMP-9 by the breast cancer cells MDA-MB231; the nature of this factor is currently under investigation in our laboratory. As the secretion process during platelet activation involves the fusion of the membrane of the storage granules with the plasma membrane, we propose that the activation and aggregation of platelet induced by the tumor cells allows this factor to be translocated to the platelet surface to act on the tumor cells in order to stimulate the secretion of the gelatinase. The granular localization of this platelet factor is thus compatible with the necessity for platelet aggregation to induce invasiveness of tumor cells.

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