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Molecular mechanisms of platelet activation and aggregation induced by breast cancer cells

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ABSTRACT

Tumor cell-induced platelet aggregation represents a critical process both for successful metastatic spread of the tumor and for the development of thrombotic complications in cancer patients. To get further insights into this process, we investigated and compared the molecular mechanisms of platelet aggregation induced by two different breast cancer cell lines (MDA-MB-231 and MCF7) and a colorectal cancer cell line (Caco-2). All the three types of cancer cells were able to induce comparable platelet aggregation, which, however, was observed exclusively in the presence of $CaCl_2$ and autologous plasma. Aggregation was supported both by fibrinogen binding to integrin αIIbβ3 as well as by fibrin formation, and was completely prevented by the serine protease inhibitor PPACK. Platelet aggregation was preceded by generation of low amounts of thrombin, possibly through tumor cells-expressed tissue factor, and was supported by platelet activation, as revealed by stimulation of phospholipase C, intracellular Ca^{2+} increase and activation of Rap1b GTPase. Pharmacological inhibition of phospholipase C, but not of phosphatidylinositol 3-kinase or Src family kinases prevented tumor cell-induced platelet aggregation. Tumor cells also induced dense granule secretion, and the stimulation of the P2Y12 receptor by released ADP was found to be necessary for complete platelet aggregation. By contrast, prevention of thromboxane A_2 synthesis by aspirin did not alter the ability of all the cancer cell lines analyzed to induce platelet aggregation. These results indicate that tumor cell-induced platelet aggregation is not related to the type of the cancer cells or to their metastatic potential, and is triggered by platelet activation and secretion driven by the generation of small amount of thrombin from plasma and supported by the positive feedback signaling through secreted ADP.

1. Introduction

Blood platelets are mostly considered for their role in hemostasis and thrombosis, but they can also be critical players in cancer spread. The distribution of malignant cells throughout the body requires their detachment from the primary tumor and the subsequent entry into the circulatory system, in a process known as intravasation [1]. Blood represents an inhospitable environment for cancer cells which are promptly attacked by the immune system and are subjected to rheological shear stresses, events that make hematogenous metastasis an overall inefficient process. Nevertheless, cancer cells exploit host's blood components to improve their chances to survive in the circulation, and platelets represent important allies for cancer dissemination [2,3]. An increasing body of evidence suggests that, by interacting with cancer cells, platelets provide protection against the immune system, support cancer cell adhesion and extravasation. Furthermore, platelets release extracellular signaling molecules that potentiate cancer cell metastatic commitment and regulate angiogenesis [4–7].

In a recent work we have reported that platelets may also support metastasis by releasing microparticles upon interaction with cancer cells [8]. We have found that two different breast cancer cell lines with different metastatic potential, the highly aggressive MDA-MB-231 cells and the low metastatic MCF7 cells, were able to stimulate platelets to release microparticles, which in turn could differently affect the metastatic aggressiveness of cancer cells by modulating migration and invasion [8]. The ability of cancer cells to induce the release of pro-metastatic platelet-derived microparticles was found to be associated to their ability to induce platelet aggregation. This observation stimulated further investigations on the mechanisms of tumor cell-induced platelet aggregation (TCIPA). The ability of cancer cells to stimulate platelet aggregation has been known for a long time. Over the past three decades, this process has been investigated in vitro using different cell

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lines, mainly derived from breast, colorectal, prostate, bladder, and melanoma tumors [9]. A correlation between the ability of cancer cells to induce TCIPA and their metastatic potential has been proposed in some studies [10–12]. Nevertheless, the molecular mechanisms and the signal transduction pathways involved in TCIPA are still poorly characterized. Depending on the cell lines used and on the general experimental setting, a different role for thrombin, ADP, TxA2, plasma, metalloproteinase, and other effectors has been proposed [13–17]. While melanoma or colorectal cancer cells have been commonly used to investigate TCIPA, detailed information about the ability of breast cancer cells to induce platelet aggregation is still limited. In particular, little is known about the modality for initiation and for subsequent amplification of platelet activation and on possible differences related to the metastatic potential of cancer cells [18,19].

In order to get further insights into the mechanisms of the previously reported ability of breast cancer cells to stimulate the release of platelet-derived microparticles, we have deeply investigated and compared platelet activation and TCIPA stimulated by two widely used breast cancer cell lines with different metastatic potential, the MDA-MD-231 and the MCF7 cells, in comparison with the better characterized Caco-2 cells. Results allow to delineate a model in which thrombin generation, platelet secretion and stimulation of the P2Y12 ADP receptor cooperate to achieve a full platelet activation and aggregation in response to all the cell lines tested.

2. Materials and methods

2.1. Materials

Thrombin, MRS2179, prostaglandin E1 (PGE1), acetylsalicylic acid (ASA), GPRP, RGDS and apyrase were from Sigma. FURA-2-AM was from Calbiochem. AR-C69931MX was provided by Astra-Zeneca. Wortmannin and U73122 were from Alexis Biochemicals. The antibodies against Rap1 (121), α-2-macroglobulin (H-8), GAPDH (V-18), Tissue Factor (H-9) and tubulin (DM1A) were from Santa Cruz Biotechnology. The antibody against phospho(Ser)PKC substrates was from Cell Signaling Technology. PP2 and PPACK were from Enzo Life Sciences. Thrombin Activity Fluorometric Assay Kit was from BioVision Incorporated. [^{14}C]serotonin was from GE Healthcare. ATP determination kit was from Biaffin GmbH & Co.

2.2. Cell culture

Human breast adenocarcinoma cell lines MCF7 and MDA-MB-231 and human colorectal adenocarcinoma cell line Caco-2 were maintained in Dulbecco modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For TCIPA experiments, cells were washed twice with PBS and detached by incubation for 15 min at 37 °C with 5 mM EDTA in PBS. Cells were recovered by centrifugation and finally resuspended in HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl and 12 mM $NaHCO_3$, pH 7.4) containing 5.5 mM glucose. The count of vital cells was determined by trypan blue staining and phase contrast microscopy analysis.

2.3. Platelet preparation

Platelet-rich-plasma (PRP) was obtained from blood withdrawn from healthy volunteers using 0.3% sodium citrate as anticoagulant, and by centrifugation at 120 ×*g* for 15 min. Washed human platelets, as well as isolated platelets, were prepared from buffy-coat bags collected the same day of the experiment, through a previously described protocol [8]. Briefly, the buffy-coat was diluted with one fourth of its initial volume using a 9:1 solution of HEPES buffer and citric acid/citrate/dextrose (152 mM sodium citrate, 130 mM citric acid and 112 mM glucose) and spun at 120 ×*g* for 15 min. A volume corresponding to one third of the upper phase was recovered, added of 1 μM PGE1 and 0.2 U/ml apyrase and then centrifuged at 720 ×*g* for 15 min to recover the platelet pellet. The platelet pellet was washed twice by resuspension in PIPES buffer (20 mM PIPES and 137 mM NaCl, pH 6.5) and, upon an additional centrifugation at 720 ×*g* for 15 min, finally resuspended in HEPES buffer in presence of 5.5 mM glucose, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, at the concentration of 0.3×10^9 platelets/ml. White and red blood cells contaminants in the platelet preparation were monitored using an automated cell counter and found to be < 0.01% and 0.002% respectively. Preliminary comparative analysis confirmed that aggregation and secretion of platelets prepared from buffy-coats bags were comparable to those of platelets prepared from whole blood (data not shown). Moreover, PKA-mediated phosphorylation in resting or PGE1-treated platelets was also comparable (data not shown). Isolated platelets were obtained by directly resuspending the platelet pellet in HEPES buffer plus 5.5 mM glucose, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, skipping the washing steps in PIPES buffer.

2.4. Platelet aggregation

Analysis of TCIPA was performed in a light transmission aggregometer from Chrono-Log Corporation, under constant magnetic stirring at 37 °C. Platelet samples (0.3 ml at 0.3×10^9/ml), left untreated or preincubated with different inhibitors, were stimulated with cancer cells (typically 10^5 cells/ml) in the absence or in the presence of different doses of platelet-free plasma or serum. The concentration of cancer cells used for platelet stimulation was selected through preliminary dose-response experiments. Although the cancer cell/platelet ratio we adopted in this work is likely higher than that found in-vivo during metastatic spread, it is in the range commonly used for TCIPA investigation [12,15,19]. The extent of platelet aggregation was continuously monitored for 30 min.

2.5. Analysis of dense granule release

Secretion of [^{14}C]serotonin from metabolically labeled platelets was performed as previously described [20]. Briefly, [^{14}C]serotonin-loaded platelets were preincubated with 5 μM imipramine and then stimulated with cancer cells in the presence of plasma (0.05% v/v). Stimulation was stopped by addition of 0.1 ml of 2% formaldehyde and 5 mM EDTA. Platelets and cancer cells were pelleted by centrifugation at 10,000 ×*g* for 5 min, and the radioactivity of [^{14}C]serotonin released in the supernatant was determined by liquid scintillation counting. For ATP quantification, platelets (0.3×10^9/ml) were stimulated with cancer cells (10^5/ml) and the reaction was stopped at different time points by adding 2 mM EDTA. Supernatant was collected upon centrifugation at 10,000 ×*g* for 5 min, and the amount of ATP in the cleared supernatant was quantified by using the luminescence-based ATP determination kit (Biaffin, Kassel, Germany), following the manufacturer's instructions.

2.6. Analysis of platelet activation

Immunoblotting analysis was performed on samples of washed platelets (0.3×10^9/ml) stimulated with the 3 different cancer cells (10^5/ml) in the presence of plasma (0.05% v/v), under stirring at 37 °C, as previously described [21]. Rap1b activation was evaluated through a pull-down assay, by selective precipitation of the GTP-bound active form of the protein, followed by immunoblotting analysis with an anti-Rap1 antibody [22]. Intracellular Ca^{2+} concentration was measured essentially as previously described [23]. Fura-2-AM loaded platelets were stimulated with cancer cells, in the presence of 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, under constant stirring at 37 °C. The Fura-2 emission fluorescence at 500 nm was monitored every 5 min using a Perkin Elmer Life Sciences LS3 spectrofluorometer.