Platelet Aggregation-Induced by Caco-2 Cells: Regulation by Matrix Metalloproteinase-2 and Adenosine Diphosphate

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Received November 8, 2005; accepted January 18, 2006

Abstract

Formation of tumor cell-platelet aggregates facilitates hematogenous metastases. However, molecular mechanisms implicated in tumor cell-induced platelet aggregation (TCIPA) in colon cancer are unclear. To investigate mechanisms of TCIPA induced by colon adenocarcinoma cells in vitro, human Caco-2 cells were used to study their interactions with platelets using aggregometry, zymography, phase-contrast microscopy, and flow cytometry. Caco-2-induced platelet aggregation in a concentration-dependent manner. This aggregation resulted in the release of matrix metalloproteinase (MMP)-2, as measured by zymography. In addition, flow cytometry showed a significant up-regulation of activated GpIIb/IIIa, total GpIIb/IIIa, GpIb, and P-selectin receptors on platelets. Inhibition of MMP-2 by phenantroline and degradation of ADP by APT102, respectively, resulted in inhibition of TCIPA. Furthermore, both phenantroline and APT102 significantly down-regulated the surface abundance of platelet receptors. Caco-2 cells aggregate platelets, at least in part, via releasing MMP-2 and ADP. Modulation of MMP-2 and ADP actions could have therapeutic value in colonic cancer.

Colorectal cancer is the fourth most common type of cancer in Western countries and the second leading cause of death from cancer in North America (Gill et al., 2004). Approximately 40 to 50% of patients who undergo potentially curative surgery finally relapse and die of metastatic disease (Andre et al., 2004). Interestingly, some patients develop thrombotic complications during the course of the disease (Tasi et al., 2004). There is strong evidence that platelets play an important role in the pathophysiology of metastasis (Nash et al., 2002; Gupta and Massague, 2004; Jurasz et al., 2004). In fact, cancer cells have the ability to induce platelet aggregation that confers several advantages to the survival of the tumor cells and its successful metastasis (Jurasz et al., 2004). Moreover, this ability to aggregate platelets correlates with the metastatic potential of tumor cells (Gasic et al., 1968; Radomski et al., 1991). There are diverse mechanisms involved in tumor cell induced-platelet aggregation (TCIPA), where platelets can be activated by cancer cells releasing ADP, thromboxane A2, and matrix metalloproteinase-2 (Jurasz et al., 2001; Alonso-Escolano et al., 2004).

ADP is stored in platelet-dense granules, and it is a potent proaggregatory agent that can be released during TCIPA induced by fibrosarcoma, breast carcinoma, and neuroblastoma cells (Bastida et al., 1986; Jurasz et al., 2001; Alonso- Escolano et al., 2004). However, the role of ADP in platelet aggregation induced by colon cancer cells is not clear (Sarrett et al., 1987; Grignani and Jamieson, 1988).

Matrix metalloproteinases (MMPs) comprise an ever-growing family of zinc- and calcium-dependent endopeptidases that are capable of degrading most components of the extracellular matrix (Sternlicht and Werb, 2001). MMPs participate in tissue remodeling in diverse physiologic situations, including embryogenesis, wound healing, and angiogenesis (Sternlicht and Werb, 2001). Moreover, overexpression of these enzymes is also implicated in the degradation of basement membrane facilitating tumor invasion. In addition, we have found previously that platelet aggregation may be mediated via the release of MMP-2 from platelets (Sawicki et al., 1997). Interestingly, we have also shown that MMP-2

ABBREVIATIONS: TCIPA, tumor cell-induced platelet aggregation; MMP, matrix metalloproteinase; FITC, fluorescein isothiocyanate; PE, phycoerythrin; TXA2, thromboxane A2.
release from platelets and cancer cells is involved in platelet aggregation induced by human fibrosarcoma HT-1080, lung carcinoma A549, and breast adenocarcinoma MCF7 cells (Jurasz et al., 2001; Alonso-Escolano et al., 2004). Although the mechanisms of MMP-mediated stimulation of platelets are not well understood, interactions between MMP-2 and platelet receptors could play an important role (Martinez et al., 2001; Galt et al., 2002).

GPIIb/IIIa is the major platelet surface transmembrane receptor, and it plays an important role in platelet aggregation (Shattil et al., 1998). In addition, platelets contain a pool of this glycoprotein stored in α-granules (Wagner et al., 1996). Furthermore, GPIb, the von Willebrand factor-binding subunit of the GPIb/IX, mediates mainly platelet adhesion (Andrews and Berndt, 2004). Finally, P-selectin, which is also stored in platelet α-granules, mediates platelet-leukocyte aggregation since it is rapidly translocated to the surface upon platelet activation (Andrews and Berndt, 2004).

We have now established an in vitro experimental model of TCIPA induced by Caco-2 cells. Using this model, we aimed at studying whether intestinal adenocarcinoma cells were able to induce platelet aggregation, molecular mechanisms responsible for the formation of platelet-human intestinal cancer cell aggregates, the abundance of different platelet receptors during TCIPA, and the regulation of Caco-2-induced platelet aggregation and the receptor abundance in the presence of several pharmacologic inhibitors of platelet aggregation.

Materials and Methods

Reagents. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Collagen was obtained from Chronolog (Havertown, PA). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against high-affinity GPIIb/IIIa (PAC-1-FITC) was purchased from Becton Dickinson Biosciences (Mississauga, ON, Canada). R-Phycoerythrin (PE)-conjugated monoclonal antibody against human platelet GPIb (CD42b-PE) and allophycocyanin-conjugated monoclonal antibody against human platelet P-selectin (CD62P) were purchased from BD Biosciences (San Jose, CA).

Expression and Purification of Human Apyrase APT102. CD39L3 was cloned from the large-insert human brain cDNA library (CLONTECH, Palo Alto, CA) by polymerase chain reaction using gene-specific primers. A soluble form of CD39L3 (APT101) was generated by removing the N-terminal 44 amino acids and the C-terminal 43 amino acids. To facilitate cloning of APT101 into vectors suitable for expression in mammalian cells, an Smal restriction site was introduced in-frame with the ATG of APT101. The resulting polymerase chain reaction product was cloned into the Smal site of pSP7T (Promega, Madison, WI). Two point mutations, R67G and T69R, were introduced into APT101 coding sequence using the quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The gene of the mutant, designated APT102, was cloned into expression plasmid vector pSEQTAG2a (Invitrogen, Carlsbad, CA). Expression plasmid was transfected into HEK 293T cell line. The recombinant protein of APT102 was secreted from HEK293 cells and purified from conditioned medium using anti-exchange chromatography, size-exclusion chromatography, and affinity chromatography.

Cell Culture. Caco-2 cells, a human intestinal adenocarcinoma cell line, were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in 75-cm² flasks in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were grown in minimum essential medium supplemented with pyruvate, sodium bicarbonate, 20% fetal bovine serum, gentamicin sulfate (0.05 mg/ml), penicillin G (0.06 mg/ml), and streptomycin sulfate (0.01 mg/ml). The medium was changed every 2 days, and cells were subcultured three times each week. When confluent, cells were detached using EDTA (7 mM) in Dulbecco’s phosphate-buffered saline and gentle shaking. EDTA was then washed away with Tyrode’s solution; afterward, Caco-2 cells were resuspended in Tyrode’s solution and counted.

Preparation of Human Washed Platelets. Blood was collected from healthy volunteers who had not taken any drugs known to affect platelet function for at least 14 days prior to the study. Washed platelet suspensions (5 × 10⁸ platelets/ml) were prepared from blood as described previously (Radomski and Moncada, 1983).

Platelet Aggregation. The interactions between platelets and tumor cells were measured by light aggregometry (Radomski et al., 1991; Jurasz et al., 2001; Alonso-Escolano et al., 2004). In brief, platelet samples (2.5 × 10⁸/ml) were placed in a whole-blood ionized calcium lumia-aggrometer (Chronolog) and incubated for 2 min at 37°C, with stirring at 900 rpm, prior to the addition of aggregating agents. For most experiments, collagen at a concentration that resulted in maximal aggregation (5 μg/ml) was used as a control agonist. TCIPA was initiated by the addition of tumor cells (10⁹-10¹⁰ cells/ml), and the reaction was monitored and analyzed by the Aggro-Link data-processing system (Chronolog) for up to 30 min. Platelet aggregation was expressed as the percentage of aggregation at a time when the highest concentration of tumor cells increased the light transmittance by 50% (Jurasz et al., 2001). To study the involvement of TXA₂ and MMP-2-dependent pathways in Caco-2-induced platelet aggregation, platelets were preincubated for 2 min with inhibitors of these pathways, such as acetylsalicylic acid (aspirin) and α-phenanthroline (Jurasz et al., 2001; Alonso-Escolano et al., 2004), and aggregation was induced with 1.5 × 10⁷ cells/ml. To study the involvement of ADP in TCIPA, a new human apyrase, APT102, was tested with the same number of tumor cells as above.

Sample Preparation for Zymography. In brief, platelets at a concentration of 2.5 × 10⁹/ml were placed into the lumi-aggregometer, and tumor cells were added at different concentrations (1.5 × 10⁵-10⁸). The aggregates of platelets and tumor cells were collected after 30 min of incubation in the aggregometer. The samples were centrifuged at 900g at room temperature for 10 min, yielding the pellet and the releasate. The pellets were then homogenized on ice three times (10 s each) using a Vibra Sonic sonicator (Sonics and Materials Inc., Danbury, CT). Both the pellet homogenate and the releasate were stored at −20°C until assayed for the presence of MMP activity by zymography.

Zymography. Gelatin zymography was used to detect the activity of MMP-2 and MMP-9. Zymographic analysis was carried out on aggregates of platelets and tumor cells, as described previously (Jurasz et al., 2001; Alonso-Escolano et al., 2004). In brief, samples were subjected to 10% SDS-polyacrylamide gel electrophoresis with a polymerized gelatin (0.2%; Sigma Chemical Co.) incorporated as a substrate for gelatinolytic proteases. After electrophoresis, the gels were washed with 2.5% Triton X-100 (three times, 20 min each) and then incubated for 48 h at 37°C in enzyme assay buffer (25 mM Tris HCl, 0.9% NaCl, 5 mM CaCl₂, and 0.05% Na₂N₃, pH 7.5). The conditioned medium of HT-1080 human fibrosarcoma cells (that contains high amounts of proMMP-2, MMP-2, proMMP-9, and MMP-9), was used as control. After 48-h development, gels were fixed and stained in 40% methanol, 10% acetic acid, and 0.1% (w/v) Coomassie Blue R-250 (Sigma Chemical Co.) for 1 h and then destained in 4% methanol with 8% acetic acid. The gelatinolytic activities were detected as transparent bands against the background of Coomassie Blue-stained gelatin. MMP-2 was identified by its mol. wt. when compared with conditioned medium of HT-1080 human fibrosarcoma cells. The intensities of the separate bands were analyzed and quantified using the ChemiDoc XRS System (Bio-Rad, Hercules, CA). The gelatinolytic activity of each band was expressed as arbitrary units of density per milligram of protein.

Microscopy of TCIPA. The structure of platelet-tumor cell aggregates was studied using phase-contrast microscopy (Jurasz et al., 2001).
Results

Caco-2 Cell-Induced Platelet Aggregation. Caco-2 cells were tested for their ability to induce platelet aggregation. When platelets were incubated in the aggregometer for 30 min at 37°C without the addition of tumor cells, no platelet aggregation was detected. However, colon adenocarcinoma cells induced platelet aggregation in a concentration-dependent manner (Fig. 1A and B). In addition, this aggregation was preceded by a lag phase of several minutes that decreased as the concentration of Caco-2 cells increased (Fig. 1A). Because the concentration of $1.5 \times 10^3$ cells/ml was sufficient to induce platelet aggregation, all remaining experiments were performed with this amount of cells.

MMP-2 Activity in Cancer Cell-Platelet Homogenates and Their Releasates. Zymography was conducted to study whether MMP-2 and MMP-9 were involved in Caco-2-induced platelet aggregation. The proMMP-2 was the major gelatinase detected, as shown by the 72-kDa band. Zymography showed a significant decrease in the MMP-2 activity in cancer cell-platelet pellets compared with unaggregated platelets ($P < 0.0005; n = 4$) when Caco-2 cells were added to induce platelet aggregation (from $1.5 \times 10^5$ to $10^6$ cells/ml) (Fig. 2A). In addition, there was a significant increase in MMP-2 activity in the releasate of aggregated platelets induced by Caco-2 in comparison with unaggregated platelets ($P < 0.0001; n = 4$), indicating the enzyme release during TCIPA (Fig. 2B). No significant differences were found in MMP-9 activity when Caco-2 cells were added to induce platelet aggregation (data not shown).

Inhibition of Caco-2-Induced Platelet Aggregation. We used selective inhibitors of three TXA$_2$, MMPs, and ADP (Sawicki et al., 1997; Jurasz et al., 2001) to characterize the mechanism of Caco-2-induced platelet aggregation. Aspirin (100 µM), APT102 (40–160 µM), and o-phenanthroline (500 µM) were used to inhibit TXA$_2$-, ADP-, and MMP-2-mediated pathways of aggregation, respectively. Aspirin did not exert any significant effect on TCIPA (Fig. 3, A and B). However, this concentration of aspirin inhibited collagen-induced aggregation by $56 \pm 7\%$ ($n = 4$) and abolished arachidonic acid-induced (100 µM) aggregation ($n = 4$). In contrast to aspirin, phenanthroline inhibited Caco-2-induced platelet aggregation, as shown by aggregometry (Fig. 3, A and B) and phase-contrast microscopy (Fig. 4). Interestingly, APT102 also inhibited aggregation induced by Caco-2 in a concentration-dependent manner and prolonged lag phase (Figs. 3, C and D, and 4).

Measurement of Platelet Receptors during Caco-2-Induced Aggregation. The interactions of platelets with...
tumor cells induced a significant ($P < 0.005; n = 4$) increase in the number of copies of activated GPIIb/IIIa (PAC-1) on platelets (Fig. 5A). Furthermore, platelet activation with Caco-2 cells ($1.5 \times 10^3$ cells/ml) resulted in a significant increase in the number of copies of total GPIIb/IIIa ($P < 0.005; n = 4$), GPIb ($P < 0.01; n = 4$), and P-selectin ($P < 0.005; n = 4$) on platelets in comparison with resting platelets (Fig. 5, B–D).

**Effects of Inhibitors on Receptors during Caco-2-Induced Platelet Aggregation.** Flow cytometry performed on platelets preincubated with aspirin (100 $\mu$M) and o-phenanthroline (500 $\mu$M) and then activated by Caco-2 cells was analyzed. Phenantroline significantly ($P < 0.01, n = 4$) inhibited Caco-2-mediated increase in total GPIIb/IIIa, activated GPIIb/IIIa, GPIb, and P-selectin (Fig. 6, A–D). In contrast, aspirin failed to prevent the increase in platelet surface abundance of these receptors ($P > 0.05$; $n = 4$) (Fig. 6, A–D). Because 40 $\mu$g/ml APT102 was found to be sufficient to inhibit aggregation induced by Caco-2 cells, the following flow-cytometry experiments conducted to study the effects of this compound on platelet receptors during TCIPA were performed using this concentration of inhibitor. Interestingly, APT102 also significantly inhibited ($P < 0.01, n = 4$) up-regulation of receptors on the surface of platelets during TCIPA (Fig. 6, A–D).

**Discussion**

Platelets have been implicated in tumor metastasis and thrombotic complications of patients with colorectal adenocarcinoma (McCarty et al., 2002; Im et al., 2004). However, the relative contribution of ADP, MMP-2, and TXA2 to colon cancer-induced platelet aggregation still remains unclear. Therefore, the aim of our investigation was to study the interactions between human intestinal cancer cells and platelets. Furthermore, we studied the expression of major receptors on the platelet surface during TCIPA. In addition, we tested the effects of different pharmacologic compounds on Caco-2-induced platelet aggregation.

First, we used human colon adenocarcinoma cells, Caco-2, to study their interactions with platelets in vitro. We showed that Caco-2 cells were able to induce platelet aggregation in...
a concentration-dependent manner, as evidenced by aggregometry.

Knowing that Caco-2 cells stimulated platelet aggregation, we next studied the molecular mechanisms implicated in this interaction. We have shown previously the requirement for activated MMP-2 to induce the MMP-2-dependent pathway both in agonist and TCIPA induced by HT-1080 and MCF7 breast cancer cells (Jurasz et al., 2001; Alonso-Escolano et al., 2004). In the present study, we have found that MMP-2 is released during platelet aggregation induced by Caco-2 cells. We have found previously that MMP-9 may inhibit platelet aggregation counteracting the effects of MMP-2 (Fernandez-Patron et al., 1999). However, Caco-2-induced platelet aggregation did not result in significant release of MMP-9, suggesting that MMP-2 is the dominant gelatinase released during Caco-2-induced platelet aggregation. However, we did not study other MMPs, such as MMP-1, which has been also involved in agonist platelet aggregation (Galt et al., 2002). Therefore, the precise role of this enzyme during TCIPA remains to be elucidated. MMP-2 has been previously involved in colorectal cancer in humans since this enzyme has been found in regions of tumor invasion (Papadopoulou et al., 2001; Ornstein and Cohn, 2002). In addition, a single-nucleotide polymorphism in the MMP-2 promoter region that increases gene transcriptional activity has been associated with colon cancer development and invasion (Xu et al., 2004).

Numerous studies have suggested that aspirin and other nonsteroidal anti-inflammatory drugs, particularly highly selective COX-2 inhibitors, could be used as chemoprophylactic agents preventing colorectal adenoma and cancer, especially in patients with familial adenomatous polyposis (Thun et al., 2002). However, aspirin did not reduce TCIPA in our study, suggesting that TXA2-mediated pathway of aggreg-
P-selectin is mainly stored in platelet α-granules against platelet GPIb and/or GPIIb/IIIa inhibited their platelets to the injured part of the vascular wall (Ruggeri, 1986). Interestingly, during the course of our investigation, human apyrase APT102 was able to attenuate platelet aggregation induced by Caco-2 cells in a concentration-dependent manner. In humans, apyrases maintain vascular fluidity and modulate inflammation by metabolically deleting extracellular ADP and ATP released by activated platelets (Marcus et al., 2003). APT102 is a human apyrase optimized by protein engineering. This protein exhibited a 4-fold higher ADPase activity than the wild-type enzyme CD39L3 and a well characterized isozyme CD39. The improved enzymatic activity was correlated with significantly higher potency to inhibit and even reverse ADP-induced platelet aggregation (Jeong et al., 2004). Therefore, taken together, MMP-2 and ADP released during the TCIPA seem to be the major pathways of platelet aggregation triggered by Caco-2.

Because platelet receptors mediate platelet adhesion and aggregation, we next studied the changes in the abundance of these receptors on platelets induced by human colon adenocarcinoma cells. In our study, we found that Caco-2 cells increased the number of copies of GPIIb/IIIa (both nonactivated and activated receptors), GPIb, and P-selectin, as measured by flow cytometry. GPIIb/IIIa is constitutively expressed as an inactive receptor on the platelet surface, but upon platelet aggregation, this receptor becomes active and binds several protein ligands (Bennett, 2001). GPIb is also constitutively expressed in platelets, and its main role is to attach several protein ligands (Bennett, 2001). GPIb is also constitutively expressed in platelets, and its main role is to attach several protein ligands (Bennett, 2001). GPIb is also constitutively expressed in platelets, and its main role is to attach several protein ligands (Bennett, 2001).

MMP-2 is crucial for the formation of Caco-2-platelet aggregates via activation of the different receptors on the platelet surface. Interestingly, APT102 also significantly reduced the number of copies of GPIIb/IIIa (both nonactivated and activated receptors), GPIb, and P-selectin on the platelet surface during TCIPA to a similar extent, suggesting that the release of ADP during TCIPA also plays an important role in regulation of platelet receptors.

In conclusion, our results show that Caco-2 cells stimulate platelet aggregation through the release of MMP-2 and ADP. The proaggregatory effects of Caco-2 are associated with an increase of platelet GPIb, GPIIIa, and P-selectin receptors. Modulation of MMP-2- and ADP-mediated pathways may represent an effective therapeutic approach to prevent or reduce tumor metastasis induced by colon adenocarcinoma.

Acknowledgments

We thank Anna Radomski for assistance in blood collection and invaluable technical help.

References


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