Inhibition of the Platelet-aggregating Activity of Two Human Adenocarcinomas of the Colon and an Anaplastic Murine Tumor with a Specific Thrombin Inhibitor, Dansylarginine N-(3-Ethyl-1,5-pentanediyl)amide

Edward Pearstein, Cynthia Ambrogio, Gabriel Gasic, and Simon Karpatkin

Departments of Medicine [E. P., C. A., S. K.] and Pathology [E. P., C. A.], and Irvington House Institute [E. P., C. A.], New York University Medical School, New York, New York 10016

ABSTRACT

Platelets are required for certain experimental metastases. Several lines of animal tumor cells aggregate platelets in vitro and in vivo. Previous studies with one of these lines, an SV40-transformed 3T3 mouse fibroblast (SV3T3) have revealed that the platelet-aggregating material is an extractable membrane-associated sialoprotein which requires divalent cation, complement, and a heat-stable plasma component for activity. Little information is available on the interaction of human tumors with platelets. We now report on the ability of two human adenocarcinomas of the colon (LoVo and HCT-8) and an anaplastic mouse tumor (Hut-20) to aggregate platelets by a different mechanism, the generation of thrombin. These spontaneous cell lines aggregate human or rabbit platelet-rich plasma after a 1- to 2-min lag period. This is often followed by a visible clot. Unlike SV3T3 cells, aggregation by LoVo, HCT-8, and Hut-20 cells is not inhibited by neuraminidase, trypsin, or cobra venom factor. These three cell lines markedly shorten the recalciﬁcation time of citrated plasma, whereas SV3T3 cells do not. Phospholipase A2 treatment inhibits the shortening of the recalciﬁcation time for the three tumors; this parallels its inhibitory effect on platelet aggregation. LoVo, HCT-8, and Hut-20 cells generate thrombin via the “tissue factor” coagulation pathway (using coagulation factor-deﬁcient substrates). Dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide, a highly speciﬁc, potent antithrombin antagonist, inhibits LoVo-, HCT-8-, and Hut-20-induced platelet aggregation at 4 to 15 μM, whereas its effect on SV3T3 cells is negligible. If platelets are required for certain human tumor metastases, dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide, or other antithrombin agents, may prove to be valuable therapeutic agents.

INTRODUCTION

A role for platelets in the hematogenous dissemination of animal tumors has been suggested by many studies (3–9, 18, 20–23, 26, 31, 32, 34, 36, 37). Certain tumor cells require platelets for the development of metastases (5, 8, 20, 32). Ultrastructural studies have demonstrated arrested tumor emboli surrounded by platelets (21, 22, 34, 37). Several tumor cells induce thrombocytopenia in vivo (5, 8, 20) and aggregate platelets in vitro (3–7, 9, 18, 23, 26, 31, 32). A correlation exists between the ability of some tumor cells to aggregate platelets in vitro and their requirement for metastases (5, 32).

We have recently studied 2 virally transformed animal cell lines (23, 31, 32). A SV3T3 line was shown to have an extractable, membrane-associated sialoprotein which aggregates platelets following a 1- to 2-min lag period. The reaction requires Ca2+, is associated with release of serotonin, and is inhibited by adenosine or indomethacin (31). Two plasma factors are also required: a heat-labile complement component and a 56° stable plasma component (23). Ten metastatic-variant derivatives of a polyoma-induced PW20 renal cell sarcoma of Wistar-Furth rats have also been studied in vivo as well as in vitro (32). They also have an extractable PAM which appears to require membrane sialic acid for platelet aggregation. Excellent correlations were obtained for metastatic potential, surface sialic acid, and PAM-induced platelet aggregation.

The purpose of this investigation was to study whether the mechanism of action of human tumors was the same as that described for the animal tumors. Two human adenocarcinomas of the colon (HCT-8 and LoVo) and one anaplastic murine tumor (Hut-20) were studied. In contrast to our studies with 2 virally transformed lines, the 3 spontaneous cell lines appear to operate via the generation of thrombin and do not require trypsin-sensitive protein or neuraminidase-digestible surface sialic acid for platelet aggregation. Of particular interest is the observation that platelet aggregation induced by all 3 spontaneous tumors can be inhibited by a highly specific synthetic thrombin inhibitor, DAPA, at approximately 10−6 M (19, 28).

MATERIALS AND METHODS

Cell Cultures. All cell lines were grown in the presence of 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). All tissue culture supplies were obtained from Grand Island Biological Co., Grand Island, N. Y.

An SV3T3 cell line was maintained in Dulbecco’s modiﬁed Eagle’s medium supplemented with 10% calf serum as described previously (31).

An anaplastic murine tumor cell line, Hut-20, was obtained through the courtesy of Dr. Addi Gazdar of the Veterans Administration Hospital, Washington, D. C. The line was grown in Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 10% Bobby calf serum.

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2 On sabbatical from the Department of Medicine, University of Pennsylvania.
3 To whom requests for reprints should be addressed.
4 The abbreviations used are: SV3T3, SV40-transformed BALB/c 3T3 fibroblast; PAM, platelet-aggregating material; DAPA, dansylarginine N-(3-ethyl-1,5-pentanediyl)amide; PRP, platelet-rich plasma; PPP, platelet-poor plasma.
A human adenocarcinoma of the colon, HCT-8, was obtained through the courtesy of Dr. Edward Cadman, Yale University School of Medicine, New Haven, Conn., and was maintained in culture in Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 10% fetal bovine serum.

Another human adenocarcinoma of the colon, LoVo, was provided by Dr. Benjamin Drewinko of the M. D. Anderson Hospital, Houston, Texas, and was grown in McCoy’s Medium 5A containing 15% fetal bovine serum.

Cells were harvested using 5 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid in Hanks’ buffered salt solution, for 10 min on ice, or with trypsin treatment [trypsin (0.5 mg/ml)-EDTA] for 3 min at 37°. Harvested cells were washed and suspended in Veronal buffer (0.03 mM sodium barbital-0.12 mM NaCl, pH 7.4) prior to use. Cells were quantitated by counting in a hemocytometer, and viability was determined by trypan blue exclusion.

The species origin of all lines was verified by karyotyping through the courtesy of Dr. M. Smith, Mt. Sinai Medical School, New York, N. Y.

PRP. Human or animal blood was anticoagulated with heparin (Liquemin-Organon, Inc., West Orange, N. J., final concentration, 5 units/ml). PRP was prepared by centrifugation at 150 x g for 5 min at room temperature. PPF was prepared from the remaining blood by additional centrifugation at 2000 x g for 15 min. PRP was incubated at room temperature for 30 min in tightly capped plastic tubes prior to use.

Platelet Aggregation. Aggregometry was performed in a Bio-Data aggregometer (Bio-Data, Willow Grove, Pa.) as described previously (23, 31, 32).

Recalcification Time. Blood was anticoagulated with sodium citrate, 0.38% final concentration, and PPP was prepared as described above. Cell suspension (0.1 ml) was incubated with 0.1 ml of 50 mM CaCl₂ for 2 min at 37° in a plastic tube. The reaction was started by the addition of 0.1 ml of PPP, and the clotting time was recorded in sec (monitored by inversion of the tube, every 30 sec). Recalcification times were also performed with human plasmas deficient for Coagulation Factors XII, IX, X, II, V, and VII (George King Biologicals, Overland Park, Kans.).

Enzyme Treatment. Tumor cells removed from tissue culture dishes with ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid were washed, suspended in Veronal buffer, and incubated either with neuraminidase (1 unit/ml; type V; Sigma Chemical Co., St. Louis, Mo.) plus 2 mM PMSF or with boiled (10 min) phospholipase A₂ (25 μg/ml; bee venom; Sigma) for 1 hr at 37°. The cells were then washed in Veronal buffer and resuspended to the proper cell count prior to utilization in aggregation and recalcification time studies. For trypsin treatment, cells in monolayer were treated with the enzyme (1.5 mg/ml) for 1 hr at 37°; the floating cells were collected and washed 3 times with Veronal buffer before use.

Treatment with Complement Inactivators. Purified cobra venom (100 units/ml; 40 to 200 μl) from Naja naja Kaouthia (Cordis Laboratories, Miami, Fla.) was incubated with 0.4 ml of PRP for various lengths of time at 37°, followed by addition of cell suspension. Cobra venom factor did not affect platelet aggregation induced in PRP with 10⁻⁶ M ADP. Furthermore, platelets isolated from cobra venom factor-treated PRP by gel filtration (35) were responsive to aggregation by PAM in the presence of fresh PPP.

Washed zymosan (Sigma) was resuspended in plasma at a final concentration of 2 mg/ml, incubated for 1 hr at 37°, and removed by centrifugation at 1000 x g for 5 min at 4°. The supernatant was assayed for its ability to support platelet aggregation.

DAPA was synthesized (28) and kindly supplied by Dr. Kenneth Mann, Mayo Foundation, Rochester, Minn. This synthetic inhibitor of thrombin has a Kᵢ for fibrinogen of 10⁻⁷ M.

Animals. Heparinized blood was collected from female New Zealand White rabbits by cannulation of the ear artery and from C57BL mice and Wistar-Furth rats by cardiac puncture. PRP and PPP were prepared from these samples as described for human plasma.

RESULTS

LoVo and HCT-8 cells aggregated platelets from heparinized PRP of 8 human donors tested and 100% of all rabbit donors tested. Hut-20 cells aggregated heparinized PRP of 60% of 18 human donors tested and 100% of all rabbit, rat, and mouse donors tested. SV3T3 cells aggregated heparinized PRP of all rabbit donors tested but did not aggregate human PRP.

Platelet Aggregation by LoVo, HCT-8, or Hut-20 Cells Is Independent of Complement

To facilitate comparisons between human lines and SV3T3-induced aggregation, rabbit PRP was often used since SV3T3 cells do not aggregate human PRP and rabbit PRP responded in a similar fashion as responder human PRP whenever compared.

We have demonstrated previously that SV3T3 PAM-induced aggregation is impaired and the lag period prolonged (23) when rabbit complement is inactivated with cobra venom factor or zymosan. No such effect was noted when LoVo, HCT-8, or Hut-20 cells (1 x 10⁴) were used as the aggregating agent, using either rabbit PRP (Chart 1) or human PRP (data not shown).

Treatment of LoVo, HCT-8, Hut-20, and SV3T3 Tumor Cells with Enzymes

Platelet Aggregation. In previous studies (31), we noted that the PAM extract of the animal tumor cell line SV3T3 was sensitive to treatment with trypsin, neuraminidase, or phospholipase A₂. These experiments were repeated with intact SV3T3 cells and compared to LoVo, HCT-8, and Hut-20 cells (Chart 2), since PAM cannot be prepared from the 3 nonvirally transformed cell lines. Although SV3T3 cells lost their platelet aggregability following treatment with trypsin, neuraminidase, or phospholipase A₂, the LoVo, HCT-8, and Hut-20 cells were only affected by phospholipase A₂ treatment (data not shown for LoVo cells). When compared to SV3T3 cells, the sensitivity to phospholipase A₂ was 25-fold greater for Hut-20 cells and similar for LoVo and HCT-8 cells.

Recalcification Time. Because clot formation was occasionally noted 10 to 15 min after platelet aggregation with the 3 spontaneous tumor cells, but not with SV3T3 cells, thrombin

Chart 1. Aggregation of rabbit PRP with SV3T3, LoVo, HCT-8, and Hut-20 cells with or without treatment with cobra venom. Aggregation induced by SV3T3 in (a) control or (b) cobra venom-treated PRP. Aggregation by LoVo, HCT-8, or Hut-20 cells in control or cobra venom-treated PRP (c); these curves were superimposable.
Tumors Aggregate Platelets via Thrombin

generation was measured, using a recalcification time. LoVo, HCT-8, and Hut-20 cells significantly shortened the recalcification time of citrated rabbit PPP. LoVo was more potent than HCT-8 or Hut-20 cells. Similar results were obtained with human PPP. This was dependent on cell concentration (Table 1). Similar results were obtained with PRP (data not shown). SV3T3 cells had a negligible effect on the recalcification time. For example, $1 \times 10^5$ cells had no effect; $1 \times 10^6$ cells shortened the recalcification time from 10.0 to 7.8 min.

Table 1 also demonstrates the effect of trypsin, neuraminidase, or phospholipase A$_2$ on the ability of tumor cells to shorten the recalcification time of rabbit PPP. As with platelet aggregation results, trypsin and neuraminidase had no effect on the recalcification time, whereas phospholipase A$_2$ did have an effect.

Table 2 demonstrates the effect of tumor cells on the recalcification times of coagulation factor-deficient human plasmas. LoVo, HCT-8, and Hut-20 cell-induced shortening of the recalcification time was noted with Factor XII-deficient and IX-deficient plasma, but not with Factor II-, V-, X-, or VII-deficient plasma. The data suggest that these cell lines were activating the coagulation system via activation of Factor VII by the extrinsic tissue pathway.

Effect of DAPA, a Specific Thrombin Inhibitor

The ability of human tumor cells to aggregate platelets via the generation of thrombin was examined by the use of a highly specific thrombin inhibitor, DAPA. Chart 3 demonstrates complete inhibition of tumor-induced rabbit platelet aggregation by DAPA at 4 to 15 $\mu$M, using $5 \times 10^5$ LoVo, HCT-8, and Hut-20 cells. Similar results were obtained with human PRP. At higher concentrations, DAPA had an effect on SV3T3 cells. However, 20-fold fewer cells and 30-fold greater DAPA concentration was required to demonstrate this effect.

DISCUSSION

Several laboratories have examined the interaction of animal tumor cells with platelets, in particular, the aggregation of platelets by tumor cells in vitro (3–9, 18, 20–23, 26, 31, 32, 34, 36, 37) and the enhancement of metastasis by platelets in vivo (5, 8, 32). Little information is available on the interaction of human tumor cells with platelets. In an early report, Gasic et al. (9) studied 7 different human tumors obtained from 29 individuals. However, 6 of 7 tumors (from 25 of the 29 individuals) were fresh tissue preparations obtained at surgery. Thus, the contribution of tissue collagen and contaminating fibroblasts to the platelet aggregation observed cannot be excluded.
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Four different neuroblastoma cell lines were grown in tissue culture. All 4 aggregated platelets. One of these, neuroblastoma IMR, had appreciable procoagulant activity when assayed by its ability to shorten the recalcification time of PPP.

We have studied the mechanism of tumor cell-induced platelet aggregation by 2 different virally transformed animal cell tumors: a SV3T3 cell line (23, 31) and a polyoma-transformed rat renal cell sarcoma (32). Both cell lines required surface sialic acid for thrombin generation; this was consistent with the observations that Vila (29) and tissue factor (10) are not inhibited by heparin and antithrombin III inhibition when bound to the platelet surface (37), thereby increasing the efficiency of thrombin-mediated platelet aggregation. This is consistent with the tissue factor coagulation pathway being operative. This suggestion was supported by recalcification studies performed with coagulation factor-deficient plasmas. These indicated that LoVo and HCT-8 cells were reacting with Factor VII to activate Factor X to Xa leading to the activation of Factor II to IIa. Similar results were obtained with a spontaneous murine tumor (Hut-20), indicating that some animal tumors may aggregate platelets in a similar fashion as that obtained for the 2 human cell lines.

One might ask the question: How does thrombin aggregate platelets in the presence of heparin and antithrombin III? We propose that thrombin is formed at the platelet membrane surface following tumor cell adherence to platelets (21–23, 34, 37), thereby increasing the efficiency of thrombin-mediated platelet aggregation. This is consistent with the observations that Vila (29) and tissue factor (10) are not inhibited by heparin (10) and antithrombin III (29), Xa is protected from heparin and antithrombin III inhibition when bound to the platelet surface (27), and binding of Xa to the platelet surface results in a 300,000-fold increase in the conversion of prothrombin to thrombin (27).

The mechanisms of induction of procoagulant activity of human tumors (12, 13, 33) as well as animal tumors (1, 2, 13, 14) have been studied by other workers. However, these studies have not concerned themselves with their interaction with platelets. With most carefully studied systems, tumor cells appear to have, almost exclusively, a serine or cysteine protease capable of activating Factor X (1, 2, 11–14, 27, 33). However, activation of the tissue factor pathway via Factor VII has also been reported for human leukemic cells (16, 17) and Yoshida ascites hepatoma cells of the rat (24). LoVo, HCT-8, and Hut-20 cells apparently operate via activation of the tissue factor pathway.

Marcum et al. (26) recently studied the interaction of Hut-20...
cells in vitro, using platelet aggregometry, and ex vivo, using an injured rabbit blood vessel perfusion technique. In vitro, Hut-20 cells aggregated heparinized human PRP after a 1-min lag period. Ex vivo, Hut-20 cells induced the formation of a platelet-tumor thrombus which adhered to the injured subendothelium of a rabbit aorta. The adherent platelet-thrombus formation could be prevented by administration of prostaglandin E1, or use of plasma deficient in Von Willebrand’s factor. Our present data support 2 mechanisms for the aggregation of platelets by tumor cells. (a) Studies on the 2 virally transformed animal tumor cells (23, 31, 32) indicate that cell surface sialoglycoprotein may be important and that complement activation is also required. Thrombin generation does not appear to be important because SV3T3 cells have a negligible effect on the recalcification time. Although DAPA can inhibit platelet aggregation induced by SV3T3 cells, this required 30-fold more inhibitor and 20-fold less cells than that required for inhibition of LoVo, HCT-8, or Hut-20 cells. The specificity of the reaction at this concentration of inhibitor and cells is therefore questionable. (£>) Our present studies on the human LoVo and HCT-8 cell lines and the murine Hut-20 cell line indicate that thrombin generation may be important. These 3 cell lines are exclusively sensitive to a highly specific synthetic thrombin inhibitor, DAPA. They are not sensitive to neuraminidase, trypsin, or complement inactivators. The 2 pathways for induction of platelet aggregation by tumor cells may be a reflection of spontaneous or opposed to viral transformation rather than a result of species differences.

The question therefore arises: Do most or all human tumor cell line aggregate platelets via the generation of thrombin? If a significant number of human cell lines do aggregate human platelets via the generation of thrombin, then highly specific synthetic thrombin inhibitors (19, 28) might be considered as possible adjuvant therapy in the treatment of human metastases. This is predicated on the supposition that platelets play a causative role in the initiation of certain tumor metastases.

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