Differentiation of Platelet-aggregating Effects of Human Tumor Cell Lines Based on Inhibition Studies with Apyrase, Hirudin, and Phospholipase

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ABSTRACT

Three different mechanisms have been detected for the aggregation of platelets by tumor cells in a homologous human system based on inhibition studies with apyrase, hirudin, and phospholipase D. In the major group, platelet aggregation induced by the SKBR3 (adenocarcinoma), SKNMC (neuroblastoma), HT29 (adenocarcinoma), and HT144 (melanoma) cell lines was inhibited by apyrase and phospholipase D but not by hirudin, suggesting that adenosine 5'-diphosphate is involved in the first step. However, since the reaction occurs only in heparinized plasma, the mechanism must differ from that of platelet aggregation which can be induced in citrated platelet-rich plasma by endogenous or exogenous adenosine 5'-diphosphate. In contrast, the Hut28 (mesothelioma) line was inhibited by hirudin and phospholipase D but not by apyrase, suggesting that the mechanism in this system involves the activation of the clotting system in the early stages. However, the coagulant-dependent mechanism observed with Hut28 can be differentiated from the similar mechanism we have observed previously with the U87MG (glioblastoma) cell line since the latter is unaffected by phospholipase D (Am J. Hematol., 11: 367–378, 1981). Phospholipase C had no effect on platelet aggregation induced by any of the human cell lines examined while both phospholipase A₂ and lyssolecithin inhibited aggregation in every case. These results suggest that two categories of human tumor cells can be defined based on whether they initiate platelet aggregation by adenosine 5'-diphosphate or coagulant-dependent mechanisms. However, within this latter category, subclassification is possible based on the inhibitory effects of phospholipase D.

INTRODUCTION

Platelets in plasma can be aggregated by a wide variety of low-molecular-weight agonists such as ADP and epinephrine or by protein agonists such as thrombin and collagen. The mechanisms of these reactions are not clearly understood. A third class of interaction involves platelet aggregation induced by tumor cells (for review, see Ref. 7). Qualitative observations of interactions between platelets and tumor cells were first made during postmortem examination over a century ago and have been confirmed numerous times in a wide variety of model systems. In 1962, Gasic and Gasic (9) observed that the incidence of tumor metastases in mice given injections of mammary adenocarcinoma TA3 tumor cells was decreased if there was a prior injection of neuraminidase, an effect they ascribed to increased "stickiness" resulting from the action of neuraminidase on the membrane glycoproteins of the tumor cells or of host endothelial cells. Subsequently, they made the important quantitative observation that this effect was due to neuraminidase-induced thrombocytopenia and that the incidence of lung tumors following injection of TA3 ascites cells was inversely proportional to the platelet count (11). They then extended these observations to show that there was a rough correlation between the ability of various tumor cells to aggregate platelets in vitro, the number of lung metastases produced in mice, and the beneficial effect of thrombocytopenia in reducing metastasis (10).

Because of the difficulty of using in vivo assays for the quantitative assessment of platelet-tumor cell interactions, most subsequent studies have utilized aggregometry in attempts to elucidate the basic mechanisms involved (12–14, 17). Surface components of the tumor cells are thought to play a major role in initiating platelet activation. Rous virus-transformed rat kidney cells produce membrane vesicles in larger amounts than do untransformed cells, and these vesicles can cause platelet aggregation and release (8). A platelet-aggregating material has been extracted with 1 M urea from SV40-transformed mouse 3T3 fibroblasts (17), and in variants of the PW20 rat renal sarcoma line, correlations have been made between the production of the platelet-aggregating material, its sialic acid content, the ability of the tumor cells to induce platelet aggregation, and their content of cell surface sialic acid (18). The mechanism by which these cell surface components effect platelet aggregation is not known but has generally been ascribed to the release of ADP from the platelets, based on the inhibitory effects of apyrase, with little contribution from the activation of the coagulation system.

The relative contribution of these 2 systems in tumor cell-induced platelet aggregation may be evaluated by using specific enzymes. Apyrase removes ADP from solution by converting it to AMP. Thus, inhibition of aggregation by apyrase implies the involvement of ADP. A similar conclusion can be drawn if inhibition by a mixture of phosphoenolpyruvate-pyruvate kinase is observed. This mixture converts ADP to ATP and, because of the speed of the reaction in comparison to apyrase, can inhibit processes in which considerable amounts of ADP are being produced. Hirudin is a polypeptide which is a specific inhibitor of thrombin, and hence, inhibition by hirudin is taken to indicate the inhibition of procoagulant-dependent reactions.

Most of the previous work has utilized animal tumor cell lines, mainly of rat or mouse origin, and human or rabbit
platelets. We have recently begun to reexamine the interaction of platelets and tumor cells (3), their attachment as mixed thrombi at the vessel wall (16), and idiosyncratic aggregation responses to tumor cells by platelets from different donors (2) utilizing homologous systems of well-characterized cell lines derived from human tumors and heparinized human PRP. Two different mechanisms of platelet aggregation have been identified in these studies. With the Hut20 line, derived from an anaplastic murine tumor, the onset of aggregation appeared to be dependent on ADP derived from the tumor cells but not from the platelets, since it occurred prior to the onset of the platelet release reaction. Aggregation occurred using platelets from donors who had ingested aspirin but was completely inhibited in the presence of apyrase and was unaffected by hirudin. On the other hand, aggregation by the U87MG (human glioblastoma) line appeared to be due to a procoagulant activity released from the tumor cells since it was completely inhibited by hirudin but was unaffected by apyrase. Furthermore, phospholipase D, which cleaves phosphatidylcholine to choline and phosphatic acid, inhibited platelet aggregation induced by Hut20 tumor cells while aggregation induced by U87MG cells was unaffected by the enzyme (3).

In the present studies with a further 6 human tumor cell lines, we have found that thrombin-mediated systems in the 2 cell lines can be differentiated on the basis of their sensitivities to phospholipase D. ADP-dependent processes appear to be the major mechanism modulating tumor cell-induced platelet aggregation in the homologous human systems examined thus far.

**MATERIALS AND METHODS**

**Materials.** All chemicals were reagent grade. Heparin sodium was obtained from Fisher Scientific Co., Pittsburgh, Pa., while the following products were obtained from Sigma Chemical Co., St. Louis, Mo.: hirudin, Grade IV, from leeches (activity, 1000 units/mg protein); phospholipase A2 from bee venom (1500 units/mg); phospholipase C type III from Bacillus cereus (80 units/mg); phospholipase D type I from cabbages (100 units/mg); phospho-ethanolamine lyase; pyruvate kinase; bovine collagen; ADP (Grade I); L-β-lysophosphatidylcholine (L-β-lysophosphatidylcholine, type III, from bovine liver). Apyrase, Grade 1, from potato (ADP activity, 500 milliunits/mg) was obtained from Sigma and was shown to be free of detectable protease activity using agar plates containing either albumin or casein (5).

**Tumor Cells and Cell Cultures.** The HT29 (adenocarcinoma), SKNM (neuroblastoma), SKBR3 (adenocarcinoma), and HT144 (melanoma) lines were provided by Dr. Jørgen Fogh, Sloan-Kettering Institute, Rye, N. Y., while the Hut23 (adenocarcinoma) and Hut28 (mesothelioma) lines were provided by Dr. Adi Gazdar, Veterans Administration Hospital, Washington, D.C.

**RESULTS**

**Aggregation by Tumor Cells.** Each of the cell lines examined produced different aggregation patterns and required different amounts of tumor cells to effect aggregation. Aggregation was dependent only on cell number and not on the degree of confluence when cells were harvested at earlier points in the growth cycle. The HT29 line aggregated platelets at a final concentration in the cuvet of 10⁶ cells/ml. The Hut28, HT144, SKBR3, and SKNM lines required 5 x 10⁶ cells/ml while the Hut23 line did not cause platelet aggregation at tumor cell concentrations as high as 10⁷/ml. Differences were also observed between the different cell lines in the effects of apyrase, hirudin, and phospholipase D on the course of aggregation. These results are described below for each of the cell lines examined.

**SKBR3.** The aggregation profile of platelets exposed to SKBR3 cells consisted of a brief phase of reversible aggregation followed immediately by a larger irreversible phase (Chart 1A, Curve a). This profile is similar to that observed previously with the Hut20 line (3). Aggregation by this cell line was completely blocked by apyrase (250 µg/ml) (Chart 1A, Curve b). The second wave of aggregation, but not the first, was inhibited by phospholipase D (Chart 1A, Curve c). Hirudin (100 units/ml) had no effect on the aggregation profile (Chart 1A, Curve d).

**SKNM.** A similar pattern of biphasic aggregation was observed with the SKNM cell line although in this case there was a prolonged delay between the reversible wave and the
Chart 1. Aggregation of platelets in human heparinized PRP by different human tumor cell lines and modification of aggregation responses. In each panel, Curve a is aggregation induced by tumor cells alone at the concentrations described below, Curve b is the aggregation produced in the presence of apyrase (120 μg/ml), Curve c is the aggregation produced in the presence of phospholipase D (10 units/ml), and Curve d is the aggregation produced in the presence of hirudin (100 units/ml). For the SKNMC line, Curve e shows the effect of phosphoenolpyruvate-pyruvate kinase. Apyrase and phospholipase D were added to the PRP in the aggregometer cuvet immediately prior to the addition of the aggregating dose of tumor cells, while hirudin was incubated with the PRP for 30 min prior to the addition of tumor cells.

A. SKBR3, 5 x 10^6 cells/ml; B, SKNMC, 5 x 10^6; C, HT144, 5 x 10^6; D. HT29, 10^7; E. Hut28, 5 x 10^6; F. Hut23, 10^7.

onset of the second wave of aggregation (Chart 1B, Curve a). Apyrase caused a delay in the onset of the first wave of aggregation and a reduction in its amplitude as well as an increase in the lag time to the second wave of irreversible aggregation (Chart 1B, Curve b). In order to clarify the possible involvement of ADP, studies were also carried out with phosphoenolpyruvate-pyruvate kinase which destroys ADP more effectively than apyrase. This combination resulted in complete inhibition of both waves of the aggregation induced by SKNMC cells (Chart 1B, Curve e). Incubation with phospholipase D resulted in the inhibition of the second wave of aggregation, but the first wave was unaffected (Chart 1B, Curve c), while hirudin had no effect on the course of platelet aggregation induced by the SKNMC line (Chart 1B, Curve d).

HT144. The HT144 line caused monophasic irreversible aggregation with a prolonged lag time (Chart 1C, Curve a). In some experiments, there appeared to be a very small first wave during this lag period, but this effect was not consistently observed. Both apyrase (Chart 1C, Curve b) and phospholipase D (Chart 1C, Curve c) completely blocked aggregation with HT144, while hirudin was without effect other than causing a slight prolongation of the lag phase preceding irreversible aggregation (Chart 1C, Curve d).

HT29. The HT29 line also showed biphasic aggregation but with no apparent dissociation between the first and second waves and with a brief lag phase preceding aggregation (Chart 1D, Curve a). Aggregation by this cell line was completely blocked in the presence of apyrase (Chart 1D, Curve b) or phospholipase D (Chart 1D, Curve c). On the other hand, neither the lag phase preceding aggregation nor the biphasic aggregation curve was affected by the presence of hirudin (Chart 1D, Curve d).

Hut28. Hut28 cells caused monophasic aggregation but with a slowly increasing base line during the prolonged lag phase preceding aggregation (Chart 1E, Curve a). Apyrase had no effect on aggregation (Chart 1E, Curve b), but it was completely inhibited by phospholipase D (Chart 1E, Curve c) and by hirudin (Chart 1E, Curve d).

Hut23. The Hut23 cell line produced no significant aggregation at tumor cell concentrations as high as 10^7/ml (Chart 1F, Curve a).

Effects of Phospholipases and Lysolceithin. As noted
above, phospholipase D showed differential effects on the aggregation produced by the various cell lines. Phospholipase C (10 units/ml) had no effect on platelet aggregation produced by any of the cell lines examined, while phospholipase A2, added at the same time as the tumor cells, completely blocked aggregation by all of the human tumor cell lines. Lysolecithin, the product of the action of phospholipase A2 on phosphatidylcholine, at a concentration of 50 μg/ml, also caused complete inhibition of aggregation with all of the tumor cell lines examined (data not shown).

**DISCUSSION**

In previous studies, we have examined platelet aggregation induced by 2 tumor cell lines, Hut20 from an undifferentiated murine tumor and U87MG derived from a human glioblastoma (3). Two different mechanisms of induction of platelet aggregation were apparent. Platelet aggregation induced by the Hut20 line appeared to be primarily dependent on ADP released from the tumor cells themselves, while aggregation induced by the U87MG line was dependent on a procoagulant activity elaborated by the tumor cell surface.

The present examination of a further 6 human tumor cell lines shows that a third mechanism of platelet aggregation by cultured human tumor cells can be recognized based on aggregation responses and the inhibitory effects of apyrase, hirudin, and phospholipase D. The results are summarized in Table 1 together with the results for the Hut20, A549, and U87MG lines examined previously (3, 16). Some groupings among the various lines appear to be possible. In all cases, aggregation occurred only with heparinized PRP and not when citrate was used as anticoagulant.

SKBR3 induces biphasic platelet aggregation that is inhibited by apyrase but not by hirudin, and phospholipase D eliminates the second wave of aggregation. This pattern is similar to that observed previously with the Hut20 line. In this case, secretion of ADP from the tumor cells initiates the first wave of aggregation, which leads to platelet activation and to a second wave of aggregation. This second wave is associated with platelet secretion which must be independent of thrombin production since it is not blocked by hirudin. SKNMC, HT144, and HT29 also probably belong in this class. With SKNMC, the lag phase was prolonged with apyrase and aggregation was completely inhibited by phosphoenolpyruvate-pyruvate kinase. With HT144 and Hut29, there was no clearly marked reversible first wave, but platelet aggregation was completely inhibited by apyrase. No effect was observed with hirudin for any of these 3 lines.

Aggregation induced by the Hut28 lines shows a rising base line prior to the onset of aggregation and is unaffected by apyrase but is completely blocked by phospholipase D and hirudin. The effects of apyrase and hirudin are identical to those seen with the U87MG line examined previously (3), suggesting that the aggregation effects of these 2 lines involves activation of the coagulation system. However, platelet aggregation by these 2 lines may be differentiated since only with the Hut28 line is aggregation inhibited by phospholipase D.

The various phospholipases have been of value in characterizing the platelet-aggregating effects of the tumor cell lines. The effects of phospholipase D on tumor cell-induced platelet aggregation have not been investigated previously, but this enzyme was able to differentiate the platelet-aggregating effects of Hut28 from those of U87MG although activation of the coagulation system appeared to be involved in each case. For those lines showing a clearly marked phase of reversible aggregation (SKBR3, SKNMC, and HT29), it may be noted that phospholipase D inhibited the second, major wave. For HT29 and Hut28, where a reversible first wave was not detectable, the aggregation response was also inhibited by phospholipase D. Little is known about the effects of phospholipase D on membranes, but the enzyme can alter calcium translocation in sarcoplasmic reticulum (6), and this may explain its differential effects on the first and second waves of tumor cell-induced platelet aggregation.

Phospholipase A2 has been reported to completely inhibit platelet aggregation induced by 2 mouse tumor cell lines (13, 17) as well as by the 5 human tumor lines which caused aggregation in the present study. We have also found that tumor cell-induced platelet aggregation is inhibited by lysolecithin, the product of the action of phospholipase A2 on phosphatidylcholine. Lysolecithin also inhibits platelet aggregation induced by ADP, epinephrine, collagen, and thrombin (15). It is known to inhibit prostaglandin synthesis (19), and this may be the basis for its antiaggregating effects, although it can also affect membrane fluidity (21) and the levels of nucleotide cyclases (1, 20).

In addition to the usual aggregating agents, platelet aggregation can be induced by the platelet-aggregating factor elaborated by IgE-sensitized basophils. Platelet-aggregating factor is lipidic in nature and is destroyed by phospholipases A2, C, and D (4). Since phospholipase C had no effect on platelet aggregation induced by any of the human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Cell concentration</th>
<th>Aggregation</th>
<th>Apyrase</th>
<th>Hirudin</th>
<th>Phospholipase D</th>
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<tbody>
<tr>
<td>SKBR3</td>
<td>Adenocarcinoma of breast</td>
<td>5 x 10⁶</td>
<td>Biphasic</td>
<td>Inhibition</td>
<td>No inhibition</td>
<td>2nd wave only</td>
</tr>
<tr>
<td>Hut20</td>
<td>Anaplastic murine tumor</td>
<td>10⁴</td>
<td>Biphasic</td>
<td>Inhibition</td>
<td>No inhibition</td>
<td>2nd wave only</td>
</tr>
<tr>
<td>SKNMC</td>
<td>Neuroblastoma</td>
<td>5 x 10⁴</td>
<td>Monophasic</td>
<td>Inhibition</td>
<td>No inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
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<td>Melanoma</td>
<td>10⁸</td>
<td>Biphasic</td>
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<td>No inhibition</td>
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<tr>
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<td>Adenocarcinoma of colon</td>
<td>10⁸</td>
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<td>Inhibition</td>
</tr>
<tr>
<td>U87MG</td>
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<td>10⁷</td>
<td>None</td>
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</tbody>
</table>

a Lines examined previously (3, 17) are included for comparison.
b Partial inhibition with apyrase, complete inhibition with phosphoenolpyruvate-pyruvate kinase.
examined, it is unlikely that platelet-aggregating factor-like material is involved in this reaction.

In summary, our results suggest that there are 2 major mechanisms by which cultured human tumor cells initiate platelet aggregation. The major mechanism, in 4 of the 6 aggregating human lines so far examined, appears to involve the secretion of ADP from the tumor cells resulting in platelet stimulation and then irreversible aggregation. This secretion may reflect damage to the tumor cells during harvesting, but the secretion of ADP from tumor cells in this group could be of physiological significance, and since aggregation does not occur in the presence of citrate, it differs from the usual mechanisms of ADP-induced platelet aggregation. The second mechanism involves the initial activation of the coagulation system and the generation of thrombin as the mediator of aggregation. Within this second group, 2 subgroups can be identified based on whether or not aggregation can be inhibited by phospholipase D. However, the different patterns of aggregation and inhibition within different groups of tumor cells, considerations of the relative importance of ADP and the coagulation system, and the observation of different patterns of inhibition with phospholipase D all suggest that no single mechanism will explain the nature of the interaction between platelets and tumor cells under all circumstances. These preliminary studies also suggest that significant differences exist between mechanisms of tumor cell-induced platelet aggregation seen previously in heterologous animal systems (8, 10, 12–14, 17, 18) and those seen in the homologous human systems studied here.

ACKNOWLEDGMENTS

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