Platelets as a Source of Growth-promoting Factor(s) for Tumor Cells

Y. Hara, M. Steiner, and M. G. Baldini

Division of Hematologic Research, The Memorial Hospital and Brown University, Providence, Rhode Island 02860

ABSTRACT

Human platelet lysate was shown to contain growth-promoting activity for four well-established malignant cell lines. Platelet lysate was able to support their cell proliferation without plasma or serum, indicating that platelets contained "survival factor" as well as mitogenic factor for these tumor cells. The growth-promoting activity was non-dialyzable, heat stable up to 56°, and partially trypsin labile, but it was completely destroyed by periodate oxidation, suggesting that a glycoprotein or glycopeptide may be the active principle. The activity was released from platelets aggregated by thrombin or collagen but not by adenosine diphosphate. This suggests that α-granules may be the principle storage site for growth-promoting activity.

INTRODUCTION

It has been suggested that platelets may play an important role in the development of cancer metastasis (24). By arresting circulating tumor cells in blood vessels, platelets allow the cells to adhere to vessel walls where they may grow (11). This hypothesis is supported by three lines of evidence: (a) involvement of platelets in tumor emboli has been reported by many investigators and is clearly documented by histological examinations of such emboli (3, 26); (b) metastatic dissemination of cancer could be reduced by induction of thrombocytopenia in the host animals (7) or by treatment with platelet antiaggregating agents such as aspirin (16) or dipyridamole (1); (c) many mouse and human tumor cell lines could aggregate platelets in vitro (8).

In recent years, platelets have also been shown to be a rich source of growth factors for nontransformed fibroblasts (14), human glial cells (27), and arterial smooth muscle cells (21). There have even been suggestions that platelets may contain growth-promoting factors for a wider variety of cells including virally transformed fibroblasts (15) and cancer-derived cell lines (4). These findings prompted us to advance the hypothesis that platelets may contain growth-promoting factors for tumor cells, which may enhance tumor metastasis by exerting growth stimulation during tumor embolization. In these studies, we have tried to confirm the presence in platelets of growth-promoting factors for malignant cells using four well-established malignant cell lines, and we have characterized these factors.

MATERIALS AND METHODS

Cells and Cell Culture. Neuro-2a, RAG, R2C, and mouse mammary tumor 060562 were obtained from the American Type Culture Collection (Rockville, Md.). In general, cells were maintained in plastic flasks (25-cm² growing surface) at 37° in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and two antibiotics, penicillin (100 units/ml) and streptomycin (100 μg/ml). For R2C, the serum supplements consisted of 15% horse serum and 2.5% fetal calf serum. Sera, culture medium, and antibiotics were all obtained from Grand Island Biological Company, Grand Island, N. Y.

Preparation of Platelet Lysate. Platelet lysate was prepared either from freshly drawn blood of healthy human donors anticoagulated with citrate dextrose solution (ACD; United States Pharmacopeia Formula A) or from outdated platelet concentrates obtained from a blood bank. After removing contaminating RBC by brief centrifugation and lysis with 1% ammonium oxalate, the platelets were washed three times with PBS containing 0.1% EDTA. The platelets obtained either from 450 ml of blood or from a comparable unit of platelet concentrate were resuspended in 10 ml of PBS and were lysed by sonication for 30 sec at the maximum power output of the low energy transducer of a Biosonic IV sonicator (Bronwill Scientific, Rochester, N. Y.). The sonicate was centrifuged at 100,000 × g for 30 min at 4° and the supernatant was tested for growth-promoting activity. Protein concentration was measured by the method of Lowry et al. (17) with bovine serum albumin as standard.

Preparation of RBC Lysate. RBC lysate was prepared from freshly drawn blood of healthy human donors. The RBC were washed three times with PBS and then resuspended in this buffer at a concentration of 3 to 5%. The cells were lysed by sonication as described above for platelets. The resultant sonicate was centrifuged at 100,000 × g for 30 min at 4°. The protein concentration of the supernatant measured as described above was adjusted to 9 to 12 mg/ml. Its growth-promoting activity was then tested.

Preparation of Human Serum. The platelet-poor plasma remaining after the preparation of platelet lysate was dialyzed against 3 changes of Ringer’s solution at 4° for 24 hr. Clotted fibrin was removed by centrifugation, and the serum was heat-inactivated at 56° for 30 min.

Assay of Growth-promoting Activity. Growth-promoting activity was measured by the ability of a sample to increase target cell number over a designated incubation period. Cells were plated at about 10⁵ cells/30-ml plastic flask containing Dulbecco’s modified Eagle’s medium supplemented with 1% fetal calf serum. After a 24-hr incubation period at 37°, the cell monolayers were washed twice with Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution, and the medium was changed to Dulbecco’s modified Eagle’s medium without fetal calf serum. At the time of medium change, Day 0, cell counts were made from triplicate flasks. To each remaining flask, platelet or RBC lysate was added in a concentration of 200 μg protein per ml. Controls contained the same volume of PBS without platelet or RBC lysate. Neuro-2a and RAG cells were counted after 4 days of incubation at 37° (Day 2 count), while mouse mammary
tumor 060562 and R2C cells were counted after 3 days of incubation (Day 3 count). Growth-promoting activity was expressed as the ratio of Day 2 or 3 cell counts over Day 0 cell counts. All cell counts were done by a Model ZB Coulter counter. All experiments were performed in duplicate or triplicate.

**Release of Growth-promoting Activity from Platelets.** Platelet pellets were prepared from ACD-anticoagulated freshly drawn human platelet-rich plasma by centrifugation at 2000 × g for 10 min and washed twice with Ca²⁺-free Tyrode's solution. They were then resuspended in Tyrode's solution containing 1.8 mM Ca²⁺ at a concentration of 1 × 10⁶ platelets/ml. Platelets were aggregated by the addition of 0.05 ml of 3 commonly used aggregating agents to each 0.35 ml of this platelet suspension in a Chrono-Log aggregometer (Chrono-Log Corp., Broomall, Pa.) at 37° by constant stirring at 1000 rpm for 5 min. Thrombin was purified by the method of Lundblad (18) from crude bovine topical thrombin (Parke-Davis, Detroit, Mich.). Aggregation was induced by addition of 2 units thrombin per ml. Bovine skin collagen (Worthington Biochemical Corp., Freehold, N. J.) was used at a concentration of 200 μg/ml. When 50 μM ADP was the aggregation inducer, human lyophilized fibrinogen (AB Kabi, Stockholm, Sweden) was added to the platelet suspension at a concentration of 1 mg/ml. Cellular material was removed by centrifugation, and the supernatant was tested for growth-promoting activity. With thrombin-treated platelets, the supernatant was heat activated at 56° for 30 min before testing growth-promoting activity.

**Modification of Platelet Lysate.** To examine the chemical properties of platelet-associated growth-promoting activity, platelet lysates were subjected to the following modifying procedures. In Treatment 1 (dialysis), platelet lysates were dialyzed against 3 changes of at least 40 volumes of 0.9% NaCl solution for 24 hr at 4° before assay of growth-promoting activity. In Treatment 2 (heat), platelet lysates were incubated at room temperature (control, 56°, 70° and 90° for 30 min and were then tested for growth-promoting activity. In Treatment 3 (trypsin digestion), platelet lysates were incubated with various concentrations of trypsin (EC 3.4.21.4, specific activity, 8,000 units/mg protein; Sigma Chemical Co., St. Louis, Mo.) at 37° (pH 8.5) for 3 hr. The reaction was stopped by addition of soybean trypsin inhibitor (Sigma) in a weight ratio of 1:1.5 (enzyme:inhibitor). In Treatment 4 (periodate oxidation), platelet lysates were incubated with 0.05 M sodium metaperiodate (Sigma) at pH 5.5 in the dark at 4° for 3 days. Periodates were then removed by dialysis, and growth-promoting activity was measured. In Treatment 5 (nonspecific protease inhibitor), phenylmethylsulfonyl fluoride (Sigma) was added to platelet lysate at a concentration of 2 mM, and its effect on growth-promoting activity was measured.

**RESULTS**

**Growth-promoting Activity of Platelet or RBC Lysate for Various Malignant Cell Lines.** As Table 1 shows, platelet lysates possessed growth-promoting activity for all 4 cell lines tested. RBC lysates, on the other hand, showed no activity for any of these cell lines. The differences in activity between controls and platelet lysates were significant at p < 0.05 (t test) in all 4 cell lines. To verify these findings, Neuro-2a (Chart 1) and RAG

![Growth-promoting activity of human platelet and RBC lysates for various malignant cell lines](chart1.jpg)

**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Platelet lysate</th>
<th>RBC lysate</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuro-2a</td>
<td>3.44±0.83</td>
<td>1.05</td>
<td>1.07</td>
</tr>
<tr>
<td>RAG</td>
<td>2.93±0.86</td>
<td>1.45</td>
<td>1.47</td>
</tr>
<tr>
<td>R2C</td>
<td>4.39±0.89</td>
<td>1.92</td>
<td>1.86</td>
</tr>
<tr>
<td>Mouse mammary tumor 060562</td>
<td>2.11±0.84</td>
<td>1.10</td>
<td>0.98</td>
</tr>
</tbody>
</table>

* Activity expressed as the ratio of Day 2 or 3 cell count (Subscript f) over Day 0 cell count (0).

Neither the platelet nor the RBC lysates alone, *i.e.*, without plasma factors, were able to support the growth of BALB/3T3 cells, a nontransformed fibroblast cell line. The greater growth-promoting activity of human serum compared to platelet lysate, measured on the basis of protein amount, was not reflected in a qualitative difference of the growing cells. The morphological appearance of the various...
tumor cell lines observed by light microscope was not noticeably different with serum or platelet lysate added as the respective growth promoter.

The existence of a possible synergism between the effect of serum and platelet lysate was examined with RAG cells. A dose-response curve of the growth-promoting activity of serum on RAG cell growth reached a plateau level at a concentration of 250 \(\mu g/ml\). Addition of platelet lysate to cultures containing this level of human serum did not show a further significant increase in growth-promoting activity.

**Dose-dependent Promotion of Cell Growth by Platelet Lysate.** Platelet lysate was added to RAG (Chart 3) and Neuro-2a (Chart 4) in a series of concentrations. Cell counts were taken after 2 days of incubation at 37°. For comparison, the response to human serum was measured under identical experimental conditions and at the same protein concentration. At 50 \(\mu g/ml\) platelet lysate, exerted detectable growth-promoting activity on both cell lines. For RAG, its activity reached a plateau at 250 \(\mu g\) protein/ml. For either cell line, human serum always showed greater growth-promoting activity.

**Release of Growth-promoting Activity from Platelets.** As plasma contains growth-promoting activity for RAG and Neuro-2a, experiments were performed with washed platelets as described in "Materials and Methods." Compared to controls obtained from the supernatant of nonaggregated platelet suspensions, thrombin-treated platelets released significant growth-promoting activity (Table 2). After heat inactivation (see "Materials and Methods"), the amount of thrombin used did not influence the cell count by itself. Collagen at very high concentration (200 \(\mu g/ml\)) induced release of a small quantity of growth-promoting activity for the Neuro-2a cell line. On the other hand, ADP up to 50 \(\mu M\) did not induce release of significant growth-promoting activity.

**Effect of Modification Procedures on Growth-promoting Activity of Platelet Lysate.** The growth-promoting activity of platelet lysate for Neuro-2a cells was nondialyzable and nontively heat stable at 56° but heat labile above 70° (Table 3). Trypsin reduced the activity in a dose-dependent manner when heated at 37° for 3 hr. Maximum reduction of stimulant activity was observed when 0.05 mg trypsin was added per mg of

\[ \begin{array}{c|c|c|c}
\text{Aggregating agent} & \text{Concentration} & \text{Target cell line} & \text{Platelets} \\
\hline
\text{Thrombin} & 2 \text{units/ml} & \text{Neuro-2a} & 2.31^b \\
\text{Collagen} & 200 \mu g/ml & \text{Neuro-2a} & 1.66^c \\
\text{ADP} & 50 \mu M & \text{Neuro-2a} & 1.29^d \\
\end{array} \]

\(^a\) Activity expressed as the ratio of Day 2 cell count (Subscript 2) over Day 0 cell count (Subscript 0).
\(^b\) Significant, \(p < 0.05\).
\(^c\) Significant, \(p < 0.10\).
\(^d\) Not significant.
platelet lysate protein. About one-half of the activity was lost by this treatment (Table 3). Periodate oxidation at pH 5.5 completely destroyed the growth-promoting activity of platelet lysate. In control experiments, the activity was resistant to exposure to pH 5.5 for the same length of time (3 days). The presence of a protease inhibitor, phenylmethylsulfonyl fluoride (2 mM), had no influence on the growth-promoting activity of the platelet lysate (Table 3).

### DISCUSSION

The presence of platelet-derived growth factors for nontransformed fibroblasts, human glial cells, and arterial smooth muscle cells has been well documented (14, 20, 21, 27). Considerable purification of these factors has already been achieved (2, 10). The existence of other platelet-derived growth-promoting factors with activities for a wider variety of mammalian cells, including transformed cells (15) and malignant cell lines (4), has been suggested. Our studies presented here demonstrate that human platelets contain growth-promoting activity for at least 4 different tumor-derived cell lines tested in tissue culture. This effect seems to be specific, as other blood cells, e.g., human RBC did not show growth-promoting or sustaining activity. All 4 lines were able to proliferate with platelet lysate as the only supplement, i.e., without plasma or serum. This finding stands in contrast to the previously made observation (22, 23), reconfirmed in this study, that platelet factor(s) alone could not stimulate cell growth in nontransformed fibroblasts. It indicates that platelet lysate contains a "survival factor" (19) for these tumor cells. At the same protein concentration, serum always showed stronger growth-promoting activity. A comparison of the dose-response curves obtained with serum and platelet lysate revealed that the saturation density with the former was higher than the one obtained by platelet lysate. A similar observation was made by other investigators in a study of a hormone-responsive rat mammary tumor (5). This evidence, we believe, indicates that these cell lines can respond to multiple growth factors present in the serum and that platelets contain some of them.

It is important to note that the tumor cells subjected either to serum- or platelet-induced growth promotion do not differ morphologically. The growth-promoting activity derived from these 2 sources did not act synergistically when tested with RAG cells.

Similar to the platelet-derived growth factor for nontransformed fibroblasts (BALB/3T3), the growth-promoting activity for tumor cells was nondialyzable. In contrast to the former, its heat resistance extended only up to 56°, whereas the growth factor for nontransformed fibroblasts was unaffected by heating up to 100° (10). The growth-promoting activity for tumor cells was only partially trypsin-sensitive. Complete destruction of the activity was never achieved by trypsin treatment only. This is consistent with the result of Kepner et al. (13) in a study of platelet-derived growth factor(s) for virally transformed fibroblasts (SV3T3), but it is different from the growth factor for nontransformed fibroblasts (BALB/3T3) and human glial cells which were completely destroyed by trypsin digestion (22). The activity for tumor cells was completely lost by periodate oxidation. This suggests that the growth-promoting activity resides in a glycoprotein or glycopeptide.

Release of growth-promoting factors for tumor cells from platelets was most prominent when thrombin was used as the aggregating agent. Collagen provided a weaker release stimulus, but ADP did not release this activity at all. Although specific experiments to determine the intracellular site of growth-promoting activity were not performed, the results obtained with different aggregating agents are consistent with a localization of this activity in α-granules. Other authors studying platelet-derived growth factor activity for nonmalignant cells have come to a similar conclusion about its intracellular distribution (12, 28). The physiological significance of release of growth-promoting factors from platelets was strengthened by the finding that tumor cells or tumor cell membrane fragments aggregated platelets and induced release of growth-promoting activity for tumor cells (9).

It has been known that in hematogenous tumor metastases only a small portion of circulating tumor cells could survive and establish metastatic foci as tumor emboli (6). Platelets are involved in the process of tumor embolization and may contribute to survival of embolized tumor cells by providing growth-promoting factors for them. Recently, Varma et al. (25) reported that low affinity Platelet Factor 4 and β-thromboglobulin had mitogenic activity for nontransformed fibroblasts (3T3). Our studies were performed with crude platelet lysate. It is not known how many different growth factors are present in platelets and how they relate to known platelet-specific substances. To clarify these problems, purification of growth factors for tumor cells is required; this work is now in progress in our laboratory.

### REFERENCES

5. Eastment, C. T., and Sibrasku, D. A. Platelet-derived growth factor(s) for a


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