Inhibitory Effects of Dipyridamole on Growth, Nucleoside Incorporation, and Platelet-activating Capability in the U87MG and SKNMC Human Tumor Cell Lines

Eva Bastida, Josefina del Prado, Lourdes Almirall, G. A. Jamieson, and Antonio Ordinas

ABSTRACT

The effects of dipyridamole on tumor cell function were examined in cultures of two lines of human origin, the SKNMC neuroblastoma line that activates platelets by a mechanism which is dependent on the release of adenosine 5'-diphosphate and the U87MG glioblastoma line that induces platelet activation by the generation of thrombin. Cells grown in the presence of dipyridamole at 1 μM showed >80% inhibition of uptake of adenosine, thymidine, and uridine with both lines. At 5 μM tumor cell growth was inhibited by 70% (U87MG) and 90% (SKNMC) but without concomitant cytotoxicity as determined by clonogenic assay (50% inhibitory concentration ~20 μM). At 10 μM dipyridamole cyclic adenosine 3′:5′-monophosphate levels increased 150% with both cell lines but no changes above baseline values were seen at 2.5 μM. The two cell lines showed different responses to being cultured in the presence of dipyridamole in terms of their ability to subsequently activate platelets. U87MG cells cultured in 10 μM dipyridamole showed a doubling of the lag time as compared with cells grown in the absence of dipyridamole but with full aggregation; with SKNMC cells the aggregation rate was reduced and cells grown in 10 μM dipyridamole showed no reversible first wave, a 5-fold increase in lag time and a 75% inhibition in total aggregation. Since therapeutic doses of dipyridamole result in plasma concentrations of ~3.5 mM these results suggest that potential antemetastatic effects of dipyridamole could be direct arising from inhibition of important steps in tumor cell metabolism or indirect by suppressing one or more of the mechanisms involved in the ability of tumor cells to activate platelets.

INTRODUCTION

Platelets may be involved in the metastatic spread of tumors (7, 26, 30), and although some laboratory findings support this hypothesis (16, 23), other results are ambiguous (12, 17). However, recent results showing a reduction in the number of lung colonies in animals treated with an antiplatelet antibody (24) confirm earlier observations about the role of platelets in the development of secondary metastases (8).

Several laboratories have found that both animal (9, 11, 14) and human tumor cells (1, 13, 22) are able to activate platelets in vitro. In our laboratory we have characterized two different mechanisms of platelet activation by tumor cell lines (2). One mechanism (ADP dependent) involves the stimulation of platelets by ADP while the other mechanism (thrombin dependent) depends on the generation of thrombin by tissue factor present on microvesicles shed by the tumor cells (3).

These experimental data suggest that the administration of antiplatelet drugs could be of help in the treatment of cancer patients. Although some few clinical studies in this respect have been completed they have been inconclusive (10, 20). Dipyridamole is one of the most used inhibitors of platelet function (21). Besides its antiplatelet phosphodiesterase activity (4) dipyridamole has also been shown to increase the synthesis of vascular prostacyclin (28) and to be a potent inhibitor of nucleoside transport (19, 25). Dipyridamole also causes inhibition of lymphoproliferation (6), killing of rat hepatoma cells (31), and enhancement of the toxicity of deoxyadenosine and deoxycoformycin to a mouse leukemia line (15).

In the present studies we report the effects of dipyridamole on the U87MG and SKNMC human tumor cell lines with regard to tumor cell proliferation, nucleoside incorporation, cAMP3 levels, and modifications in tumor cell proaggregant and procoagulant activities.

MATERIALS AND METHODS

Cell Culture. Two human tumor cell lines were used in the present study, the U87MG line derived from a glioblastoma and the SKNMC line derived from a neuroblastoma. Both had been kindly provided by the late Dr. Jörgen Fogh of the Sloan Kettering Institute, Rye, NY. U87MG and SKNMC cells were grown in 95% air-5% CO2 as monolayers in polycarbonate flasks (Nunc Corp., Denmark) using minimal essential medium supplemented with 1% nonessential amino acids, gentamycin (50 mg/ml), 2 mM glutamine, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. The concentration of fetal calf serum was 15 and 10% for the U87MG and SKNMC cell lines, respectively. The cells were passaged twice weekly.

For the metabolic studies the tumor cells were harvested by trypsinization (0.25%) for 2 min and the viability was determined by trypsin exclusion; in no case was viability less than 90%. For platelet aggregation experiments and for the procoagulant activity tests the cells were harvested in the absence of proteases by decanting the culture medium and then washing the monolayers twice with Hank’s balanced salt solution without Ca2+ and Mg2+. Washed cell monolayers were treated for 1 h with 5 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid in HBSS at 37°C. The detached cells were then centrifuged (100 × g for 10 min) and the cell pellets were resuspended in the same solution to yield a cell concentration of approximately 1 × 106 cells/ml. Viability was measured by trypan blue exclusion and in no case was viability less than 90%.

Inhibition of Incorporation of Labeled Nucleosides. The 3H-labeled nucleosides studied here were thymidine (20 μCi/μmol), adenosine (17.8

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2 To whom requests for reprints should be addressed.

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µCi/µmol), and uridine (27.1 µCi/µmol) (New England Nuclear, Boston, MA), made up as solutions of 33 µCi/ml before use. For measuring the incorporation of labeled nucleosides, 3 x 10^4 tumor cells were seeded into 2.5 cm macrowell plates (Costar, Cambridge, MA) containing 2.5 ml of cell culture medium. Aliquots of 0.5 ml of medium without or with appropriate concentrations of dipyridamole were immediately added to the wells. For each experiment triplicate wells were run for controls and for dipyridamole-treated cells. After 24 h of incubation at 37°C the labeled nucleosides were added to the culture medium in appropriate volumes to give a final concentration of 1 µCi/ml. Incubation was then continued for 1 h at 37°C. After this period of time the medium was removed, the wells were maintained on ice, and the cell monolayers were washed twice with cold HBSS and three times with 3 ml of cold trichloroacetic acid (5% w/v) allowing each aliquot of TCA to stand for 10 min in contact with the cell monolayers. Finally and at room temperature the monolayers were washed twice with absolute ethanol (10 min each wash). Any remaining liquid was evaporated under nitrogen and 1 ml of 0.3 N NaOH was added to dissolve the residue. After 1 h at 37°C 750 µl of the solution were pipetted from each well, neutralized with 150 µl of 1.5 N HCl, and placed in a scintillation vial to which 9 ml of scintillation fluid (Aquasol II; New England Nuclear, Boston, MA) were added.

Growth Inhibition Studies. Logarithmically growing U87MG and SKNMC tumor cells were harvested and seeded into 25 cm² flasks at concentrations of 2 x 10^4 cells/flask. Triplicate samples were used for each experiment. In certain experiments a fixed concentration of dipyridamole was added to the medium and the cells were incubated for different periods of time. In other experiments graded concentrations of the drug were added to the medium and the cells were harvested after 24 h of incubation. In either case the cells were harvested by trypsinization (0.25%) for 2 min and the viability was determined by the trypan blue exclusion test.

Clonogenic Assay. Clonogenic assays were performed as described by Saimon et al. (27). Briefly logarithmically growing U87MG and SKNMC cells were seeded at 500 cells/25 cm² flask. Immediately after this dipyridamole in various concentrations from 2 to 30 µM was added and the cells were allowed to grow. After 7 days incubation the medium was discarded and the cells were stained with a saturated solution of crystal violet. Colonies consisting of more than 50 cells were counted and the number of surviving colonies in the dipyridamole-treated group was calculated as a percentage of the number of colonies found with untreated cells. The 50% inhibitory concentration values for the cytotoxicity of dipyridamole were calculated by linear regression analysis.

Intracellular cAMP Levels. U87MG and SKNMC cultures grown in the presence and absence of dipyridamole were trypsinized after 24 h of incubation. An aliquot was withdrawn from the cell suspension in order to determine the cell recovery and the remainder was centrifuged. Cell pellets were resuspended in HBSS and sonicated in a Branson sonifier equipped with a microtip (Branson Sonic Power Co., Danbury, CT). The sonicate was treated with 2 ml of cold 5% TCA and the protein precipitate was removed by centrifugation at 10,000 x g for 10 min. The supernatant fractions were extracted 5 times each with 3 volumes of diethyl ether to remove TCA and the extracted aqueous samples were then evaporated under nitrogen at 50°C. The residues were redissolved in 0.2 ml of acetate buffer (pH 5.4) prior to assay. cAMP content of the tumor cells was determined by radioimmunoassay according to a standard technique (29) using a commercially available kit (Amersham Corporation, Arlington Heights, IL). Recovery of cAMP was 92 ± 10% (SD) as determined by using added [3H]cAMP (New England Nuclear, Boston, MA) and calculated concentrations were corrected accordingly.

Platelet Aggregation. Blood from healthy donors who had not taken platelet-affecting drugs for at least 10 days before phlebotomy was used. Blood was drawn by venipuncture and anticoagulated with heparin (5 U/ml). Platelet-rich plasma was prepared by centrifugation at 100 x g for 10 min and platelet aggregation was measured by aggregometry (22).

Clotting Assays. The recalcification time was measured in a Fibrometer (Becton Dickinson, Rutherford, NY). The procoagulant activity of the tumor cells was measured using the one-stage assay. Pooled, citrated, platelet-poor normal human plasma (100 µl) was incubated with 100 µl of the tumor cell suspension at 37°C in the control buffer for 1 min followed by addition of 100 µl of 25 mM CaCl₂ and the clotting time was recorded.

RESULTS

Effect of Dipyridamole on Tumor Cell Growth. Two different types of experiments were performed in order to evaluate the effects of dipyridamole on tumor cell growth. In one case the number of cells was calculated in U87MG and SKNMC cultures during growth in the presence of a fixed amount (5 µM) of dipyridamole (Chart 1). The growth of both cell lines was reduced compared with the control cultures and the SKNMC line was more sensitive than U87MG to the presence of dipyridamole at all of the time points examined.

In a second type of experiment a dose-response curve was constructed to evaluate the effect of dipyridamole on tumor cell proliferation (Chart 2). Tumor cells (1 x 10⁶/ml) without and with different concentrations of dipyridamole were seeded into 25 cm² flasks and allowed to incubate at 37°C for 24 h. At the end of this period cultures were harvested and the cell count and cell viability were determined. Dipyridamole inhibited cell proliferation in both tumor cell lines in a dose-dependent fashion and maximum inhibition was reached at approximately 5 µM. Again SKNMC cells were more sensitive to dipyridamole than were U87MG cells at all concentrations examined. None of the concentrations studied displayed a cytotoxic effect since the viability of the cells cultured in the presence of dipyridamole was in the same range as that of controls (approximately 90%) as measured by trypan blue exclusion.

Cytotoxic Effects of Dipyridamole on U87MG and SKNMC Tumor Cell Cultures. Determination of cytotoxicity by clonogenic assay indicated that the 50% inhibitory concentration for dipyridamole was 23 µM for U87MG cells and 19 µM for SKNMC cells (Chart 3). These cytotoxic concentrations are much higher than the drug concentrations which gave maximal inhibition of cell growth in both lines (~5 µM).

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Chart 1. Effects of 5 µM dipyridamole on growth of U87MG cells (B) and SKNMC cells (C) at different harvest times. Points, means of triplicate experiments; bars, SE.
Effects of Dipyridamole on Cultured Tumor Cells

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Chart 2. Effect of dipyridamole concentration on growth of U87MG (●) and SKNMC (○) cells. Points, means of triplicate assays; bars, SE.

Effect of Dipyridamole on Nucleoside Incorporation. The effect of dipyridamole on the incorporation of [3H]adenosine, [3H]thymidine, and [3H]uridine was examined for both U87MG and SKNMC cells. Concentrations of dipyridamole ranging from 10⁻⁵-10 μM were added to the culture medium and incubated for 24 h with the growing cells followed by 1 h incubation with the labeled nucleosides. Incorporation of [3H]-labeled nucleosides for the U87MG line was inhibited 10-20% at dipyridamole concentrations as low as 10⁻³ μM and reached about 80% inhibition for [3H]thymidine and [3H]uridine at 0.1 μM (Table 1). Inhibition of nucleoside incorporation into SKNMC cells required higher concentrations of the drug. Dipyridamole at 1 μM inhibited incorporation of the three nucleosides tested approximately 80%. At a concentration of 10 μM there was complete inhibition of nucleoside incorporation with both cell lines.

Effect of Dipyridamole on Intracellular cAMP Levels. Baseline values for cAMP in cells grown in the absence of dipyridamole were 3.8 ± 0.6 pmols/10⁶ cells and 4.6 ± 0.3 pmole/10⁶ cells for U87MG and SKNMC cells, respectively (Table 2). Culturing the cells in the presence of 2.5 μM dipyridamole caused no significant increase in these values. However, at 10 μM dipyridamole concentrations cAMP values were increased about 150% to a value of 6.2 ± 1.1 for U87MG and 8.2 ± 2.1 for SKNMC; values were significant at the level, P <.05. The SKNMC cells which initially displayed higher cAMP levels than did U87MG cells also displayed higher values after being cultured in the presence of dipyridamole.

Platelet-Aggregating Activity of Tumor Cells Cultured in the Presence of Dipyridamole. Platelet aggregation was induced with 1 x 10⁵ U87MG cells/ml and with 1 x 10⁶ SKNMC cells/ml. These cell concentrations were chosen since they are the minimum necessary to give maximal platelet aggregation with control tumor cells grown in the absence of dipyridamole. U87MG cells grown in the presence of 2.5 μM dipyridamole were as effective as control cells as there was no change in either the lag time or the maximal aggregation (Chart 4A). U87MG cells grown in the presence of 10 μM dipyridamole were only slightly less effective as shown by a moderate prolongation of the lag time and a slight reduction in maximal aggregation response.

Effects on platelet aggregation were more marked using SKNMC cells that had been cultured in the presence of dipyridamole. A clear diminution in the first wave of aggregation together with some reduction in maximal aggregation was observed when SKNMC cells grown in the presence of dipyridamole at 2.5 μM were used as compared to control cells (Chart 4B). SKNMC cells cultured in dipyridamole at a concentration of 10

Table 1
Inhibition of [3H]-labeled nucleoside incorporation into tumor cells

Dipyridamole was added to the culture followed by incubation for 24 h. One h before harvesting [3H]-labeled nucleosides were added. Results are means of triplicate experiments and are expressed as percentage inhibition of uptake in comparison with controls grown in the absence of dipyridamole.

<table>
<thead>
<tr>
<th>Dipyridamole (μM)</th>
<th>Adenosine</th>
<th>Thymidine</th>
<th>Uridine</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁵</td>
<td>U87MG</td>
<td>SKNMC</td>
<td>U87MG</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>20</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>10⁻³</td>
<td>65</td>
<td>80</td>
<td>80</td>
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<tr>
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<td>75</td>
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</tr>
<tr>
<td>10</td>
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<td>90</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2
Effect of dipyridamole on intracellular cAMP levels

<table>
<thead>
<tr>
<th>Dipyridamole (μM)</th>
<th>cAMP (pmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U87MG</td>
</tr>
<tr>
<td>0</td>
<td>3.8 ± 0.6³</td>
</tr>
<tr>
<td>2.5</td>
<td>4.1 ± 0.5³</td>
</tr>
<tr>
<td>10</td>
<td>6.1 ± 1.1³</td>
</tr>
</tbody>
</table>

³ Mean ± SE of three different sets of replicate experiments.

Experiments were performed on triplicate cultures in a 60 mm tissue culture dish. Incubations were carried out at 37°C in a water bath. Results are expressed as means of triplicate experiments ± SE. The level of significance was determined using the Student t-test. Results are expressed as percentage inhibition of uptake in comparison with controls grown in the absence of dipyridamole.
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**A**

- CONTROL
- 2.5 µM
- 10 µM

**B**

- CONTROL
- 2.5 µM
- 10 µM

**Chart 4.** Representative tracings of the platelet-aggregating activity of tumor cells cultured in presence of 2.5 and 10 µM dipyridamole. A, platelet-aggregation tracings obtained with 1 x 10⁶ U87MG cells/ml; B, platelet-aggregation tracings obtained with 1 x 10⁶ SKNMC cells/ml.

µM were unable to induce the first wave of reversible aggregation and showed significantly less maximal aggregation (Chart 4B).

**Procoagulant Activity of Tumor Cells Cultured in the Presence of Dipyridamole.** We have already demonstrated that U87MG tumor cells (but not SKNMC cells) display a strong procoagulant activity that is able to significantly shorten the recalification time of pooled normal platelet-poor plasma (3). In the present work the one-stage recalification time was reduced from 210 ±12 to 60 ±4 s by U87MG cells and this value was unaffected when U87MG cells grown in the presence of dipyridamole. We have already demonstrated that dipyridamole has an inhibitory effect on the growth of both tumor cell lines studied, that this effect is time and concentration dependent, and that concentrations with maximal inhibitory effect show no concomitant cytotoxicity. The plasma levels of dipyridamole when given at therapeutic doses as an antiplatelet drug reach a maximum value of approximately 3.5 µM (21). This concentration is in the same range as the concentrations we have found to inhibit the growth of U87MG and SKNMC cells (5 µM) and far below the cytotoxic concentrations determined by clonogenic assay (~20 µM). The results suggest that a potential effect of the drug could be directed to the inhibition of tumor cell growth.

Incorporation of nucleoside into tumor cells was also markedly inhibited by dipyridamole at concentrations well below therapeutic plasma levels. Nucleoside transport is the initiating step in the utilization of exogenous nucleosides for the salvage pathway and it is known that the activities of the nucleoside salvage enzymes are increased in rat and human hepatomas (5, 18). Our results demonstrate that dipyridamole at concentrations below 1 µM is a powerful inhibitor of nucleoside incorporation in both tumor cell lines studied.

The observed increase of intracellular cAMP in the tumor cells induced by dipyridamole could affect their ability to cause platelet aggregation. It is interesting to note that the platelet-aggregating ability of the U87MG line which activates platelets by a thrombin-dependent mechanism is less affected when the tumor cells are cultured in dipyridamole than is the SKNMC line that operates through an ADP-related mechanism. It is important to point out that the procoagulant activity of the U87MG tumor cells was unaffected after culturing the cells in the presence of dipyridamole. The procoagulant activity displayed by this cell line is caused by the presence of tissue factor in the tumor cell membrane (3). Therefore this lack of effect on the generation of the procoagulant activity suggests that dipyridamole does not alter the structure of the U87MG cell membrane with regard to the exposure of tissue factor.

The above results suggest that the effect of dipyridamole on tumor cell metastasis could be direct by inhibiting important steps in tumor cell metabolism such as nucleoside incorporation or could be indirect by suppressing one or more of the mechanisms involved in the capability of tumor cells to activate platelets.

**REFERENCES**


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