Dipyridamole Decreases Platelet-Derived Growth Factor Levels in Human Serum

Kazuhiko Takehara, Gary R. Grotendorst, Richard Silver, and E. Carwile LeRoy

Mitogenic activities on confluent fibroblasts by sera from patients with scleroderma and normal controls were studied. In experiments using eight different fibroblast strains (one human fetal lung fibroblast, one foreskin fibroblast, three adult skin fibroblast, and three scleroderma fibroblast), pooled scleroderma serum showed lower mitogenic activity by 3H-thymidine incorporation assay than pooled normal serum. Individual serum investigations revealed that 11 of 33 patients (33%) showed low mitogenic activities; all 11 patients were receiving dipyridamole. Of 15 patients, 11 (73%) receiving dipyridamole showed low mitogenic activities; none of 18 patients not receiving dipyridamole showed low mitogenic activities. Approximately 70% of this serum activity was inhibited by the addition of anti-platelet-derived growth factor (PDGF) antibody indicating that most of this serum activity seemed to be derived from PDGF. Western blot analysis of extracts of normal sera before and after administration of dipyridamole with antihuman PDGF antibody showed a large decrease in the amount of immunoreactive PDGF present. These data indicate that dipyridamole is an effective drug to lower release of PDGF during blood clotting.

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The role of platelet-derived growth factors (PDGF) in the pathogenesis of vascular disease has been an active area of research. Several growth-promoting and chemotactic factors for connective tissue cells have been shown to be contained in the alpha granules of the platelet. As described in the response to injury hypothesis of Ross, PDGF-like factors from various cell types are released into the vessel wall and recruit the underlying connective tissue to proliferate at the intimal surface forming a fibromuscular lesion. It has been extremely difficult to test the role of platelets in vascular disease processes in vivo. One reason has been the difficulty of neutralizing platelet function in the intact organism without compromising the individual's general blood clotting mechanisms.

Scleroderma represents another vascular disease in which there is a proliferation of connective tissue surrounding the blood vessel. Previous studies have reported that sera from some scleroderma patients contain elevated levels of mitogenic activity for fibroblastic cells. For this reason, we measured the levels of fibroblast mitogenic activity in scleroderma patients. We found many with significantly decreased levels of fibroblast mitogenic activity compared with that of the normal controls. All of the patients with decreased levels of mitogenic activity were being treated with dipyridamole. This effect was maintained as long as dipyridamole treatment was continued, but the level of serum mitogenic activity returned to normal after the treatment was terminated. Immunologic tests with specific IgG to human PDGF confirmed that the majority of the mitogenic activity present was PDGF-related and that PDGF levels were decreased in sera collected from individuals receiving dipyridamole therapy. Thus, dipyridamole may be useful in preventing the continued aggregation and release reactions of platelets in the treatment of vascular diseases where platelets may play a potential role. Additionally, these data suggest that dipyridamole treatment could be used to develop an animal model having altered release of platelet factors in order to determine the roles of these factors in normal and pathological states in vivo.

Methods

Sera

Sera from 33 scleroderma patients (23 females, 10 males, mean age 46.9 years, range 26 to 74) at the Medical University of South Carolina were studied. All patients satisfied American Rheumatism Association preliminary clinical criteria. As controls, sera from 16 healthy donors (10 females, 6 males, mean age 36.7 years, range 22 to 59) were also studied. Blood was allowed to clot at room temperature for 1 to 2 hours, was centrifuged at 3000 rpm for 20 minutes, and the sera were stored at -20°C prior to use. After thawing, they were heat-inactivated (56°C for 30 minutes). In some experiments, pools of 4 to 7 different individual sera were used. All procedures had informed consent and institutional approval.
Fibroblast Culture

Human fetal lung fibroblasts (MRC-5) were purchased from American Type Culture Collection (Rockville, Maryland). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Quality Biological Incorporated, Gaithersburg, Maryland) with 10% fetal calf serum (FCS, Armour Pharmaceutical Company, Kankakee, Illinois, lot number Y 68305). Incubations were maintained in T75 flasks (Falcon Products, Cockeysville, Maryland), at 37°C in 90% air, 10% CO₂. Human foreskin fibroblasts were grown from explants of newborn foreskins. Human adult skin fibroblasts and scleroderma fibroblasts were grown from explants of forearm skin biopsies from healthy donors or from patients with scleroderma, respectively. In some experiments, cells stored under liquid nitrogen were used.

Growth Assays

Confluent fibroblasts were trypsinized and 4 x 10⁴ cells were placed in 16 mm culture plates (Costar, Cambridge, Massachusetts) in DMEM with 10% FCS. Cells were incubated for 4 to 6 days until confluent, and then the medium was changed to DMEM containing test serum (5%, 10%, 20%). After 16 hours, cells were labeled for 2 hours with 2 μCi/ml of ³H-thymidine (Amersham, Arlington Heights, Illinois). The cell layers were washed three times with phosphate-buffered saline and five times with ice-cold, 5% trichloroacetic acid, and dissolved in 1 ml of 0.1 N NaOH and 0.1% sodium dodecyl sulfate (Sigma Chemical Company, St. Louis, Missouri). An aliquot of this extract was counted in a Beckman scintillation counter. The background was measured by adding serum-free medium with 2 mg/ml bovine serum albumin.

Anti-Platelet-Derived Growth Factor Antibody

Purified PDGF was obtained from human platelets as described before. Antibodies to PDGF were prepared in goats using purified human PDGF as an antigen. Goats were immunized with 20 μg of purified PDGF in Freund’s complete adjuvant via multiple Intradermal injections. After 28 days, the goats were rechallenged with 20 μg of pure PDGF In Freund’s incomplete adjuvant. Immune serum was collected 7 days after injection of antigen. The serum used in these experiments was collected from the fourth immunization cycle. The IgG fraction of immune serum was prepared by chromatography on DEAE-Affigel Blue Sepharose (0.02 M NaCl; 0.02 M Tris; pH 8.0) (Biorad, Richmond, California). Anti-PDGF IgG was used in all experiments described. Western blots were performed as described by Towbin et al. using affinity purified rabbit antigoat IgG (alkaline phosphatase conjugated) (Kirkgaard and Perry Laboratories, Incorporated, Gaithersburg, Maryland).

Platelet Extracts

Platelet extracts were obtained by acid-ethanol extraction and ether precipitation. The ether-precipitated pro-

Figure 1. ³H-thymidine uptake of human fetal lung fibroblasts (MRC-5) after 16 hours. Scleroderma (SD) serum showed smaller mitogenic effects at 20%, 10%, or 5% concentrations than normal (NL) control serum. Values are means ± SEM of triplicate wells. FCS = fetal calf serum.

Results

Serum Mitogenic Activity

Initially, we tested pooled normal and scleroderma serum for mitogenic activity using the ³H-thymidine incorporation assay described under Methods. Scleroderma serum showed less mitogenic activity on fetal lung fibroblasts (MRC-5) than normal control serum at concentrations of 5%, 10%, and 20% (Figure 1). This lowered mitogenic activity of scleroderma serum was reproduced in all experiments using eight fibroblast strains (see Methods). Data at 10% serum concentration are shown in Table 1. No significant differences were shown between human sera before or after heat inactivation.

The mitogenic activity of 33 sera from patients with scleroderma and 16 sera from normal controls was measured by ³H-thymidine incorporation assay on foreskin fibroblasts after stimulation with 20% serum. As shown in Figure 2, the mean of scleroderma patients’ stimulation index (SI 4.0 ± 1.8) was significantly lower than the control group (SI 5.1 ± 1.1) by analysis of Student’s t test (p < 0.05). Sera from 11 of 33 scleroderma patients gave responses under the normal range (< mean of normal controls = 2.0). Scleroderma patients grouped into a normal mitogen group (n = 22, SI ≥ 2.96) and a low mitogen group (n = 11, SI < 2.96) showed no significant differences in age, duration, and frequencies of truncal sclerosis or of involvement of lung, esophagus and kidney. However, when patients with scleroderma were grouped into those receiving dipyridamole and those not taking dipyridamole, all 11 patients who showed serum low mitogenic activity were receiving dipyridamole. Sera from 11 of 15 (73%) dipyridamole-treated patients showed low mitogenic activity, while sera from none of 18 patients not taking dipyridamole showed low mitogenic activity. The mean of the stimulation indexes of the group taking dipyridamole
Table 1. Serum Effects on 3H-Thymidine Uptake of Confluent Fibroblasts

<table>
<thead>
<tr>
<th>Cell</th>
<th>10% Fetal calf serum</th>
<th>10% Scleroderma serum</th>
<th>10% Normal serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRC-5 (fetal lung fibroblasts)</td>
<td>8091 ± 560</td>
<td>15084 ± 1274</td>
<td>23909 ± 182</td>
</tr>
<tr>
<td>MRC-5</td>
<td>-</td>
<td>15567 ± 1242</td>
<td>26357 ± 1950^*</td>
</tr>
<tr>
<td>Foreskin fibroblasts</td>
<td>20294 ± 507</td>
<td>36611 ± 1339</td>
<td>52952 ± 2211</td>
</tr>
<tr>
<td>Adult skin fibroblasts (1)</td>
<td>594 ± 71</td>
<td>1764 ± 696</td>
<td>4275 ± 84</td>
</tr>
<tr>
<td>Adult skin fibroblasts (2)</td>
<td>1501 ± 211</td>
<td>6187 ± 193</td>
<td>7284 ± 132</td>
</tr>
<tr>
<td>Adult skin fibroblasts (3)</td>
<td>377 ± 120</td>
<td>704 ± 74</td>
<td>1126 ± 176</td>
</tr>
<tr>
<td>Scleroderma fibroblasts (1)</td>
<td>60 ± 11</td>
<td>249 ± 2</td>
<td>339 ± 2</td>
</tr>
<tr>
<td>Scleroderma fibroblasts (2)</td>
<td>810 ± 13</td>
<td>2684 ± 693</td>
<td>3576 ± 119</td>
</tr>
<tr>
<td>Scleroderma fibroblasts (3)</td>
<td>1321 ± 118</td>
<td>5148 ± 198</td>
<td>8372 ± 348</td>
</tr>
</tbody>
</table>

Values are cpm/well, mean ± SEM.

*10% scleroderma and normal pooled serum without heat inactivation.

Figure 2. Individual serum effects on 3H-thymidine incorporation of foreskin fibroblasts at 20% serum concentration. Values are expressed as stimulation index (SI): SI = (cpm 20% test serum-background)/(cpm 20% FCS-background). The background was measured by serum-free medium with 2 mg/ml bovine serum albumin. The total of the scleroderma group (SI 4.0 ± 1.8) was significantly lower than that of the normal control group (SI 5.1 ± 1.1) (p < 0.05). The scleroderma dipyridamole (+) group (SI 2.6 ± 1.3) showed significantly less mitogenic effect when compared with both the normal control group and the scleroderma dipyridamole (-) group (SI 5.2 ± 1.1) (p < 0.001). No significant difference was shown between the normal control group and the scleroderma dipyridamole (-) group.
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Figure 3. The effect of dipyridamole on mitogenic activities in the sera and in platelet extracts. In two scleroderma patients (•), lowered mitogenic activity increased to the normal range after discontinuing dipyridamole. In three normal controls (○), dipyridamole (300 mg/day for 3 days) reduced mitogenic activities in the sera. In two normal controls, mitogenic activities in platelet extracts from $5 \times 10^7$ platelets were compared before and after taking dipyridamole; no significant difference was observed. The 100% in the figure represents the effect of 10% pooled normal serum (a), or that of 20 mg/ml of purified PDGF (b), respectively.

The 100% In the figure represents the effect of 10% pooled normal serum (a), or that of 20 mg/ml of purified PDGF (b), respectively.

(SI = 2.6) was significantly lower than that of those not receiving dipyridamole (SI = 5.2) and that of normal control group (SI = 5.1) ($p < 0.001$).

Platelet counts measured at the same time when blood was drawn or within 2 months, did not show any significant difference between the group taking dipyridamole ($n = 14$, $271 \pm 102 \times 10^4$) and those individuals not taking dipyridamole ($n = 16$, $307 \pm 88 \times 10^4$). These findings are similar to those reported by Emmons et al.20 and Rajah et al.21 which showed that dipyridamole had no significant effect on the circulating platelet count either during or after therapy.

Table 2. Serum Mixing Experiments

<table>
<thead>
<tr>
<th>Serum</th>
<th>3H-Thymidine uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% NL</td>
<td>20472 ± 1467</td>
</tr>
<tr>
<td>5% NL + 2% pooled SD</td>
<td>25851 ± 3093</td>
</tr>
<tr>
<td>5% NL + 5% pooled SD</td>
<td>36759 ± 4357</td>
</tr>
<tr>
<td>5% NL + 10% pooled SD</td>
<td>48436 ± 1745</td>
</tr>
<tr>
<td>5% NL + 2% pooled NL</td>
<td>27196 ± 328</td>
</tr>
<tr>
<td>5% NL + 5% pooled NL</td>
<td>48025 ± 188</td>
</tr>
<tr>
<td>5% NL + 10% pooled NL</td>
<td>64267 ± 4633</td>
</tr>
</tbody>
</table>

Either 2%, 5%, or 10% of pooled scleroderma (SD) serum or pooled normal (NL) serum was added to 5% serum obtained from normal individuals. Values are means ± sd.

Serum Mixing Experiments

To determine whether lowered mitogenic activities observed in the sera from scleroderma patients are due to the presence of inhibitors in the sera, either pooled scleroderma serum or pooled normal serum (2%, 5%, 10%) was added to 5% normal serum, and the mitogenic activities in these mixed sera were tested. As shown in Table 2, the data from these experiments indicate that pooled sclero-

Table 3. Dipyridamole Effect on 3H-Thymidine Incorporation by Foreskin Fibroblasts

<table>
<thead>
<tr>
<th>Serum</th>
<th>3H-Thymidine Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Serum</td>
<td>21677 ± 324</td>
</tr>
<tr>
<td>+ DP 50 ng/ml</td>
<td>21803 ± 479</td>
</tr>
<tr>
<td>+ DP 200 ng/ml</td>
<td>19333 ± 1585</td>
</tr>
<tr>
<td>+ DP 1 µg/ml</td>
<td>20782 ± 1342</td>
</tr>
<tr>
<td>Fetal calf serum</td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>6392 ± 141</td>
</tr>
<tr>
<td>+ PDGF 10 ng/ml</td>
<td>24905 ± 1326</td>
</tr>
<tr>
<td>+ PDGF 10 ng/ml + DP 50 ng/ml</td>
<td>28049 ± 3124</td>
</tr>
<tr>
<td>+ PDGF 10 ng/ml + DP 200 ng/ml</td>
<td>25461 ± 1392</td>
</tr>
<tr>
<td>+ PDGF 10 ng/ml + DP 1 µg/ml</td>
<td>21394 ± 2208</td>
</tr>
</tbody>
</table>

Values are the means of triplicate wells ± SEM.

DP = dipyridamole, PDGF = platelet-derived growth factor.
Scleroderma serum contains decreased levels of mitogens and does not decrease the level of mitogenic activity found in normal serum.

**Reversibility of the Dipyridamole Effect on Decreased Serum Mitogenic Activity**

In two scleroderma patients, serum mitogenic activities were compared before and after stopping dipyridamole. The sera obtained while the patients were receiving dipyridamole (400 mg/day) showed low mitogenic effects; sera obtained 2 weeks after stopping dipyridamole showed normal mitogenic effect (Figure 3). Additionally, in three normal controls, mitogenic activities in the sera were compared before and after taking dipyridamole (300 mg/day) for 3 days. Dipyridamole reduced the mitogenic effect in the serum by 76%, while it did not reduce mitogenic activities in the platelet extracts.

**Dipyridamole Effects on Foreskin Fibroblast Cultures**

To ensure that the dipyridamole was not altering the growth response of the fibroblast, we tested the effect of dipyridamole on the growth response of fibroblasts to serum and platelet-derived growth factor. The presence of dipyridamole at concentrations comparable to those found in treated patients' sera did not affect 

% suppression = 100 x

\[
\frac{1 - \left( \frac{10\% \text{ test serum with antibody-background}}{10\% \text{ test serum without antibody-background}} \right)}{1 - \left( \frac{10\% \text{ test serum with antibody-background}}{10\% \text{ test serum without antibody-background}} \right)}
\]

This antibody did not crossreact with bovine PDGF but suppressed approximately 70% activity in human serum. Therefore, most of the serum mitogenic activity to confluent fibroblasts in human serum was derived from PDGF-related growth activities. FCS = fetal calf serum, SD = scleroderma serum, NL = normal control serum, ab = antibody, (−) = without, (+) = with.

**Evidence that Serum Mitogen is PDGF-Related**

The major mitogenic factor contained in serum for fibroblastic cells is the platelet-derived growth factor. One means of determining whether the mitogenic activity present in both the low mitogen (dipyridamole-treated patients) sera and normal sera is PDGF-like, is to test the ability of anti-PDGF antibody to neutralize the mitogenic activities contained in these sera. Anti-PDGF antibody (60 μg/ml) was incubated with DMEM containing 10% test serum for 1 hour before changing media. This concentration of anti-PDGF antibody gave maximal effect of decreasing mitogenic activities. As shown in Figure 4, anti-PDGF antibody had a slight effect on 

\[
\text{Figure 4. Anti-PDGF antibody effect on serum mitogen to foreskin fibroblasts. The percent suppression was expressed as follows:}
\]

\[
\text{% suppression} = 100 \times \left( 1 - \frac{10\% \text{ test serum with antibody-background}}{10\% \text{ test serum without antibody-background}} \right)
\]

\[
\text{This antibody did not crossreact with bovine PDGF but suppressed approximately 70% activity in human serum. Therefore, most of the serum mitogenic activity to confluent fibroblasts in human serum was derived from PDGF-related growth activities. FCS = fetal calf serum, SD = scleroderma serum, NL = normal control serum, ab = antibody, (−) = without, (+) = with.}
\]

\[
\text{Figure 5. Immunodetection of PDGF in Western blot of sera from normal control before and after taking dipyridamole. Samples were electrophoresed under nonreducing conditions in a 12% acrylamide gel in the presence of SDS and transferred to nitrocellulose paper as described in Methods. Immunoreactive PDGF peptides were detected with antihuman PDGF IgG (goat). Lane 1. Normal human serum; Lane 2. Normal human serum after dipyridamole treatment (3 days at 300 mg/day).}
\]
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Oedema and control serum were derived from PDGF. Fetal calf serum has low levels of PDGF compared to human sera and the antibody used does not appear to cross-react with bovine PDGF. Preimmune IgG had no effect on the mitogenic activity of either normal or scleroderma sera (data not shown). These data indicate that treatment with dipyridamole can lower the level of PDGF present in serum.

To confirm this, we quantitated the level of PDGF present by a Western blot assay using anti-human PDGF IgG. Crude PDGF was prepared by acid-ethanol extraction and ether precipitation from 1-ml aliquots of serum prepared from a normal control before and after taking dipyridamole. The ether-precipitated proteins were redissolved in 1 ml of 1 M acetic acid and 200 μl was lyophilized and electrophoresed on a 12% acrylamide gel containing SDS under nonreducing condition. The proteins were then electrotransferred to nitrocellulose paper and the PDGF present was detected immunologically. The data shown in Figure 5 indicate that the PDGF level decreased to approximately 75% of normal after administration of dipyridamole. These data support the results of our mitogenic assays which indicate that there is 50% to 80% decrease in PDGF in the sera of individuals treated with dipyridamole.

Discussion

Using a 3H-thymidine incorporation assay of confluent fibroblast cultures, we found less mitogenic activity in scleroderma serum than in control serum regardless of the source of fibroblasts. This result was confirmed using eight different fibroblast strains (one human fetal lung fibroblast, one foreskin fibroblast, three adult skin fibroblasts, three scleroderma fibroblast). Decreased serum mitogenic activity on confluent foreskin fibroblasts was demonstrated in 11 of 33 patients with scleroderma, all of whom were receiving dipyridamole. Approximately 70% of mitogenic activity in all sera tested was blocked by preincubation with anti-PDGF antibody, indicating that most of these mitogenic activities were derived from PDGF-related growth activities. We also observed increases of serum mitogen after stopping dipyridamole in the patients who were taking dipyridamole; we observed decreases of serum mitogen in normal controls after taking dipyridamole. Thus, dipyridamole, a known antplatelet drug, may block the release of PDGF from platelets, explaining why sera from scleroderma patients taking dipyridamole showed low mitogenic effects.

Antiplatelet drugs have been used for the treatment of patients with scleroderma, because of a vascular hypothesis for its pathogenesis.11–13 Kahaleh et al.22 reported that elevated levels of circulating platelet aggregates and β-thromboglobulin in the patients with scleroderma and these platelet abnormalities were reduced by treatment with dipyridamole (400 mg/day) and aspirin (150 mg every third day) for 2 to 4 months. However, in a randomized, double-blind, controlled study,23 no clinical or objective laboratory improvement was noted in the patients who were taking dipyridamole (225 mg/day) and aspirin (975 mg/day). This difference might be due to the use of different dosages of dipyridamole and aspirin, suggesting that further investigation of antiplatelet drugs is necessary. Patient selection may also be important in these studies.

In recent years, the effects of scleroderma serum on fibroblast replication have been reported.14–16, 24–26 Some studies14–16 suggested that sera from patients with scleroderma showed elevated levels of mitogenic activity for adult skin fibroblasts, while others24–26 reported lower levels or similar levels of mitogenic activity for foreskin fibroblasts as compared to normal control sera. These differences may be due to the source of fibroblasts; however, our data were consistent with lower mitogenic activity in scleroderma serum regardless of the source of fibroblasts.

The role of PDGF in connective tissue formation, fibrosis, or wound repair has been studied for years.1–3, 6–8 PDGF has been found to be the principal mitogenic factor for fibroblasts, smooth muscle cells, and fibroblastic cell lines,1–3 and chemoattractant for vascular smooth muscle cells and fibroblasts.6–8 Addition of PDGF to a model wound system stimulated the formation of connective tissue in vivo.67 Thus PDGF appears to play an important role in connective tissue formation. The present study showed that most of the mitogenic activity in human serum for fibroblasts was PDGF-related activity, and this activity could be decreased by 78% by taking 300 mg per day of dipyridamole for 3 days. Apparently, dipyridamole caused this by blocking platelet aggregation and PDGF release from platelets without changing the total mitogenic activities in the platelets. The most surprising observation is that this occurred when blood underwent maximal clotting as in the serum collection tubes. These data suggest that the effects of dipyridamole on the progression of vascular diseases needs to be carefully evaluated. Additionally, it is our hope that dipyridamole treatment may be applied to animal models of fibrosis and wound healing enabling the further investigation of the role of platelets and platelet-derived factors in these processes in vivo.

Acknowledgment

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Index Terms: dipyridamole • serum mitogen • PDGF • scleroderma
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