Inhibitory Effects of a Novel Antiplatelet Agent, E5510, on Collagen-Induced Platelet-Derived Growth Factor Release and Aggregation of Human Platelets In Vitro

Ken-ichi Nomoto, Takao Saeki, Motoji Kogushi, Hiroko Kobayashi, Tohru Fujimori and Isao Yamatsu

Department of Cardiovascular Disease Research, Eisai Tsukuba Research Laboratories, 1-3, Tokodai, 5-chome, Tsukuba, Ibaraki 300-26, Japan

Received July 16, 1992 Accepted October 12, 1992

ABSTRACT—E5510, 4-cyano-5,5-bis(4-methoxyphenyl)-4-pentenoic acid, is a new anti-platelet-aggregation agent under development. We examined the inhibitory efficacy of E5510 on PDGF-release from washed human platelets. E5510 concentration-dependently inhibited collagen-induced PDGF release from human platelets. PDGF release was reduced to below the detection limit (0.47 ng/ml) by preincubation of platelets with 0.04μM or higher concentrations of E5510. Total growth factor release from platelets was also measured by a bioassay with cultured smooth muscle cells. E5510 almost completely abolished the mitogenic effect of collagen-induced platelet releasates at concentrations of 0.04μM or higher. Our data suggest that the release of PDGF and other growth factors was inhibited by E5510 at the same concentration that inhibited platelet aggregation.

Keywords: E5510, Platelet (human), Platelet-derived growth factor, Smooth muscle cell, Cell proliferation

Restenosis after successful percutaneous transluminal coronary angioplasty (PTCA) remains the major problem limiting the long-term efficacy of this procedure. Approximately 30–40% of atherosclerotic coronary arteries treated by PTCA undergo restenosis (1). This restenosis seems to be principally due to the proliferation of neointimal smooth muscle cells (SMCs) (2); however, the factors responsible for this have not been clearly identified. Findings about intimal hyperplasia have been largely derived from animal studies, which have attempted to investigate the “response to injury” in models of atherosclerosis (3). Some of these results may be applicable to balloon angioplasty. Balloon angioplasty causes the denudation of endothelial cells which is followed by platelet adhesion and aggregation, probably resulting in the release of platelet-derived growth factor (PDGF) as well as other growth factors from platelets. PDGF is both mitogenic and chemotactic for vascular medial smooth muscle cells. As evidence for this concept, Friedman et al. (4) have reported inhibition of aortic intimal thickening after balloon injury in rabbits made thrombocytopenic with anti-platelet antisera. Similarly, Moore et al. (5) have reported marked suppression of aortic intimal thickening in complicated atherosclerotic lesions caused by repeated endothelial injury after thrombocytopenia. It is thought, therefore, that SMCs stimulated by PDGF or other growth factors derived from platelets undergo proliferation and migration into the intima, resulting in restenosis. Thus, drugs that inhibit the release of PDGF and other growth factors from platelets may provide a potential approach for preventing clinical restenosis after angioplasty. E5510 is a new anti-platelet aggregating agent under development by Eisai Co., Ltd. E5510 has a wide spectrum of inhibitory effects on human platelet aggregation, exhibiting potent inhibition of platelet aggregation induced by collagen, arachidonic acid, ADP, platelet activating factor, epinephrine, and thrombin (6). In addition, it inhibits both the adhesion of platelets to collagen and thrombin-induced ATP secretion through multiple modes of action such as inhibition of cyclooxygenase and phosphodiesterase (6, 7). Thus, E5510 inhibits the release of ATP from platelet dense granules. These results strongly suggest that E5510 also inhibits PDGF release from the alpha-granules of platelets.

The present study was undertaken to examine the ability of E5510 to inhibit collagen-induced PDGF release from human platelets.
MATERIALS AND METHODS

Cell culture
Rat smooth muscle cells (RSMCs) were isolated from the aortas of Sprague-Dawley rats (Japan SLC, Inc., Shizuoka, Japan) by enzymatic digestion with 0.3% collagenase type I (Sigma, St. Louis, MO, USA) and 0.05% elastase type III (Sigma). The cells were cultured in Eagle’s MEM medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (Flow Laboratories), L-glutamine (2 mM, Gibco Oriental Co., Ltd., Tokyo, Japan), penicillin (100 units/ml, Gibco), and streptomycin (100 μg/ml, Gibco). The cells were harvested with a trypsin-EDTA solution (0.05% trypsin and 0.53 mM EDTA, Gibco) and then subcultured at a 1:5 dilution (at approximately 5-day intervals). RSMCs between passage 10 and 13 were used for the present studies. Cell cultures (75 cm² flasks, 4 x 10⁴ cells/cm²) were incubated at 37°C in a humidified atmosphere of 5% CO₂-95% air.

Preparation of human washed platelets
Fresh blood (100 ml) was drawn from the antecubital vein of three healthy male human volunteers who had received no medication for more than one week, and 3.8% trisodium citrate solution (Citrál®, Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan) at 10% of the total volume was immediately added to prevent blood coagulation. The blood thus collected was immediately subjected to centrifugal separation at 150 x g for 15 min at room temperature (22–25°C) to separate the supernatant platelet-rich plasma (PRP). After adding acid citrate dextrose (ACD-A® solution, Terumo Co., Ltd., Tokyo, Japan) at 15% volume to the PRP, it was further centrifuged at 2000 x g for 10 min at room temperature, and the supernatant plasma was discarded. The sedimented platelets were washed twice with 0.1% EDTA/saline, and then suspended in Ca²⁺-free Tyrode solution to give a final platelet concentration of 4 x 10⁴ cells/ml, as determined by a Coulter Counter (Coulter Electronics Inc., FL, USA). Silicon-coated glassware and polypropylene tubing were used for preparing PRP and in the following experiments.

Measurement of platelet aggregation
Platelet aggregation was measured with an aggregometer (Hematracer, Nicho Bioscience Co., Ltd., Tokyo, Japan) by the turbidimetric method of Born and Cross (8). E5510 was first dissolved in dimethyl sulfoxide (Sigma) to 50 mM and then diluted to 1 mM with 1% BSA/saline; this stock solution was then various concentrations with saline so that the final concentrations in the assay mixtures were 0.03, 0.04, 0.05, 0.1 and 0.3 μM E5510. As a control, the vehicle (saline) was administered in place of test compound solution. Test compound solution (25 μl) or saline as the control was added to 200 μl of washed platelet suspension in a cuvette and preincubated in the aggregometer at 37°C for 3 min with constant stirring at 1000 rpm. To induce platelet aggregation, 25 μl of a solution of collagen (Collagen Reagent Horm®, 0.1%, Hormon-Chemie, Munich, FRG) was added to the reaction mixture, and platelet aggregation was measured for 6 min after the addition of collagen. The concentration used was the lowest concentration that would induce maximum platelet aggregation in the absence of E5510. The resultant final concentration of collagen in the test solution was 1 μg/ml (data not shown). Change in light transmission detected by the aggregometer, after the addition of collagen, was automatically recorded. The intensity of platelet aggregation was measured according to the previously reported method (6), and the effect of E5510 was expressed as % inhibition compared to the control.

Preparation of human platelet releasate
After platelet aggregation was measured for 6 min, the reaction was terminated by adding 2 mM EGTA. The mixture was centrifuged at 10,000 x g for 3 min at room temperature, and the resultant supernatant fraction (200 μl), the releasate, was isolated. The releasate was stored in aliquots at -20°C.

Measurement of PDGF content in the platelet releasate
PDGF levels were determined by a platelet-derived growth factor [125I] assay system (Amersham Japan, Tokyo, Japan) based on a radioimmunoassay method. This assay system recognizes PDGF-AB and PDGF-BB. Fortunately, it is well-known that PDGF purified from human platelets contains 70% PDGF-AB and 30% PDGF-BB (9). The detection limit of the assay was 0.47 ng/ml.

Assessment of proliferative responses of RSMCs to collagen-induced platelet releasate and PDGF
Total growth factor release from platelets was assessed in the cultured RSMCs bioassay. Cultured RSMCs were harvested and then plated at 8 x 10⁴ cells/0.5 ml onto 24-well plates (16 mm wells, Falcon®, Nippon Becton Dickinson Co., Ltd., Tokyo, Japan), in Eagle’s MEM supplemented as described above, and allowed to grow for 72 hours. They were then made "quiescent" by a 48-hr incubation in fresh Eagle’s MEM without FCS. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂-95% air with media changes every day.

The mitogenic response of the collagen-induced releasate of washed human platelets (10% v/v) or recombinant human PDGF-AB (Boehringer Mannheim Yamanouchi Co., Ltd., Tokyo, Japan) was determined as previously
described by Rains and Ross (10) with some modifications. Briefly, increasing doses of recombinant human PDGF-AB or collagen-induced releasate of human platelets were added to wells. Plasma derived serum or calf CMS I (calf serum incubated with CM-Sephadex to remove PDGF) were not added to the wells because PDGF alone could elicit a significant mitogenic response. After a 22-hr incubation at 37°C, the cells were pulsed with 0.25 μCi/well [³H]-thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA, USA) for 2 hr and then washed 3 times with phosphate-buffered saline. This was followed by 15-min fixation in 10% trichloroacetic acid at 4°C. Cells were then dissolved in 0.5 N NaOH and placed in ACS-II (Amersham Japan, Tokyo, Japan) scintillation fluid. Counting was performed with a scintillation counter. Proliferative responses of platelet releasates in 200 μl washed platelets + 25 μl saline + 25 μl collagen and in 200 μl washed platelets + 50 μl saline were defined as 100% and 0%, respectively.

RESULTS

Inhibitory effects of E5510 on platelet aggregation in vitro

The inhibitory effect of E5510 on collagen-induced platelet aggregation in washed human platelets is shown in Fig. 1. The inhibition by E5510 was concentration-dependent, with approximately 50% inhibition at 0.03 μM and approximately 100% inhibition at 0.04 μM or higher concentrations.

Effect of E5510 on PDGF release from washed human platelets

The inhibitory effect of E5510 on collagen-induced PDGF release from washed human platelets of two subjects (subjects 2 and 3) is illustrated in Fig. 2. E5510 concentration-dependently inhibited the collagen-induced

![Fig. 1. Antiaggregatory effect of E5510 on collagen-induced washed human platelet aggregation. The inhibitory effect of E5510 on platelet aggregation was evaluated by comparison with the aggregation intensity induced by collagen in the presence of saline. Each point (0.03-0.05 μM) is the mean±S.E. of three different measurements each from 3 subjects (n=9), and each point from 0.1 and 0.3 μM is the mean±S.E. of three different measurements each from 2 subjects (n=6).

Fig. 2. Effect of E5510 on platelet releasate level of PDGF. Each bar represents the mean±S.E. of triplicate measurements, each from two subjects. The detection limit of this assay was 0.47 ng/ml.
PDGF release. In the absence of E5510, the PDGF content of collagen-induced platelet releasates was about 7 ng/ml. Preincubation of washed platelets with 0.03 μM E5510 reduced the PDGF concentration of the platelet releasate to approximately 3 ng/ml. The PDGF content of the platelet releasate was decreased to below the detection limit (0.47 ng/ml) by preincubation of the platelets with 0.04 μM or higher concentrations of E5510.

**Effects of E5510 on the mitogenic response to PDGF and collagen-induced platelet releasate**

Although PDGF exists in three dimeric forms: homodimers (PDGF-AA and PDGF-BB) and heterodimer (PDGF-AB), PDGF-AB is the major isoform found in human platelets. We used RSMCs in this bioassay because PDGF-AB is a good mitogen for RSMC (11). Recombinant human PDGF-AB elicited a significant mitogenic response at concentrations of 0.78 ng/ml and higher, and the peak response (24,477 dpm) occurred at a concentration of approximately 25 ng/ml (Fig. 3). In the absence of E5510, the mitogenic activity of collagen-induced platelet releasates was 23,081 dpm in terms of the radioactivity of [3H]-thymidine incorporated by SMCs (data not shown). The incorporation of [3H]-thymidine was similar to the maximum response to recombinant human PDGF-AB.

Preincubation of washed platelets with increasing concentrations of E5510 diminished the mitogenic effects of collagen-induced platelet releasates in a concentration-dependent manner. The mitogenic activity of collagen-induced platelet releasates was reduced to approximately 60% at 0.03 μM E5510. Preincubation of washed platelets with 0.04 μM and higher concentrations of E5510 abolished the mitogenic effect of collagen-induced platelet releasates (Fig. 4). E5510 had no effect on basal [3H]-thymidine incorporation of RSMCs, even at E5510 concentrations up to 0.3 μM (Fig. 4).

**Relationship between inhibitions of platelet aggregation and release of mitogenic factors from human platelets by E5510**

Figure 5 shows the relationship between inhibition of platelet aggregation and inhibition of mitogenic activity by E5510. E5510, at concentrations of 0.03 μM and higher, almost completely inhibited platelet aggregation and the release of growth factors responsible for the mitogenic activity of RSMCs. Inhibition of aggregation and inhibition of mitogenic activity were strongly correlated \((r = -0.905)\).
DISCUSSION

The pathological features of the neointimal response after angioplasty are 1) migration of medial SMCs into the intima, 2) proliferation of SMCs within the intima, and 3) increase of fibrous components (2). Platelets are thought to play a major role in the formation of arterial intimal lesions by releasing growth factors that stimulate SMC growth, at sites of endothelial denudation (3). PDGF released from platelets may especially play an important role in SMC migration (12-14). In this regard, PDGF has been shown to be chemotactic for mesenchymal cells in vitro (13).

In this study, the release of PDGF from collagen-stimulated platelets was inhibited by E5510 at the same concentration as that which caused inhibition of platelet aggregation. Within 30 min after balloon injury in vivo, platelet factor 4 (PF4), one of the constituents in the alpha-granules of platelets, can be detected throughout the intima and media (15). Because PF4 and other platelet-secreted proteins such as PDGF reside in the same granule population (16) and are secreted in response to the same stimuli, they also enter the vessel wall after the removal of the endothelial cells. Therefore, E5510 may have clinical potential for the inhibition of PDGF release from platelets adherent to the subendothelial components after balloon angioplasty, at the same plasma concentrations that can cause inhibition of platelet aggregation.

In the absence of E5510, the PDGF content of the collagen-induced platelet releasate was estimated to be approximately 7.6 ng/ml because the collagen releasate of washed human platelets was added to the wells at a volume of 10%. The [3H]-thymidine incorporation was 23,081 dpm under these conditions (data not shown). The [3H]-thymidine incorporation of 0.78 and 1.56 ng/ml of human recombinant PDGF-AB was 8140 and 9442 dpm, respectively. These results suggest that E5510 not only inhibits PDGF release, but also simultaneously inhibits the release of other platelet-secreted proteins such as epidermal growth factor (EGF) and transforming growth factor-beta (TGF-beta) contained in the same granule population. The relative importance of the EGF and TGF-beta compared with PDGF is unknown, but Majack et al. (17) found that TGF-beta regulated the expression of thrombospondin (TSP) protein in confluent cultured SMCs, which was mediated via the induced synthesis of an intermediary PDGF-AA. The extracellular matrix molecule TSP is a glycoprotein that mediates cell attachment, spreading, and migration (18). The formation of the neointima is a multistep process involving SMC migration and proliferation. SMC may attach to this matrix molecule, facilitating the process of migration. Zabrenetzky et al. (19) reported that TSP facilitates chemotactic migration of cultured cells. Therefore, TGF-beta, which is secreted from platelets, may play an important role in extracellular changes to facilitate SMC migration into the intima, via the production of TSP. Further studies are needed to clarify the possible involvement of TGF-beta in neointimal formation.

In conclusion, our data suggested that E5510 dose-dependently inhibits the release of PDGF and other growth factors from human platelets. Therefore, E5510 may be an effective candidate for preventing restenosis after angioplasty via inhibition of the release of growth factors from platelets.

Acknowledgments

We would like to thank Dr. Wendy Gray for her help with preparation of this manuscript. We also thank Mrs. Shoko Iizumi and Miss Setsuko Suzuki for their technical assistance.

REFERENCES

1 Schwartz, L., Bourassa, M.G., Lesperance, J., Aldridge, H.E., Kazim, F., Salvatori, V.A., Henderson, M., Bonan, R. and David, P.R.: Aspirin and dipyridamole in the prevention of restenosis after percutaneous transluminal coronary angioplasty. N. Engl. J. Med. 318, 1714-1719 (1988)
2 Liu, M.W., Roubin, G.S. and King, S.B., III: Restenosis after coronary angioplasty: Potential biologic determinants and role of intimal hyperplasia. Circulation 79, 1374-1387 (1989)
3 Ross, R. and Glomset, J.A.: The pathogenesis of atherosclerosis. N. Engl. J. Med. 295, 369-377, 420-425 (1976)
4 Friedman, R.J., Steimerman, M.B., Wenz, B., Moore, S., Gauldie, J., Gent, M., Tiell, M.L. and Spaet, T.H.: The effect...
of thrombocytopenia on experimental atherosclerotic lesion formation in rabbits. Smooth muscle cell proliferation and re-endothelialization. J. Clin. Invest. 60, 1191–1201 (1977)

5 Moore, S., Friedman, R.J., Singal, D.P., Gauldie, J., Blajchman, M.A. and Robert, R.S.: Inhibition of injury induced thromboatherosclerotic lesions by anti-platelet serum in rabbits. Thromb. Haemostas. 35, 70–81 (1976)

6 Fujimori, T., Harada, K., Saeki, T., Kogushi, M., Akasaka, K., Yamagishi, Y. and Yamatsu, I.: Pharmacological properties of the novel anti-platelet aggregating agent 4-cyano-5,5-bis (4-methoxyphenyl)-4-pentenoic acid. Arzneimittelforschung 37, 1143–1148 (1987)

7 Fujimori, T., Harada, K., Saeki, T., Kogushi, M., Katayama, K. and Satoh, M.: E5510, a novel antiplatelet drug with multiple modes of action. Cardiovasc. Drug Rev. 9, 264–284 (1991)

8 Born, G.V.R. and Cross, M.J.: The aggregation of blood platelets. J. Physiol. (Lond.) 168, 178–195 (1963)

9 Heldin, C.H. and Westmark, B.: Platelet-derived growth factor: three isoforms and two receptor types. Trends Genet. 5, 108–111 (1989)

10 Raines, E.W. and Ross, R.: Purification of human PDGF. Methods Enzymol. 109, 749–773 (1985)

11 Ferns, G.A.A., Reidy, M.A. and Ross, R.: Balloon catheter de-endothelialization of the nude rat carotid: Response to injury in the absence of functional T lymphocytes. Am. J. Pathol. 138, 1045–1057 (1991)

12 Fingerle, J., Johnson, R., Clowes, A.W., Majesky, M.W. and Reidy, M.A.: Role of platelets in smooth muscle cell prolifera-

13 Ferns, G.A.A., Rains, E.W., Spruget, K.H., Motani, A.S., Reidy, M.A. and Ross, R.: Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. Science 253, 1129–1132 (1991)

14 Jewien, A., Bowen-Pope, D.F., Lindner, V., Schwartz, S.M. and Clowes, A.W.: Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. J. Clin. Invest. 89, 507–511 (1992)

15 Goldberg, I.D. and Stemerman, M.B.: Vascular permeation of platelet factor 4 after endothelial injury. Science 209, 611–612 (1980)

16 Stein, B., Fuster, V., Israel, D.H., Cohen, M., Badimon, L., Badimon, J.J. and Chesebro, J.H.: Platelet inhibitor agents in cardiovascular disease: An update. J. Am. Coll. Cardiol. 14, 813–836 (1989)

17 Majack, R.A., Majesky, M.W. and Goodman, L.V.: Role of PDGF – A expression in the control of vascular smooth muscle cell growth by transforming growth factor-beta. J. Cell Biol. 111, 239–247 (1990)

18 Roberts, D.D., Sherwood, J.A. and Ginsburg, V.: Platelet thrombospondin mediates attachment and spreading of human melanoma cells. J. Cell Biol. 104, 131–139 (1987)

19 Zabrenetzky, V.S., Kohn, E.C. and Roberts, D.D.: Suramin inhibits laminin- and thrombospondin-mediated melanoma cell adhesion and migration and binding of these adhesive proteins to sulfatide. Cancer Res. 50, 5937–5942 (1990)