logically important peptides at the cell membrane surface. Alternatively, the enzyme may function as a processing enzyme, acting on a putative precursor containing the Arg-Arg doublet.

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1 Turner, A. J., Hooper, N. M., and Kenny, A. J., Methods Neurosci. *11* (1989) 189.
2 Turner, A. J., in: Neuropeptides and Their Peptidases, p. 183. Ed. A. J. Turner. Ellis-Horwood, Chichester 1987.
3 Erdos, E. G., and Skidgel, R. A., FASEB J. *3* (1989) 145.
4 Goldstein, A., Tachibana, S., Lowney, L. I., Hunkapiller, M., and Hood, L., Proc. natl Acad. Sci. USA *76* (1979) 6666.
5 Goldstein, A., Fischli, W., Lowney, L. I., Hunkapiller, M., and Hood, L., Proc. natl Acad. Sci. USA *78* (1981) 7219.
6 Tachibana, S., Araki, K., Ohya, S., and Yoshida, S., Nature *295* (1982) 339.
7 Satoh, M., Yokosawa, H., and Ishii, S., J. Neurochem. *52* (1989) 61.
8 Jones, D. H., and Matus, A. I., Biochim. biophys. Acta *356* (1974) 276.
9 Levy, M., Fishman, L., and Schenkein, I., Methods Enzymol. *19* (1970) 672.
10 Schenkein, I., Levy, M., Franklin, E. C., and Frangione, B., Archs Biochem. Biophys. *182* (1977) 64.

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Purification of platelet-derived endothelial cell growth inhibitor and its characterization as transforming growth factor-β type 1

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Abstract. In 1986, Brown and Clemmons (Proc. natl Acad. Sci. USA *83* (1986) 3321) showed that platelets contain a substance, platelet-derived growth inhibitor (PDGI), that inhibits in vitro endothelial cell replication. Although platelets are rich in transforming growth factor β (TGF-β), PDGI was considered not to be related to TGF-β, on the basis of its reported properties (extraction from platelets at neutral pH, binding to heparin-Sepharose). However, we purified PDGI to near homogeneity and showed that on the basis of HPLC retention behavior, in vitro growth inhibitory activities with several cell types, receptor binding, and immunoneutralization of growth inhibitory activity with specific anti-TGF-β type 1 antibodies, PDGI is most probably identical with TGF-β type 1.
Key words. Platelet-derived growth inhibitor; transforming growth factor-β type 1; bovine aortic endothelial cells.

The growth of cells in culture and in vivo is modulated by various effectors, some of which are growth factors and others growth inhibitors. The equilibrium between stimulatory and inhibitory signals is essential for normal control of cell proliferation and cell differentiation. The significance of growth factors in these processes is well established, but the role of growth inhibitors is less clear. The regulation of vascular endothelial cell proliferation by growth factors and inhibitors is likely to be physiologically important in new capillary blood vessel formation. This process, often termed angiogenesis or neovascularization, is a complex and tightly regulated sequence of events involving migration, proliferation and maturation of endothelial cells[1]. Endothelial cell proliferation and angiogenesis are virtually absent in healthy adult tissue, but occur in controlled manner during tissue growth (embryogenesis, menstrual cycle, placenta formation) and repair (wound healing). Furthermore, unregulated neovascularization is a hallmark of pathological states (tumor growth, chronic inflammations, retinopathies, etc.)[2,3].

Many factors are known which stimulate endothelial cell proliferation and/or angiogenesis. They include the fibroblast growth factors basic and acidic FGF[4,5], platelet-derived endothelial cell growth factor[6], and vascular permeability factor/endothelial cell growth factor VPF/VEGF[7,8]. These mitogens also stimulate angiogenesis, but there are additional angiogenic factors that stimulate neovascularization without inducing endothelial cell proliferation[9]. Likewise, a number of molecules with potent growth inhibitory activity for endothelial cells have been characterized, i.e. TGF-β[10-12], tumor necrosis factor-α[13-15], interleukin-1[16,17], interferons[15,18,19] and heparin[20,21]. In addition, there are numerous reports describing less well-characterized endothelial cell and angiogenesis inhibitors[22-34]. In view of the potential importance of negative regulatory factors in the biological control of neovascularization, it would be of considerable interest to determine the chemical nature of novel endothelial cell growth or angiogenesis inhibitors. Brown and Clemmons[22] described an apparently novel endothelial cell growth inhibitory factor from platelets, which they named platelet-derived growth inhibitor (PDGI). The reported properties (extraction at neutral pH, binding to heparin) suggested that this factor was not related to the major endothelial cell growth inhibitor

present in platelets, TGF-β[35]. Here we demonstrate, however, that this factor is most probably identical with TGF-β type 1.

Materials and methods

Basic FGF (bFGF) and TGF-β were isolated from bovine brain[11,12] and human platelets[35], respectively, as described. Clinically outdated human platelets were obtained through the courtesy of the Swiss Red Cross in Zürich.

Purification of PDGI

Platelets were extracted as described by Brown and Clemmons[22]. A hundred outdated 10-unit packs (1 unit corresponds to 5×10^9 thrombocytes) of human platelets were combined (about 5 l) and centrifuged at 5000 × g for 30 min to remove residual plasma. The platelets were washed once with 0.15 M NaCl/10 mM Na_2HPO_4/pH 7.4 and recentrifuged as described above. The washed platelets (about 700 ml) were resuspended in 2 volumes of 0.9 M NaCl/10 mM Na_2HPO_4/pH 7.4 and frozen/thawed three times. After centrifugation at 20,000 × g for 30 min, the supernatant was dialyzed overnight at 4 °C against 10 mM Na_2HPO_4/pH 7.4 (40 l) using a Spectrapore membrane (molecular weight cut off 6–8000 Da, diameter 31.8 mm). The retentate was centrifuged again at 20,000 × g for 30 min, then shaken overnight with 16.7 g DEAE Sephadex A 50 (Pharmacia) at 4 °C. The DEAE Sephadex A 50 beads were packed into a column (5 × 60 cm), and washed with 10 mM Na_2HPO_4/pH 7.4 until the absorbance of the eluate at 280 nm reached a minimal value. Bound proteins were eluted with 1 M NaCl/10 mM Na_2HPO_4/pH 7.4.

The heparin-Sepharose chromatography was performed according to Brown and Clemmons[22], with minor modifications. The 1 M salt eluate from the DEAE Sephadex A 50 column was diluted about 10 times with 10 mM $(NH_4)_2CO_3$/pH 7.4 and loaded onto a heparin-Sepharose column (1.5 × 5 cm, CL-6B Pharmacia) preequilibrated with 0.1 M NaCl/10 mM $(NH_4)_2CO_3$/pH 7.4. After the sample had been loaded, the column was washed with 0.1 M NaCl/10 mM $(NH_4)_2CO_3$/pH 7.4 (rather than with 0.1 M and 0.25 M NaCl as was done by Clemmons) until the absorbance of the eluate at 280 nm reached a minimal value. Bound protein was eluted with 1 M NaCl/10 mM $(NH_4)_2CO_3$/pH 7.4. Fractions of 2 ml were collected.

Reverse-phase high performance liquid chromatography (RPLC) was carried out at room temperature using an LKB HPLC instrument (model 2152 HPLC controller and model 2150 pumps) coupled to a variable wavelength Kratos Spectroflow 773 detector. The following chromatography columns were used: a) a semi-preparative Vydac C4 column (25 × 1 cm, 5-µm particles, 30 nm pore size, The Separation Group, Hesperia, CA) and b) an analytical C8 column (RP-300, 22 × 0.46 cm, 10-µm particles, 30-nm pore size, Brownlee, Santa Clara, CA). The mobile phase used was 0.1% trifluoroacetic acid (TFA)/acetonitrile. Further details are given in the legends to the figures.

Determination of protein concentration

Protein concentrations were estimated by determination of RPLC peak areas at 210 nm, using areas of known amounts of protein chromatographed under the same conditions as standards. Alternatively, protein concentration was determined by the Biorad protein assay[36]. BSA was used as a standard for both methods.

Heat treatment

Heat-lability was tested by boiling lyophilized PDGI samples and appropriate controls dissolved in DMEM/pH 7.4 for 10 min. The inhibitory activities of boiled samples were determined by assessing the effects of 10-µl aliquots on the growth of endothelial cells (see below).

Cell cultures

Endothelial cells. Adult bovine aorta endothelial (ABAE) cells were provided by Dr D. Gospodarowicz, San Francisco and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (Hyclone Sterile Systems, Logan, UT) in the presence of bFGF as described previously[37–39]. They were plated for basal and bFGF-induced growth inhibition assays (see below).

Chicken embryo fibroblasts. Chicken embryo fibroblasts (CEF), kindly provided by H. Gehring, Zürich, were prepared as described[40]. 1,250,000 cells/5 ml were plated in Eagle's minimal essential medium (MEM) with 10% fetal calf serum in a Corning plastic flask. After incubation for 72 h at 37 °C in humidified 5% CO_2 atmosphere the cells were trypsinized and plated in MEM with 10% fetal calf serum for basal growth inhibition assays (see below).

Opossum kidney epithelial cells. Warnock opossum kidney (WOK) cells (wild type) were provided by Prof. H. Murer and J. Forgo, Zürich and were cultured in DMEM-Ham's F 12 (Flow) (1:1) with 10% fetal calf serum as described[41] and plated for basal growth inhibition assays (see below).

Growth inhibition assays

a) Inhibition of bFGF-induced cell growth. ABAE cells were seeded in 24-multiwell plates containing 0.5 ml medium per well at a density of 8000 cells per well. 10-µl aliquots of appropriately diluted test samples (with DMEM/0.5% BSA) and bFGF[1 ng/ml]were added on day 0 and day 2 after plating the cells. The cells were incubated for five days, trypsinized and counted in a model ZM Coulter particle counter. Further details are contained in the figure legends.

b) Inhibition of basal cell growth. ABAE, WOK cells or CEF were seeded in 24-multiwell plates containing 0.5 ml medium per well at densities of 16,000 cells per well. 10-µl aliquots of appropriately diluted test samples (with DMEM/0.5% BSA) were added on day 0 and day 2 after

plating the cells. The cells were incubated for six days and counted as described above.

Radioreceptor binding assay

The assay was performed by A. B. Roberts, Bethesda, MD, USA, as previously described[42]. Briefly, confluent human lung carcinoma A549 cells were incubated with 100 pM[^{125}I]-TGF-β and unlabelled aliquots of TGF-β or test samples, respectively, and cell-bound[^{125}I]-TGF-β was measured.

Antibody neutralization

The assay was performed by A. B. Roberts, Bethesda, MD, USA, as previously described[43]. Briefly, growth of mink lung epithelial CCL64 cells was inhibited by aliquots of test samples in the presence or absence of antibodies specific for TGF-β type 1[43] (those antibodies do not recognize TGF-β type 2). Cell growth was measured by determination of 5'-[^{125}I]iodo-2'-deoxyuridine incorporated into the cells.

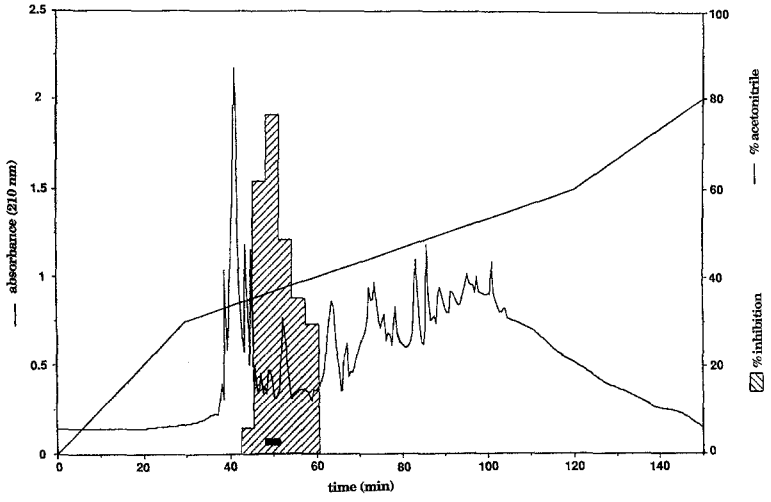

Figure 1. Purification of heparin-Sepharose purified PDGI by semipreparative RPLC on a C4-column. The PDGI fraction eluted from heparin-Sepharose chromatography was pumped directly onto a semipreparative Vydac C4 reverse-phase column and eluted with the 0.1% trifluoroacetic acid (TFA)/acetonitrile mobile phase at a flow rate of 1.5 ml/min using a 120-min-gradient of 30% to 60% acetonitrile. Fractions of 4.5 ml were collected at room temperature. 150 µl of each fraction was lyophilized in the presence of 50 µg BSA in a Speed Vac Concentrator, redissolved in 50 µl DMEM and tested for inhibitory activity by adding 10-µl aliquots to cells. The % inhibition is expressed relative to that of maximally inhibited ABAE cells (inhibited by 10 ng/ml TGF-β type 1). The horizontal bar indicates the fraction used for further purification.

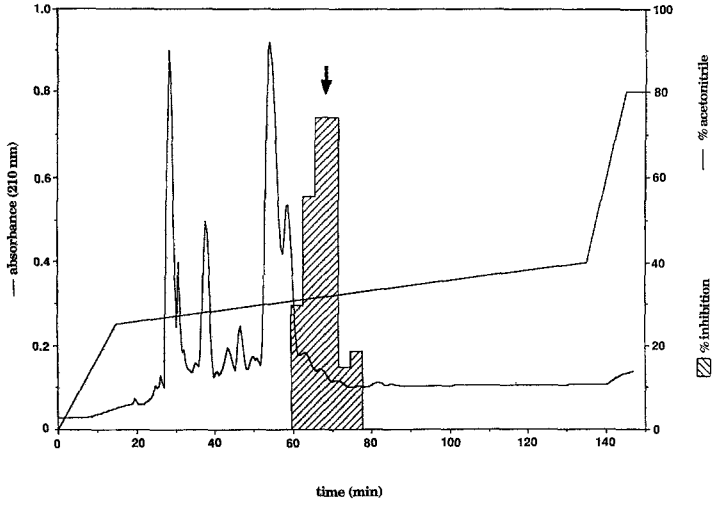

Figure 2. Purification of PDGI on an analytical RPLC C8 column. The fraction from the semipreparative C4-column containing the highest biological activity (fig. 1, horizontal bar) was diluted five-fold with 0.1% TFA, pumped onto an analytical C8-column and eluted with the 0.1% TFA/acetonitrile mobile phase at a flow rate of 0.7 ml/min using a 120-min-gradient of 25% to 40% acetonitrile. Fractions of 2.1 ml were collected at room temperature. 150 µl of each fraction were lyophilized in the presence of 50 µg of BSA, redissolved in 50 µl DMEM and assayed for inhibitory activity as described (fig. 1). The arrow indicates the retention time of authentic TGF-β type 1 chromatographed under identical conditions.

Results

Purification of PDGI. The purification of PDGI from one hundred 10-unit packs of human platelets was achieved in 5 steps. The first step, consisting of platelet extraction, yielded 5 g of protein. The second step, DEAE batch adsorption elution, yielded 2.5 g of protein. Heparin-Sepharose chromatography provided significant purification: only 1% (25 mg) of the proteins present in the DEAE Sephadex eluate bound to heparin-Sepharose. The eluted proteins were further purified in two steps with RPLC (figs 1, 2). After chromatography on a semi-preparative Vydac C4-column (fig. 1) the PDGI fraction (marked by the horizontal bar in fig. 1) contained 50 µg protein. Further purification on an analytical RPLC C8-column yielded approximately 1 µg of highly purified inhibitory protein (fig. 2).

Characterization of PDGI. Highly purified PDGI (fig. 2) inhibits the basal and the FGF-stimulated growth of ABAE cells cultured in serum-containing medium (fig. 3). This inhibition is dose-dependent from 0.1 to 10 ng/ml PDGI for basal growth, and from 0.6 to 10 ng/ml PDGI when the growth of cells is stimulated by bFGF [1 ng/ml]. Furthermore, PDGI similarly inhibits the basal growth of CEF in a dose-dependent manner from 0.16 to 10 ng/ml (data not shown).

PDGI and TGF-β type 1 were compared with respect to potency in the endothelial cell growth inhibition assay. PDGI and TGF-β produced qualitatively identical (parallel) dose-response curves (fig. 4). Making the assumption that PDGI and TGF-β are identical, the specific activity of PDGI was calculated from the dose-response curve and the protein concentration as 2000–4000 units/µg protein, depending on the batch of material (one unit is defined as the quantity of material needed to elicit half-maximal inhibition of endothelial cell growth). The specific activity of authentic TGF-β type 1 is 6400 units/µg protein (half-maximal response at 0.2 ng/ml). Thus, the PDGI preparation is estimated to be approximately 30–60% pure. PDGI and TGF-β were also compared in a heat-inactivation assay. As shown in figure 4, heating inactivated PDGI and TGF-β similarly.

As a further test of comparison, PDGI binding to the TGF-β receptor was tested in human lung carcinoma cells A549 using a TGF-β radioreceptor binding assay. In this assay, PDGI competed in a manner identical to that of human TGF-β type 1 standard (fig. 5). Furthermore,

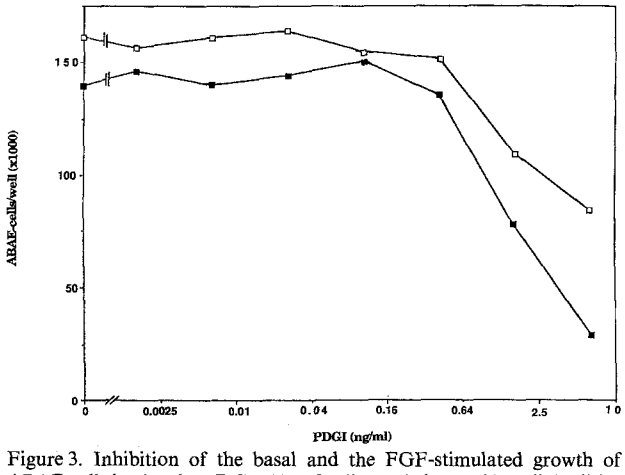

Figure 3. Inhibition of the basal and the FGF-stimulated growth of ABAE-cells in vitro by PDGI. ABAE cells, seeded at 16,000 cells/well for basal and at 8000 cells/well for FGF-stimulated growth, were grown in the presence of various doses of PDGI for 6 and 5 days, respectively. Points represent means of duplicate determinations (variations less than 10%). Symbols: open and closed squares represent FGF-stimulated and basal growth, respectively.

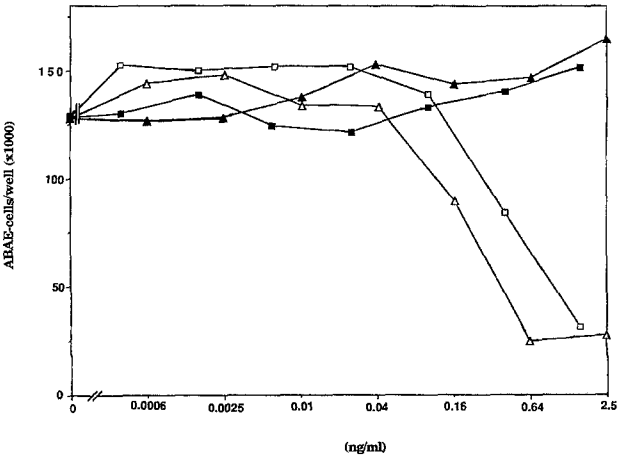

Figure 4. Comparison of endothelial cell growth inhibitory activities of PDGI and TGF-β type 1 on basal growth before and after heat inactivation. ABAE cells, seeded at 16,000 cells/well, were grown for 6 days in the presence of various doses of PDGI, PDGI boiled, TGF-β type 1 and TGF-β type 1 boiled. The protein concentration of the RPLC-fractions were determined as described above. Points represent means of duplicate determinations (variations less than 10%). Symbols: squares denote PDGI, triangles indicate TGF-β; open and closed symbols represent normal and heat-inactivated samples, respectively.

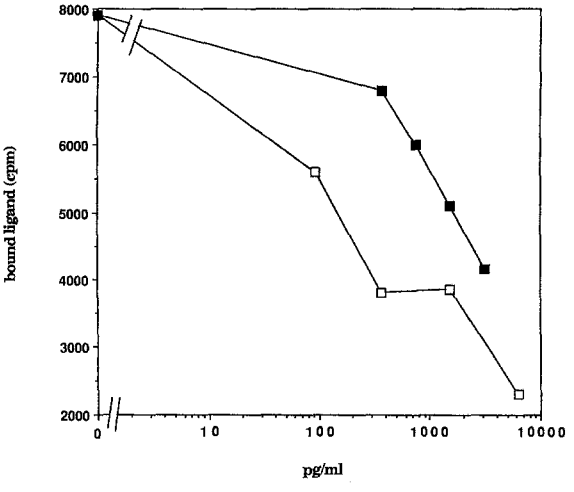

Figure 5. Competition of PDGI with human TGF-β in the radioreceptor binding assay. A549 cells were incubated with [^{125}I]-TGF-β and various doses of unlabeled TGF-β or PDGI at 40 °C for 3 h. The total cell-associated radioactivity was determined. Data represent means of triplicate determinations (variations less than 10%). Symbols: open squares denote TGF-β, closed squares indicate PDGI.

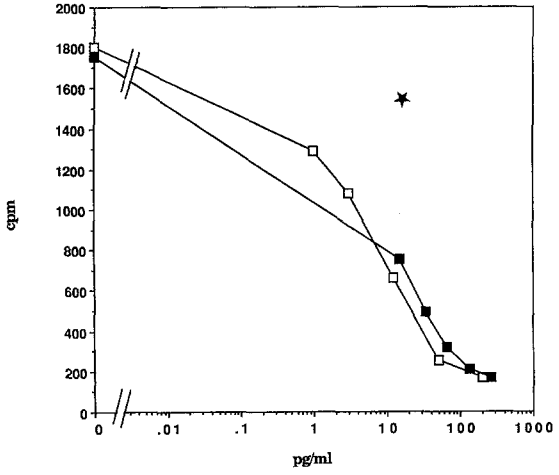

Figure 6. Growth inhibition of CCL-64 cells by PDGI and TGF-β in vitro. CCL-64 cells, seeded at 500,000 cells/well were grown for 22 h in the presence of various doses of PDGI or authentic TGF-β in the presence or absence of antibodies specific for TGF-β type 1. Points represent means of triplicate determinations (variations less than 10%). Symbols: open squares denote TGF-β, closed squares indicate PDGI, star represents PDGI in the presence of antibodies specific for TGF-β type 1.

the growth of mink lung epithelial cells CCL64 was inhibited similarly by human TGF-β type 1 and PDGI (fig. 6), and the PDGI activity in this assay was substantially blocked with antibodies which react specifically with TGF-β type 1 but not TGF-β type 2[42] (fig. 6). Finally, under highly resolutive RPLC conditions PDGI and TGF-β type 1 show indistinguishable retention behavior (fig. 2). All these data suggest close biological and chemical similarity between PDGI and TGF-β.

Discussion

Clemmons et al.[22] provided evidence that human platelets contain an endothelial cell growth inhibitor with characteristics apparently different from those of known platelet-derived inhibitors, most notably TGF-β, which occurs in high concentrations in platelets.

Our data suggest that PDGI is identical to a TGF-β-like protein. The following evidence is presented. Both are potent inhibitors for ABAE cells as previously described[45] possessing the same intrinsic biological activity. They also inhibit CEF growth but do not affect the basal growth of WOK cells (data not shown). Furthermore, they show the same binding behavior to A549 cells (parallel binding curve). Both peptides are acid-stable (they retain their bioactivity when boiled under acidic conditions[44]), are heat-labile at neutral pH, and coelute on a reverse-phase C8-column. Finally, the inhibitory activity of PDGI was neutralized by a highly specific antibody against TGF-β type 1 which does not cross-react with TGF-β type 2.

Our procedure for purification of PDGI is identical to that of Clemmons[22] except that heparin-Sepharose was washed with buffer containing 0.1 M NaCl after sample loading, rather than with 0.25 M NaCl[22], prior to elution of activity with 1 M NaCl. We have observed in some experiments that a minor portion of inhibitory activity was eluted from the heparin column by washing with 0.25 M NaCl. However, this material was shown to be indistinguishable from that eluted with 1 M NaCl when subjected to RPLC (data not shown). This suggests that PDGI binds only weakly to heparin-Sepharose (elution at or near 0.25 M NaCl) which is in agreement with the known weak binding of TGF-β to heparin-like substances.

The data presented here suggest that PDGI is identical to TGF-β type 1. Although we cannot entirely rule out the possibility that PDGI is a distinct molecular entity that is masked by TGF-β in all chromatography steps, or is undetected after RPLC because of inactivation, there was no experimental evidence to support such a notion and we consider this possibility to be unlikely. In conclusion, our results appear to invalidate the postulated existence of a new inhibitor in human platelets as proposed by Brown and Clemmons[22].

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1 Joseph-Silverstein, J., and Rifkin, D. B., Sem. Thromb. Hemostas. *13* (1987) 504.
2 Folkman, J., in: Important Advances in Oncology, p. 42. Eds V. De Vita, S. Hellman, and S. A. Rosenberg. Lippincott, Philadelphia 1985.
3 Folkman, J., Cancer Res. *34* (1974) 2109.
4 Esch, F., Baird, A., Ling, N., Ueno, N., Hill, F., Denoroy, L., Klepper, R., Gospodarowicz, D., Böhlen, P., and Guillemin, R., Proc. natl Acad. Sci. USA *82* (1985) 6507.
5 Gimenez-Gallego, G., Rodkey, J., Bennett, C., Rios-Candelore, M., DiSalvo, J., and Thomas, K., Science *230* (1985) 1385.
6 Ishikawa, F., Miyazono, K., Hellman, U., Drexler, H., Werbstedt, C., Hagiwara, K., Usuki, K., Takaku, F., Risau, W., and Heldin, C. H., Nature *338* (1989) 557.
7 Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D., and Ferrara, N., Science *246* (1989) 1306.
8 Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Conolly, D. T., Science *246* (1989) 1309.
9 Folkman, J., and Klagsbrun, M., Science *235* (1987) 442.
10 Frater-Schröder, M., Müller, G., Birchmeier, W., and Böhlen, P., Biochem. biophys. Res. Commun. *137* (1986) 295.
11 Baird, A., and Durkin, T., Biochem. biophys. Res. Commun. *138* (1986) 476.
12 Heimark, R., Twardzik, D., and Schwartz, S., Science *233* (1986) 1078.
13 Sato, N., Goto, K., Haranaka, N., Satomi, H., Nariuchi, Y., Mano-Hirano, Y., and Sawasaki, Y., J. natl Cancer Inst. *76* (1986) 1113.
14 Frater-Schröder, M., Risau, W., Hallmann, R., Gautschi, P., and Böhlen, P., Proc. natl Acad. Sci. USA *84* (1987) 5277.
15 Stolpen, A., Guinan, W., Fiers, W., and Pober, J., Am. J. Path. *123* (1986) 16.
16 Gaffney, E. V., and Tsai, S. C., Cancer Res. *46* (1986) 3834.

17 Tsai, S. C., and Gaffney, E. V., Cancer Res. *46* (1986) 1471.
18 Heyns, A., Eldor, I., Vlodavsky, N., Kaiser, N., Fridman, R., and Panet, A., Exp. Cell Res. *161* (1985) 297.
19 Böhlen, P., Frater-Schröder, M., Michel, T., and Jiang, Z. P., in: Angiogenesis, p. 119. Eds D. Rifkin and M. Klagsbrun. Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor 1987.
20 Wright, T. C. Jr, Johnstone, T. V., Castellot, J. J., and Karnovsky, M. J., J. cell. Physiol. *125* (1985) 499.
21 Willems, C., Astaldi, G. C. B., DeGroot, P. G., Janssen, M. C., Gonsalvez, M. D., Zeijlemakerr, W. P., Van Mourik, J. A., and Van Aken, W. G., Exp. Cell Res. *139* (1982) 191.
22 Brown, M. T., and Clemmons, D. R., Proc. natl Acad. Sci. USA *83* (1986) 3321.
23 Brem, H., and Folkman, J., J. exp. Med. *141* (1975) 427.
24 Lutty, G. A., Thompson, D. C., Gallup, J. Y., Mello, R. J., Patz, A., and Fenselau, A., Invest. Ophthalm. vis. Sci. *23* (1983) 52.
25 Seyedin, S. M., Thomas, T. C., Thompson, A. Y., Rosen, D. M., and Piez, K. A., Proc. natl Acad. Sci. USA *82* (1985) 2267.
26 Williams, G. A., Eisenstein, R., Schumacher, B., Hsiao, K. C., and Grant, D., Am. J. Ophthalm. *97* (1984) 366.
27 Takigawa, M., Shirai, E., Enomoto, M., Hiraki, Y., Fukuya, M., Suzuki, F., Suiio, T., and Yugari, Y., Cell Biol. int. Reports *9* (1985) 619.
28 Schumacher, B., Grant, D., and Eisenstein, R., Arteriosclerosis *5* (1985) 110.
29 Homandberg, G., Williams, J., Grant, D., Schumacher, B., and Eisenstein, R., Am. J. Physiol. *120* (1985) 327.
30 Glaser, B. M., Campochiaro, P. A., Davis, J. L. Jr, and Sato, M., Archs Ophthalm. *103* (1985) 1870.
31 Lutty, G. A., Mello, R. J., Chandler, C., Fait, C., Bennett, A., and Patz, A., J. cell. Sci. *76* (1985) 53.
32 Lee, A., and Langer, R., Science *221* (1983) 1185.
33 Eisenstein, R., Goren, S. B., Schumacher, B., and Choromokos, E., Am. J. Ophthalm. *88* (1979) 1005.
34 Rastinejad, F., Polverini, P. J., and Bouck, N. P., Cell *56* (1989) 345.
35 Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D., and Sporn, M. B., J. biol. Chem. *258* (1983) 7155.
36 Bradford, M. M., Analyt. Biochem. *72* (1976) 248.
37 Gospodarowicz, D., Cheng, J., Lui, G. M., Baird, A., and Böhlen, P., Proc. natl Acad. Sci. USA *81* (1984) 6963.
38 Böhlen, P., Esch, F., Baird, A., and Gospodarowicz, D., EMBO J. *4* (1985) 1951.
39 Böhlen, P., Baird, A., Esch, F., Ling, N., and Gospodarowicz, D., Proc. natl Acad. Sci. USA *81* (1984) 5364.
40 Sharma, C. P., and Gehring, H., Biochem. biophys. Res. Commun. *39* (1986) 1243.
41 Koyama, H., Goodpasture, C., Miller, M. M., Teplitz, R. L., and Riggs, A. D., In Vitro *14* (1978) 239.
42 Danielpour, D., Dart, L. L., Flanders, K. C., Roberts, A. B., and Sporn, M. B., J. cell. Physiol. *138* (1989) 79.
43 Frolik, C. A., Wakefield, L. M., Smith, D. M., and Sporn, M. B., J. biol. Chem. *259* (1984) 10995.
44 Sporn, M. B., Roberts, A. B., Wakefield, L. M., and deCrombrugghe, B., J. Cell Biol. *105* (1987) 1039.
45 Anzano, M. A., Roberts, A. B., and Sporn, M. B., J. cell. Physiol. *126* (1986) 312.

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Preferential hydrophobic interactions are responsible for a preference of D-amino acids in the aminoacylation of 5′-AMP with hydrophobic amino acids

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Abstract. We have studied the chemistry of aminoacyl AMP to model reactions at the 3′ terminus of aminoacyl tRNA for the purpose of understanding the origin of protein synthesis. The present studies relate to the D, L preference in the esterification of 5′-AMP. All N-acetyl amino acids we studied showed faster reaction of the D-isomer, with a generally decreasing preference for D-isomer as the hydrophobicity of the amino acid decreased. The β-branched amino acids, Ile and Val, showed an extreme preference for D-isomer. Ac-Leu, the γ-branched amino acid, showed a slightly low D/L ratio relative to its hydrophobicity. The molecular basis for these preferences for D-isomer is understandable in the light of our previous studies and seems to be due to preferential hydrophobic interaction of the D-isomer with adenine. The preference for hydrophobic D-amino acids can be decreased by addition of an organic solvent to the reaction medium. Conversely, peptidylation with Ac-PhePhe shows a preference for the LL isomer over the DD isomer.
Key words. Aminoacylation and peptidylation of 5′-AMP; stereoselectivity.

We would like to understand how the process of protein synthesis came into existence. One of the most challenging aspects of that problem is that of chirality. Although chemical syntheses of amino acids generally proceed to give equal amounts (racemic mix) of both optical isomers (D and L), biological systems almost exclusively use L-amino acids in protein synthesis. This in spite of the fact that D-amino acids can participate in each step of protein synthesis [1–3]. It is our general assumption that the origin of a biochemical system is based on the set of possible chemical reactions and that by studying the relevant ones, we can understand the origin of that biochemical system. In the case of protein synthesis, the relevant chemical reactions concern 5′-AMP.

Each amino acid is first activated by ATP, yielding the aminoacyl adenylate anhydride, with the amino acid covalently attached to the phosphate of 5′-AMP. The amino acid is then passed to become an ester of the ribose of the AMP residue which is at the 3′ terminus of every tRNA. The amino group of the aminoacyl tRNA then