4. For screening purposes, Ames *et al.* (*3*) suggested that the animals be treated with the enzyme inducer Aroclor 1254. We did not do this because the highly lipophilic Aroclor 1254 or its metabolites might have been retained in the subcellular preparations and its vicinal dichlorinated constituents activated by conjugation with glutathione, as occurs with 1,2-dichloroethane [U. Rannug, A. Sundvall, C. Ramel, *Chem. Biol. Interact.* **20**, 1 (1978)]. However, as with preparations from untreated animals, kidney S9 and liver S9 from Aroclor 1254–treated rats activated cysteine, and kidney S9 from Aroclor 1254–treated rats activated glutathione.
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Human Platelet–Derived Growth Factor (PDGF): Amino-Terminal Amino Acid Sequence

Abstract. *Human platelet-derived growth factor (PDGF) obtained from outdated human platelets was subjected to amino-terminal amino acid sequence analysis by automated Edman degradation. Despite the apparent presence of limited proteolytic degradation of the protein derived from this method, the sequence analysis reveals two primary peptide sequences and suggests that active PDGF is composed of two, possibly homologous, peptides linked by a disulfide bond or bonds.*

Platelet-derived growth factor (PDGF) is a heat-stable (100°C), cationic (isoelectric point, *p*I, 9.8) polypeptide (*1*) that circulates in blood stored in the α granules of platelets (*2*). It is released into serum during blood clotting, and it represents the major polypeptide growth factor of human serum. It is a potent mitogen for cultured fibroblasts, smooth muscle cells, and glial cells (*3–5*). Platelet-derived growth factor was isolated originally from whole human serum (*6*) and subsequently from clinically outdated human platelets (*1, 5, 7–9*) and from human platelet-rich plasma (*10*).

Unreduced, active PDGF exhibits multiple molecular weight forms ranging in size from 28,000 to 35,000 daltons, as judged by analytical sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*1, 7–10*). Reduction of PDGF produces inactive, lower molecular size polypeptides ranging from 12,000 to 18,000 daltons (*5, 7, 8*). Whether these polypeptides represent two or more subunits held together in native PDGF by disulfide bonds, as has been suggested (*7, 8*), or whether they represent pieces of a single polypeptide produced by proteolytic fragmentation during the handling and fractionation of the outdated platelets but still held together by disulfide bonds, has not been established.

Elucidation of the primary structure of PDGF will help to provide an understanding of the molecular basis for its function and its relation to other polypeptide growth factors, serum proteins, and platelet factors. Here we report the results of amino-terminal amino acid sequence analysis of both active PDGF and the major, inactive peptides derived from it by disulfide reduction.

PDGF was purified by a modification of a previously described procedure (*1, 8*). Two major bands of PDGF activity, corresponding to protein bands of 35,000 (PDGF-I) and 32,000 (PDGF-II) daltons, were recovered from the final stage of purification (SDS-PAGE). Biologic activity during purification was assessed by the ability of PDGF to stimulate DNA synthesis in cultures of BALB/c-3T3 (clone A30) cells (*1*). The specific activity of purified PDGF is estimated at about 3000 units per microgram of protein, and that of the platelet lysate is about 0.03 unit per microgram of protein. One unit of PDGF activity is defined as the amount required to induce 50 percent of the cells to synthesize DNA (*1*). The stained protein eluted from the gels seems to retain full biologic activity.

Amino-terminal amino acid sequence analysis was performed with automated Edman degradation on a gas-phase sequenator designed and constructed at Caltech (*11, 12*). Analysis of phenylthiohydantoin amino acids released by the sequenator was accomplished by reverse-phase high-performance liquid chromatography on a cyano column (IBM Instruments) (*13*).

Sequence analysis of several preparations (80 to 400 pmole) of both PDGF-I and PDGF-II revealed the presence of three to four detectable amino acid sequences in all of them. Although the relative amounts of the different sequences varied somewhat between the PDGF-I and PDGF-II preparations, they were not sufficiently unalike to allow unambiguous determination of the individual sequences. Therefore, the purified PDGF fractions were reduced, alkylated, and fractionated by SDS-PAGE. This procedure yielded two major fractions, with apparent molecular sizes of approximately 18,000 (fraction A) and 14,000 (fraction B) daltons, and one minor fraction, with an apparent molecular size of 12,000 daltons (fraction C) (Fig. 1).

Sequence analysis of fractions A, B, and C revealed that together they exhibited all of the sequences present in the unreduced PDGF preparations. Moreover, although none of the three fractions gave a single, clean sequence, each by itself gave a simpler mixture of sequences than that present in either PDGF-I or PDGF-II. Quantitative analysis of these sequence data established the individual sequences shown in Fig.

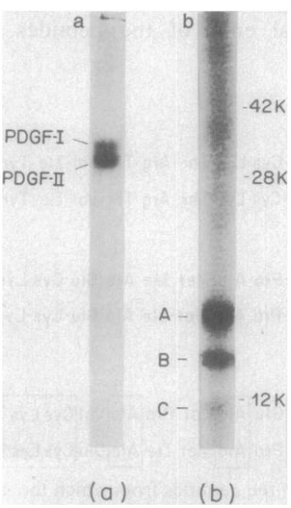

Fig. 1. SDS-PAGE of unreduced (a) and reduced or alkylated (b) PDGF. (a) The two major forms of unreduced PDGF, PDGF-I, and PDGF-II. The specific activity of PDGF-I and -II is about 3000 units per microgram of protein. (b) The sample of the reduced or alkylated PDGF demonstrates the presence of reduced fractions A, B, and C. Fractions A and B are the major reduced fractions of PDGF. Fraction C is a minor component and is present only in some reduced PDGF preparations. For reduction, purified PDGF (10 to 20 μg) was dissolved in 50 μl of 0.5*M* ammonium bicarbonate containing 20 m*M* dithiothreitol. The reduced preparation was kept for 2 hours at room temperature, and 50 μl of 40 m*M* iodoacetamide in 0.5*M* ammonium bicarbonate was added. After 5 minutes, the preparation was dialyzed against 0.02 percent SDS, lyophilized, dissolved in 25 μl of Laemmli sample buffer (*9*) containing 2 percent SDS, and subjected to SDS-PAGE (16 percent gels, 2 mA per gel). The gels were stained overnight at room temperature with 0.1 percent Coomassie brilliant blue R-250 in 10 percent acetic acid and 10 percent methanol and destained with 10 percent acetic acid and 10 percent isopropyl alcohol. The stained proteins were eluted (*8*), dialyzed at room temperature against 0.1 percent SDS, and lyophilized.

2A. In essence, two major sequences (*1a* and *2a*) and two minor sequences (*1b* and *2b*) were observed. Moreover, the two minor sequences represent shortened forms of the major sequences in which three (sequence *1b*) and five (sequence *2b*) amino acids are missing from the amino terminal ends of sequences *1a* and *2a*, respectively, presumably by limited proteolysis of the longer peptides *1a* and *2a*. Therefore, the amino-terminal sequence analysis reveals that active PDGF exhibits two major peptide sequences and contains no other sequences that are not derivatives of these two.

Table 1 lists the sequences detected in the various peptide preparations. While there is some correlation with the apparent size of the peptide fractions on SDS-PAGE and the observed amounts of the amino-terminal-shortened sequences, the shortness observed at the amino-terminal ends is not sufficient to account for the range of apparent molecular size (12,000 to 18,000 daltons) seen on the gels. This range of sizes suggests the existence of size differences at the carboxyl-terminal ends or of partial glycosylation of the peptides.

A fundamental question about the structure of the active PDGF molecules (that is, the unreduced 32,000 to 35,000 dalton forms) is whether they are composed, in the absence of any proteolytic degradation, of a single long polypeptide, of two identical peptides linked by disulfide bridges, or of two nonidentical peptides linked by disulfide bridges. Our data cannot distinguish between these choices.

If native PDGF is a single long polypeptide, then the apparent subunit structure observed in the sequencing studies could result from a proteolytic split near the middle of the peptide with the two fragments being held together by disulfide bridges. Such proteolysis, as well as that observed at the amino- and carboxyl-terminal ends of the molecule, is not unexpected in view of the derivation of the PDGF from units of outdated human platelets. However, it must not result in inactivation of the PDGF activity since all of the active, unreduced PDGF seems to break down into the smaller peptides upon reduction.

Table 1. Population of sequences *1a*, *1b*, *2a*, and *2b* in various PDGF fractions.

Fraction	Molecular size (daltons)	Percent*			
		1a	*1b*	*2a*	*2b*
PDGF-I	35,000	40	10	25	25
PDGF-II	32,000	20	10	35	35
PDGF-A	18,000	60	Tr†	30	10
PDGF-B	14,000	Tr	Tr	75	25
PDGF-C	12,000	50	50	Tr	Tr

*These percentages were determined from quantitative analysis of the phenylthiohydantoin amino acids released during the automated Edman degradation of the various PDGF fractions and are accurate to ±5 percent. †Tr, trace.

If native PDGF is composed of two identical peptides linked by disulfide bridges, then the observed sequences require that 20 to 30 amino acid residues must have been clipped from at least one end of both peptides in the native PDGF to give the observed sequence heterogeneity in PDGF-I and in fraction A (that is, the highest molecular weight unreduced and reduced preparations, respectively). Such degradation must not result in inactivation of the PDGF. Indeed, the occurrence of even more extensive degradation present in PDGF-II cannot have much effect since the specific activities of PDGF-I and PDGF-II are approximately equal (*8*).

If native PDGF is composed of two nonidentical peptides linked by disulfide bridges, then the observed sequences can be generated from it by much less extensive degradation, at least at the amino-terminal ends of the peptides. While further sequence analysis of the PDGF peptides will be required to decide whether this is the case, examination of the two major observed sequences for homology suggests that two distinct peptides do comprise the native PDGF. Many multi-subunit proteins, including immunoglobulins, hemoglobin, and acetylcholine receptor (*14*), are composed of nonidentical but homologous subunits. Alignment of PDGF sequences *1a* and *2a* as shown in Fig. 2B reveals a similar degree of homology. The two sequences are identical in 8 of 19 positions without adding any gaps due to an insertion or deletion (or both). However, while the homology could indicate an evolutionary relationship between two separate peptides, it might also indicate the presence of two related domains such as those that exist within immunoglobulin light chains and heavy chains. The junctions between these domains are known to be particularly susceptible to proteolytic cleavage compared to the rest of the peptides, and cleavage at such a point in a single, long polypeptide in native PDGF could give the appearance of the presence of two homologous peptides.

A limited search of the known sequences of other growth factors and other serum proteins has not revealed any significant homology between them and the PDGF sequences. The only interesting observation from this search is that the Glu-Glu (glutamic acid) sequence at positions 3 and 4 in PDGF sequence *1a* is also present at positions 3 and 4 in platelet factor 4 (*15*) and β-thromboglobulin (*16*), two peptides that, like PDGF, are stored in the α granules of platelets.

While the sequence data from our study is admittedly limited, it should be sufficient to assist those interested in cloning the gene or genes encoding PDGF by serving as the basis for the synthesis of oligonucleotide probes (*17*).

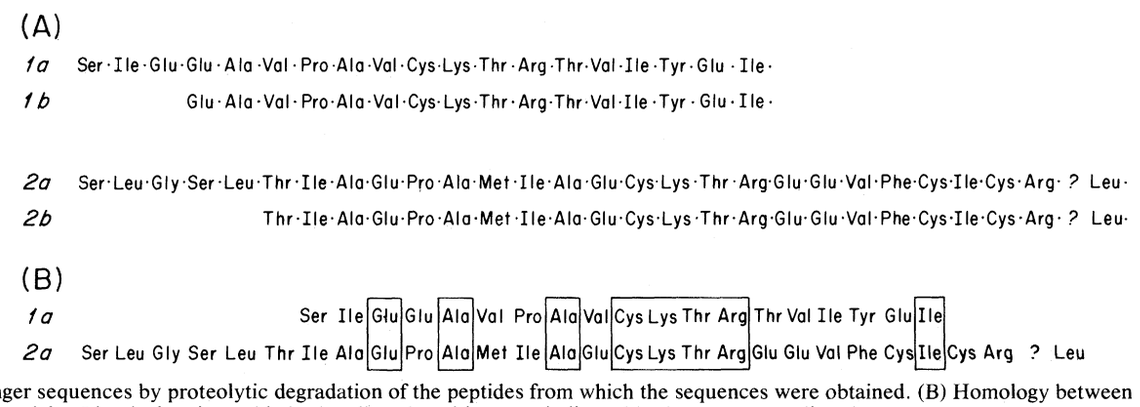

Fig. 2. (A) The amino-terminal amino acid sequences of the peptides derived from reduction, alkylation, and SDS-PAGE of active PDGF. Sequences *1b* and *2b* are identical to sequences *1a* and *2a*, respectively, except for the apparent removal of three and five amino acids, respectively, from the amino-terminal ends of the longer sequences by proteolytic degradation of the peptides from which the sequences were obtained. (B) Homology between PDGF sequences *1a* and *2a*. Identical amino acids in the aligned positions are indicated by boxes surrounding them.

This is a particularly powerful approach to obtaining the complete primary sequence of proteins such as PDGF that are available in such small quantities that obtaining the complete sequence from the protein itself is difficult at best. The utility of the method is illustrated by the recent success in cloning the complementary DNA's of the acetylcholine receptor subunits with the use of synthetic DNA probes based on amino-terminal sequence data (*18*). It can also be used to synthesize polypeptides (*19*) for the generation of antibodies to PDGF that might be useful in functional studies, cloning experiments, and radioimmunoassay of PDGF.

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Cytotoxicity of a Perfluorocarbon Blood Substitute to Macrophages in vitro

Abstract. *Murine macrophage and macrophage-depleted splenocyte cultures were incubated under ambient oxygen with a commercially available perfluorocarbon blood substitute. The perfluorocarbon preparation was found to be selectively cytotoxic to macrophages. This finding may be significant in view of the preliminary therapeutic usage of these preparations. In addition, perfluorocarbons may be useful as a means of selectively removing macrophages from tissue and organ cultures.*

Liquid perfluorocarbons have the interesting property of dissolving a far greater volume of oxygen than other liquids at comparable partial pressures. Whereas normal saline or blood plasma dissolves about 3 percent oxygen (based on the volume ratio of gas to liquid) and whole blood about 20 percent, pure perfluorocarbon can dissolve 40 percent or more (*1*). This phenomenon has led to the development of stable, aqueous emulsions of these chemicals for use as oxygen transport fluids. They have been tested in experimental animals as breathing liquids and as substitutes for red blood cells (*2, 3*). Recently, perfluorocarbon emulsions have been used therapeutically as blood supplements in patients who required emergency surgical treatment or who refused blood transfusions (*4*). These initial clinical studies have shown little toxicity with the exception of a report of pulmonary crisis believed to be due to complement activation (*5*).

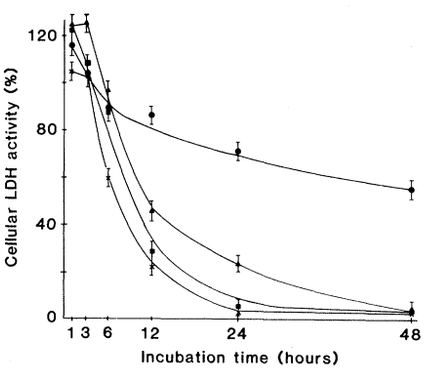

Fig. 1. Percentage decrease in cellular LDH activity of macrophage cultures incubated with (●) 5 percent, (▲) 10 percent, (■) 15 percent, and (x) 20 percent FC-43. For each assay, 1×10^6 cells in 1 ml were cultured in a 2.0-cm^2 multiwell tissue culture plate. At the end of the incubation the cells were washed, lysed, and assayed for LDH by measuring the reduction of pyruvate to lactate, using reduced nicotinamide-adenine dinucleotide (NADH) as the coenzyme (*9*). Activity was calculated from the rate of the change in absorbance at 340 nm as measured in a Beckman Acta III spectrophotometer. The percent activity is expressed in terms of the ratio of LDH activity of the fluorocarbon-incubated culture over a control culture containing no fluorocarbon for that time point. Error bars denote the standard error of the mean for two assay determinations.

Reasoning that the particulate nature of perfluorocarbon emulsion might lead to its uptake by the reticuloendothelial system, we were concerned about adverse effects that perfluorocarbon therapy might have on macrophage function. Animal studies have shown that a large percentage of the administered material is retained in the liver and the spleen with an elimination half-life of several weeks (*6*).

We studied the effect on murine leukocytes in vitro of a commercially available preparation of artificial blood containing as principal ingredients FC-43 (perfluorotributylamine, 25 percent, weight/vol-