Accumulation of PDGF B and Cell-binding Forms of PDGF A in the Extracellular Matrix

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Abstract. PDGF is a powerful mitogen initially identified within platelets, but also shown to be produced by a wide variety of cell types. PDGF is encoded on two separate genes. These give rise to three polypeptides, PDGF B and two forms of PDGF A (SA and LA), resulting from alternative splicing of the PDGF A gene primary transcript. We report that in CHO cells transfected with PDGF gene constructs and producing moderate levels of PDGF homodimers, much of the PDGF LA and B produced, but little if any SA, is found in the matrix laid down beneath the cells.

Immunoreactive PDGF in cells, and in matrix below expressing cells, was visualized by laser confocal microscopy. Western blotting of protein in matrix extracts, cell extracts, and secreted into the growth medium was used to demonstrate that the range of PDGF A polypeptides seen in the matrix was overlapping with those reported previously to be cell associated in cell types such as NIH3T3 and COS 7. This suggests that attachment to matrix or cell surface may be alternative fates for these polypeptides, with fate dependent on the characteristics of the producing cells.

Immunoreactive PDGF A and B could be partially released by incubation of matrix material with heparin but not with other glycosaminoglycans. Digestion of matrix with chondroitin ABC lyase but not heparitinase or collagenase displaced some PDGF from its attachment sites. The results indicate attachment of PDGF to matrix proteoglycans, at least partly through the glycosaminoglycan moieties, and perhaps to additional components. The significance of matrix deposition for PDGF action is discussed.

PDGF is a mitogenic growth factor isolated from platelets, where it is found primarily as a heterodimer of A and B chains (reviewed in Ross et al., 1986; Heldin and Westermark, 1990). The A and B polypeptides, which are encoded by separate genes, are two members of a family of related polypeptides, which also includes vascular endothelial cell growth factor (VEGF,[1] VPF; Keck et al., 1989; Leung et al., 1989), a recently described placental factor (Maglione et al., 1991), and a connective tissue growth factor produced by vascular endothelial cells (Bradham et al., 1991).

Molecular cloning of the PDGF A and B genes has been reported by several groups (Betsholtz et al., 1986; Rao et al., 1986). Alternative splicing of the PDGF A gene transcript (Tong et al., 1987; Collins et al., 1987; Sánchez et al., 1991) results in the production of at least two functional polypeptides. The most common form of PDGF A mRNA lacks the gene's sixth exon and encodes a 196 amino acid form of PDGF A (SA). Expression of the SA gene in cell types such as 3T3 and COS 7 results in the production of a homodimer of molecular weight 30 kD which is predominantly secreted from the cells. An alternative mRNA includes an additional exon and encodes a PDGF A polypeptide of 211 amino acids, LA. This polypeptide has been reported to be much less efficiently secreted than the SA product. The extended COOH terminus of the LA polypeptide includes a stretch of very basic amino acids. In recent reports, it has been suggested that in cell types such as NIH 3T3, PDGF LA molecules remain attached to cells via this additional basic region. An analogous sequence in the COOH terminal region of PDGF B appears to explain why some processing products of this protein are cell-attached (Ostman et al., 1991; LaRochelle et al., 1990*a*).

It has recently been reported that cellular binding of PDGF can be blocked (Khachigian et al., 1992) and cell-bound PDGF released from cells (Raines and Ross, 1992) by the basic peptide from the COOH terminus of PDGF LA. Binding of PDGF polypeptides to cell-surface heparan sulphate proteoglycan (HSPG) via this basic amino acid sequence has been proposed (Raines and Ross, 1992; Khachigian et al., 1992; Khachigian and Chesterman, 1992).

In contrast with results reported for 3T3 and other cell types, a report on PDGF expression in CHO cells found little evidence for surface localization or major cell association of PDGF. Thyberg et al. (1990) described localization of small amounts of PDGF BB in the Golgi, lysosomes, and ER of

1. *Abbreviations used in this paper*: HSPG, heparan sulfate proteoglycan; VEGF, vascular endothelial cell growth factor.

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expressing CHO cells, while PDGF SA was present at lower levels, and in locations consistent with packaging of the protein for secretion. In this report, we demonstrate that PDGF LA produced by CHO cells is predominantly deposited into extracellular matrix beneath the cells, with relatively little cell-associated material being found. In CHO cells expressing PDGF B, substantial amounts of this homodimer are also associated with matrix material, while PDGF SA is secreted into the growth medium as in other cell types.

Materials and Methods

PDGF Gene Isolation and Vector Construction

PDGF SA, LA, and B coding regions were isolated by polymerase chain reaction amplification from cellular RNA as described previously (Sánchez et al., 1991). The genes were then inserted into expression vectors to allow inducible expression in mammalian cells. The vectors used were derived from pSVL (Pharmacia-LKB, Uppsala, Sweden), a vector which includes the SV-40 late promoter and splice region and SV-40 polyadenylation signals, together with plasmid sequences to allow replication in *Escherichia coli* and antibiotic selection. pSVL was first treated to remove NaeI sites within pBR322 sequences, as shown in Fig. 1. Subsequently, the SV-40 promoter was removed and the promoter from the human metallothionein IIA gene, contained on a BglII fragment excised from pMetCAT (McNeall et al., 1989) was inserted at the cut site (Fig. 1). PDGF coding sequences were inserted into the polylinker region of the vector, as shown in Fig. 1, to yield the vectors pMetPDGF-SA, pMetPDGF-LA, and pMetPDGF-B.

Cell Culture and Transfection Procedures

CHO K1 cells were routinely cultured in a 1:1 mixture of DME and Ham's F12 medium containing 10% FCS, as described previously (McNeall et al., 1989). For induction and collection of secreted protein, cells were transferred to serum-free medium. Expression of inserted genes under control of the metallothionein promoter was induced by addition to the medium of cadmium chloride (1 μM) and zinc chloride (50 μM) for 18 h before cell harvest.

CHO cells were transfected with DNA by calcium phosphate co-precipi-

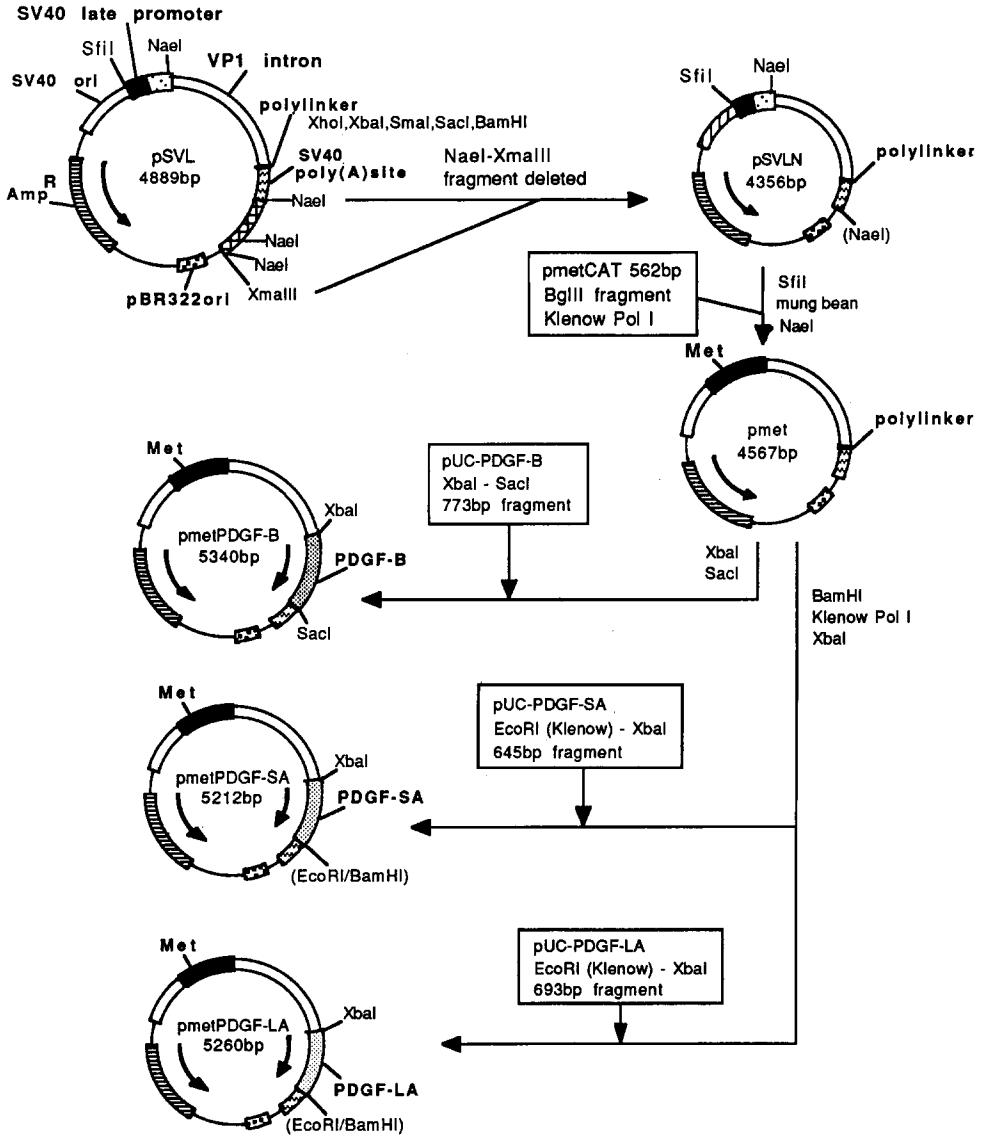


Figure 1. Construction of metal-inducible expression vectors containing PDGF genes. The vectors used were derived from pSVL (Pharmacia-LKB) which contains the SV-40 late promoter and splice region followed by a polylinker and SV-40 polyadenylation region. Excess NaeI sites in pBR322 sequences were removed by deletion of the NaeI to XmaIII 535-bp fragment to yield the plasmid pSVLN. This was subsequently digested with NaeI and SfiI; mung bean nuclease digestion was used to generate blunt ends (Maniatis et al., 1982). This vector was ligated to a 562-bp fragment containing the human metallothionein IIA promoter. The fragment was generated by BglII digestion of pMet CAT (McNeall et al., 1989) followed by Klenow DNA polymerase I to generate blunt ends. The vector generated after ligation, pMet, was the recipient for coding sequences from the genes PDGF B, PDGF SA, and PDGF LA. To insert PDGF B coding sequences, pMet was digested in the polylinker with XbaI and SacI. This was ligated to a 750-bp XbaI/SacI fragment cut from a pUC clone containing PDGF B coding sequences inserted at the SmaI site (Sánchez et al., 1991). pMet PDGF-LA contained a 610-bp fragment from a pUC clone of PDGF-LA, generated by EcoRI digestion, end filling with Klenow polymerase, and then digesting with XbaI (Sánchez et al., 1991). Coding sequences for PDGF-SA were generated by the same strategy from a pUC clone containing PDGF-SA. pMet was cut with BamHI, end filled with Klenow DNA polymerase I, and then cut with XbaI before ligation to the above two fragments. All procedures used methods outlined in Maniatis et al. (1982).

tation (Ausubel, 1990). Cells were cotransfected with the PDGF expression vector of interest (9 μg), pSV2neo (1 μg), and pSV2pac (4 μg) to allow selection of transfected cells for resistance to G418 (400 μg/ml; Geneticin; Sigma Immunochemicals, St. Louis, MO) and puromycin (10 μg/ml; Geneticin; Sigma Immunochemicals). The double transfection and selection procedure was utilized to increase the proportion of surviving transfectants expressing introduced genes at high levels (Wirth et al., 1988). Colonies of resistant cells (100–200 colonies per 75 mm culture dish) were collected and frozen in bulk, without cloning.

RNA Assays

Cytoplasmic RNA was isolated as described by Gough (1988). PDGF A and B mRNAs were detected in total cytoplasmic RNA by ribonuclease protection assay using an RPA II ribonuclease protection assay kit (Ambion Inc., Austin, TX). Complete coding sequences for PDGF SA and B, generated by polymerase chain reaction (Sánchez et al., 1991), were cloned into Bluescribe M13$^+$ (Vector Cloning Systems, San Diego, CA) and riboprobes were transcribed in a 20 μl reaction using the Riboprobe transcription system (Promega Biotec, Madison, WI) according to the manufacturer's instructions. After transcription, the DNA template was removed by adding 1 U of RNase-free DNase (Promega Biotec) and incubating the mixture for a further 15 min at 37°C. Probes were purified by electrophoresis on a 5% polyacrylamide/7 M urea gel as detailed in instructions for the Ambion RPA kit. After ribonuclease digestion, protected RNA fragments were separated on the same gel system and detected by autoradiography in comparison with HinfI-digested pBR322 DNA, end-filled with Klenow polymerase I and [^{32}P] dATP (Maniatis et al., 1982). A parallel RNase protection assay was carried out using a probe from the glyceraldehyde phosphate dehydrogenase gene (Tso et al., 1985) to standardize the amounts of RNA added to the original reactions.

Protein Assays

PDGF A and B polypeptides were detected by Western blotting (Ausubel, 1990). Secreted protein was harvested by incubating 60 mm plates of cells overnight in 2 ml of serum-free medium with or without metal ions to induce PDGF expression. The medium was collected, and debris removed by brief centrifugation (500 *g*, 10 min). The supernatant was, for some experiments, concentrated tenfold in Amicon Centricon 10 microconcentrators, by spinning for 1 h at 6,500 rpm in a Sorvall GSA rotor (DuPont, Newton, CT). Control experiments showed that no PDGF was lost during this procedure. The remaining cells were washed with PBS, and collected in PBS by scraping with a rubber spatula. Cells were washed in PBS, collected by centrifugation, and resuspended in 100 μl Laemmli sample buffer (no mercaptoethanol) (Laemmli, 1970). While some extracellular matrix material would be collected with the cells after scraping, the two wash/centrifugation steps were included to separate all but the more closely associated material from the collected cells. The cell extract was passed through a 26 G needle ten times and heated to 98°C for 10 min. Debris was removed by spinning the sample for 5 min in an Eppendorf microcentrifuge (Brinkman Instruments, Inc., Westbury, NY). Aliquots of cellular and secreted proteins were separated by electrophoresis in 15% polyacrylamide gels containing SDS, under non-reducing conditions. The stacking gel was 6% polyacrylamide. The separated proteins were transferred to nitrocellulose membrane (0.2 μm) by electroblotting, as described by Towbin et al. (1979). After blocking with 1% instant non-fat dry milk powder in TBS (12 mM Tris-HCl, pH 7.4, 120 mM NaCl) for 2 h at 37°C, filters were incubated with polyclonal antibody specific for either PDGF AA or BB homodimers (rabbit anti-human, obtained from Genzyme Corp., Boston, MA, and diluted to 20 μg/ml in blocking buffer). After overnight incubation at 4°C, unbound antibody was removed by washing the filter four times in blocking buffer. Filters were then incubated with sheep anti-rabbit IgG coupled to alkaline phosphatase (Silenus Laboratories, Hawthorn, Australia), diluted 1 in 20,000 in blocking buffer. The filters were further developed to show bound enzyme activity using a commercial kit (Immunoselect; Bethesda Research Laboratories, Gaithersburg, MD).

The standards used were recombinant PDGF SA homodimer expressed in *E. coli* and PDGF B homodimer expressed in yeast (Genzyme Corp., Boston, MA), diluted in 10 mM acetic acid and 0.1% BSA according to the manufacturer's instructions. Prestained protein mixes (Biorad Laboratories, Richmond, CA) were used as molecular weight markers.

For immunofluorescent labeling, cells were grown on glass coverslips overnight in serum-containing medium, with or without metal induction. Cells were fixed and antibody treated as described by Thyberg et al. (1990) using the same rabbit antibodies directed against PDGF AA and BB as described above, and fluorescein-conjugated swine anti-rabbit IgG (DAKOPATTS A/S, Copenhagen, Denmark). In these experiments, antibodies against PDGF-AA were used at 10 μg/ml. Fluorescence due to immunoreactive PDGF was viewed in a laser scanning confocal microscope (model MRC 500; BioRad Laboratories, Cambridge, MA). Polyclonal antibodies prepared in rabbit against mouse laminin were a gift from Ms A. Dalton (CSIRO). Rhodamine-phalloidin for actin labeling was from Molecular Probes, Inc., Eugene, OR) and was used according to the manufacturer's instructions.

Matrix Studies

Cells were cleared from attachment to extracellular matrix materials by lysis with Triton X-100/NH_4OH as described by Bashkin et al. (1989). Matrix remaining was treated with enzymes or other additives for 60 min at 37°C in PBS (2.5 ml) containing $CaCl_2$ (133 μg/ml) and $MgCl_2$ (100 μg/ml). PBS was then removed from the plates. To examine the sizes of proteins remaining at the surface, Laemmli sample buffer was added (300 μl per 100 mm plate; Laemmli, 1970) and the matrix was dispersed by scraping with a rubber spatula. This material was collected, and heated at 98°C for 10 min. Debris was removed by microfuging and protein in the supernatant was analyzed by polyacrylamide gel electrophoresis, carried out under nonreducing conditions, followed by Western blotting as described above. The supernatant material, removed after enzyme or other treatment of the matrix, was concentrated tenfold. Aliquots were diluted in 3× concentrated Laemmli sample buffer and heated and analyzed as described above. ^{35}S-labeling of sulphated proteoglycans being deposited into the extracellular matrix was carried out as described by Brunner et al. (1991). Sodium [^{35}S] sulphate (400 mCi/mmole) was obtained from DuPont.

Results

PDGF Production in Transfected Cells

CHO cells in culture were transfected separately with the expression vectors pMetPDGF SA, pMetPDGF LA, and pMetPDGF B, together with selectable marker genes as described in Materials and Methods. Uncloned, antibiotic-resistant cell populations from each of the three transfections (CHO SA, CHO LA, and CHO B, respectively) were tested for PDGF expression by Western blotting of material associated with cells, and secreted into the medium. Fig. 2 shows PDGF homodimers detected for these CHO SA and CHO LA cell populations. Untransfected CHO cell samples showed no immunoreactive material in either medium or cell extracts. CHO SA and CHO LA cell samples were not detected in Western blots when these were probed with antibody directed against PDGF BB (not shown). In CHO SA cultures tested with antibody to PDGF AA, >80% of the immunoreactive material detected was found in the medium. Three protein bands were seen running at 37, 34, and 32 kD compared with molecular weight standards, with the smaller bands predominating. The lowest band had an electrophoretic mobility similar to that of the standard PDGF AA produced in *E. coli* (28.6 kD, running at 32 kD in our gel system) (Fig. 2). In some experiments, material of a lower molecular weight, corresponding to that of monomer, was also seen. Little cell-associated PDGF SA was seen, and that detected was larger (39 and 42 kD), indicating that this was unprocessed or partially processed material. Production of immunoreactive PDGF SA by the CHO SA culture was estimated by comparison with the PDGF AA standard at 200–400 ng per 10^6 cells per day following heavy metal induction. Amounts of PDGF produced were estimated from four to five different samples for this and other cell lines, but provide an approximation of relative levels only. Production was not rigorously quantitated using a PDGF standard curve. Our

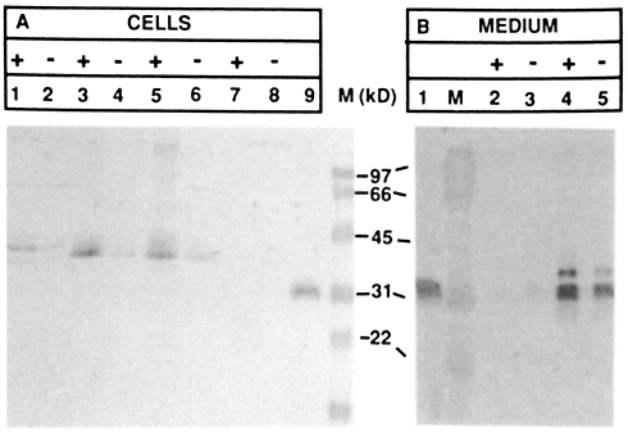


Figure 2. Production of PDGF A polypeptides in CHO cells. CHO cells expressing PDGF SA and LA genes (CHO SA and CHO LA, respectively) were tested for the relative localization of immunoreactive PDGF A in cell extracts and secreted into the medium, with or without induction of gene expression by heavy metals as described in Materials and Methods. Analysis was by Western blotting after separation of proteins on a nonreducing SDS–polyacrylamide gel. Medium samples are 10 μl of 10× concentrated material (5% of total collected per 5 × 10^6 cells); cell extract samples are 10 μl (10% of total per 5 × 10^6 cells). (Lanes *A-9* and *B-1*) 20 ng PDGF AA; (lanes *M*) protein markers whose sizes are indicated in kD. (*A*) Lanes *1* and *2*, CHO LA cells, plus and minus induction as indicated; lanes *3* to *6*, duplicate samples for CHO SA cells; lanes *7* and *8*, untransfected CHO cells. (*B*) Lane *2* and *3* CHO LA; lanes *4* and *5*, CHO SA. Samples shown are from two gels run in parallel.

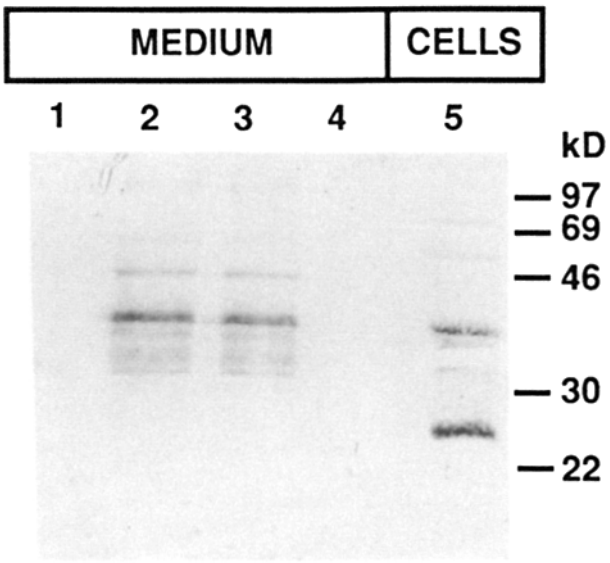


Figure 3. Production of PDGF B by CHO B cells. Protein secreted into the medium and retained by CHO B cells was collected and analyzed as described for Fig. 2, except that antibody specific for PDGF BB was used in Western blotting procedures. (Lane *1*) 0.5 ng PDGF BB (recombinant); (lanes *2* and *3*) duplicate lanes of medium from metal-induced CHO B cells; (lane *4*) medium from metal-induced CHO cells (control); (lane *5*) metal-induced CHO B cells extract. Medium samples were 12 μl 10× concentrated (6% of total collected from 5 × 10^6 cells); cell extracts were 5 μl samples (5% of total from 5 × 10^6 cells).

results compare with a previous report of PDGF production in CHO cells at 700 ng/10^6 cells/d (Ostman et al., 1988) using a different expression vector.

In contrast to our results with PDGF SA, a lower level of PDGF LA (around 20 ng per 10^6 cells/d) was detected, either secreted into the medium by or associated with CHO LA cells. Of the immunoreactive material detected, more was associated with cells than in the medium. The apparent molecular weight of this material (predominantly 42 kD) was consistent with it being a partially processed form of PDGF LA. Secreted material was similar to that from CHO SA cells, with the 34-kD form predominant. (This material is difficult to detect in Fig. 2, and was seen only at a low level in replicate experiments.) Our estimates of relative production of PDGF SA and LA in transfected cells depend on an assumption that the antibody being used detects the LA and SA forms with equal efficiency.

Fig. 3 shows the pattern obtained when CHO B cells were examined. Moderate amounts of material reacting with antibody to PDGF BB were detected, with a variety of forms (predominantly 26 and 38 kD) found to be cell associated, while 38-kD material and a series of bands down to 30 kD were found in the medium. These can be compared with the standard PDGF BB produced in yeast (32 kD). As for Fig. 2, CHO cell extracts and medium did not react with anti-PDGF BB antibody. No antibody binding was detected in control experiments in which CHO B samples were probed with anti-PDGF AA antibody. Total PDGF BB production detected in CHO B cells was of the order of 80–120 ng of homodimer per 10^6 cells in 24 h. Our results differ from those reported by Thyberg et al. (1990), who examined the fate of metabolically labeled PDGF BB, produced in CHO cells over a 6-h labeling period. They found a higher proportion of cell-retained (although apparently not cell surface-localised) material than reported here. In experiments where we have cultured CHO B cells in the same, serum-free medium for up to 5 d before harvest, we have found a net change in the distribution of PDGF BB between cells and supernatant, with a greater proportion of immunoreactive material being found in medium after the longer culture period (Martins, R., and M. Sleigh, unpublished data). This may reflect relatively inefficient processing of PDGF BB by CHO cells, and suggests that the observed distribution between cells and supernatant (we have not investigated matrix PDGF in this context) will depend when sampling occurs.

RNA being expressed in the three transfected cell cultures was examined by RNase protection assay using total cytoplasmic RNA. Fig. 4 shows the results obtained for CHO SA and CHO LA cultures. In both cases, approximately tenfold induction of RNA resulted from heavy metal treatment of the cultures. High levels of PDGF-specific RNA were also observed in CHO B cells (results not shown), but in these cells, metal induction of the RNA was limited to fivefold.

The protected region of probe is larger in hybrids with SA than with LA RNA. This results in some loss of radioactive label from LA compared with SA RNA hybrids. After correcting for this, the level of PDGF RNA produced by CHO SA cells appeared to be no more than fivefold greater than that expressed in CHO LA cells. This difference is less than

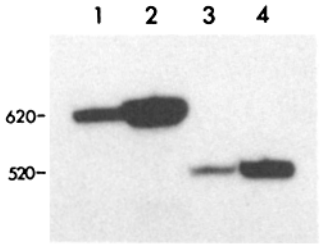


Figure 4. Expression of PDGF A RNA in CHO SA and CHO LA cells. Ribonuclease protection was used to assess PDGF RNA levels in CHO SA (lanes *1* and *2*) and CHO LA cells (lanes *3* and *4*). RNA from cells with (lanes *2* and *4*) or without (lanes *1* and *3*) metal induction was incubated with a radiolabeled probe from the SA gene as described in Materials and Methods. Amounts of RNA used were first standardized to give comparable band intensities following RNase protection assay using a GAPDH probe. After RNase digestion of the mRNA/probe hybrids, SA RNA yields a protected fragment of 620 bases and LA a fragment of 520 bases, since the LA gene diverges from the SA transcript used as a probe beyond exon 5 sequences.

would be expected from the results shown in Fig. 2, where 10–20-fold more immunoreactive PDGF was detected for CHO SA than for CHO LA cultures.

Localization by Immunofluorescence of PDGF LA and B Homodimers Made in CHO Cells

Previous published reports have suggested that in NIH3T3 and COS 7 cells, the primary site for PDGF LA localization is cellular (La Rochelle et al., 1990*a*; Ostman et al., 1991). In Fig. 2, we thus would have expected to find more PDGF LA in cell extracts than we observed. In addition, there is an apparent discrepancy between total PDGF LA protein detected relative to PDGF SA, given the ratios of RNA production in the two cell types, as discussed above. This led us to further examine the location of immunoreactive PDGF in producing cells by fluorescence microscopy. The results are shown in Fig. 5. Some immunoreactive material was evident associated with CHO SA cells (Fig. 5 *b*) as would be expected from published data and the results of Fig. 2. No material was detected in these samples at the basal level of the cells where they contact the culture dish (Fig. 5, upper right frame of *b*). For PDGF LA, on the other hand, most of the fluorescence detected was found in this plane (Fig. 5 *c*). These findings are consistent with immunoreactive material associated either with the basal surface of producing cells, or deposited into extracellular matrix. Since the material detected appeared to spread beyond the cell boundaries, the latter appears more likely. This was further explored by staining equivalent cultures for the matrix protein laminin and for PDGF A in parallel. Coverslips from PDGF LA cultures were stained with rabbit anti-mouse laminin polyclonal antibody, followed by FITC-labeled anti-rabbit antibody. These results confirm that immunofluorescent material produced by CHO LA cells has a similar localization to laminin at the cell-plate interface, suggesting a matrix localization for PDGF LA (Fig. 5, *f* and *g*).

As predicted from the Western assays (Fig. 2), and compared with the total amount of fluorescence detected for CHO LA cells, only small amounts of immunoreactive material were found associated with CHO SA and LA cells. In both cases this was located in granular regions within the cells. CHO cells containing no PDGF LA gene gave similar staining for laminin, but no staining with the PDGF antibody (not shown).

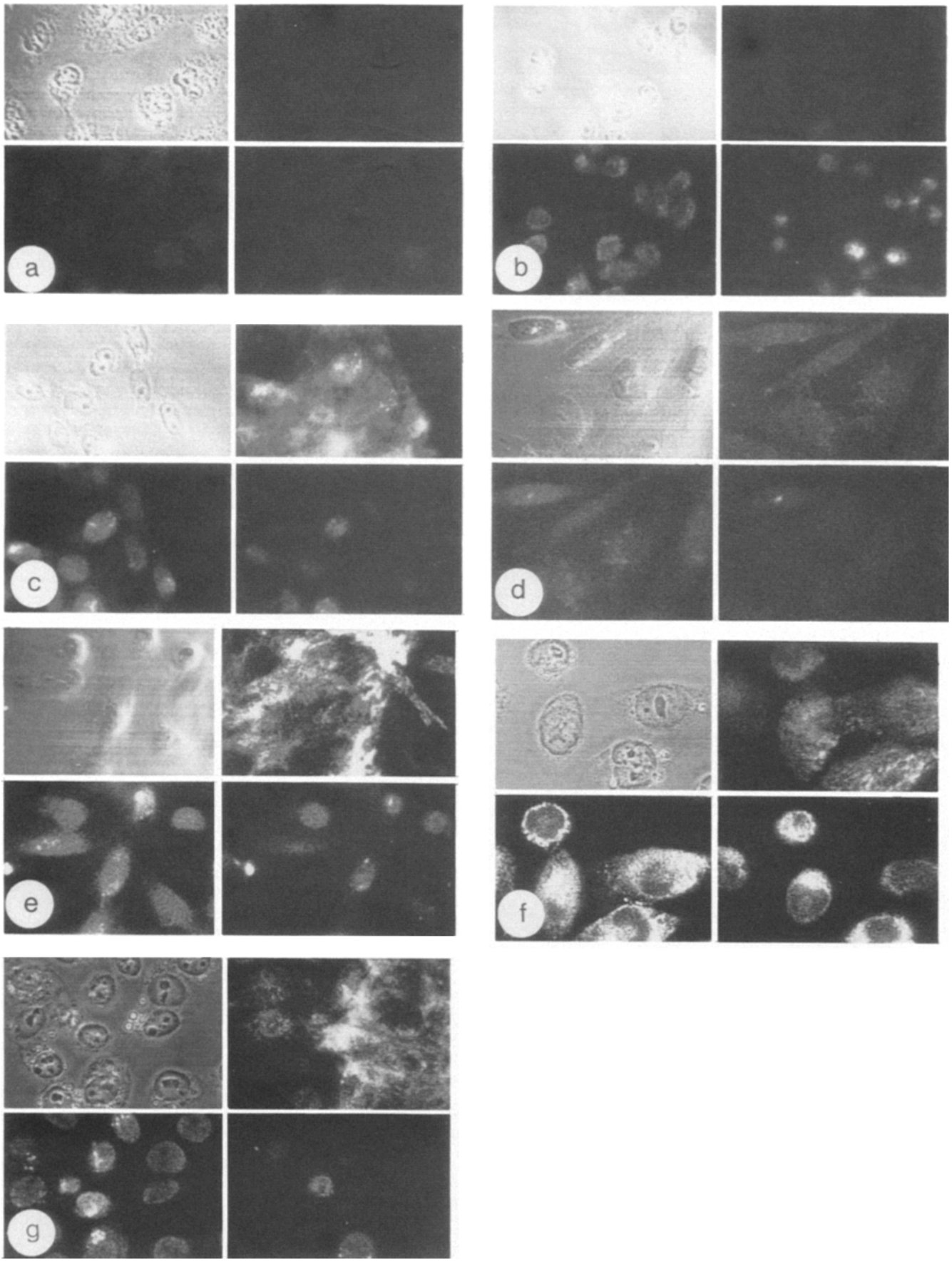
A somewhat similar pattern to that described for PDGF LA was seen for PDGF B-expressing cells (Fig. 5 *e*). The bulk of immunoreactive material detected was in a similar location to that shown for PDGF LA in Fig. 5 (*c* and *g*), presumably in or on the matrix below cells. For both PDGF LA and B, examination of transfected cells that had not undergone induction indicated similar relative distribution of fluorescence between matrix and cells as seen in induced cells. However, the levels detected were lower, as expected.

Analysis of Matrix-Associated PDGF LA

To expose underlying matrix material and examine how PDGF was bound within it, CHO LA cells were lysed by treatment with Triton X-100 and NH_4OH, as described by Bashkin et al. (1989). PDGF LA was shown by fluorescence microscopy to be retained in the attached matrix after this treatment (data not shown).

The matrix material was extracted with Laemmli sample buffer, and PDGF LA dimers present were detected by Western blotting. As shown in Fig. 6 *A*, substantial amounts of PDGF LA were observed, with sizes of homodimers in the range 48–34 kD. The major bands were 40 and 44 kD. No significant immunoreactive material of higher molecular weight was present. Little if any fully processed material (30 kD) was detected. The matrix material prepared by cell lysis with Triton and shown in Fig. 6 reveals a different although overlapping pattern of protein bands compared with material displayed from cell extracts (Fig. 2, lane *Al*). This indicates effective separation of cellular and extracellular material by the procedures used. The relative amounts of material found in the matrix extracts compared with cell extracts are also consistent with the distribution of material observed in immunofluorescence studies. Assuming that our extraction method liberates all PDGF for detection by Western blotting, the results shown in Fig. 6 indicate PDGF LA equivalent to ~120 ng of the PDGF SA standard localized in the matrix by 10^6 cells in 24 h under conditions where 20 ng of equivalent cell-associated material was detected. The total of ~700 ng produced per plate of 5×10^6 cells compared with 1–2 μg for CHO SA under equivalent conditions is consistent with the relative amounts of PDGF A mRNA produced by the two cell types (Fig. 4). As stated previously, estimation of PDGF levels involves several assumptions and is useful as a guide only.

Since the Triton method lyses cells during their removal, there is some chance that PDGF contained within cells or associated with the cell surface may contaminate the matrix during the procedure (Bashkin et al., 1989; Brunner et al., 1991). An alternative approach is the use of 2 M urea, which removes the cells intact (Gospodarowicz et al., 1983). This method was tested as a control for the Triton procedure. The urea method was not particularly effective in CHO LA cell removal using published conditions and even with more extended urea treatment, cells and cell debris remained associated with the surface. This was demonstrated by staining for actin on coverslip surfaces with rhodamine-phalloidin after removal of cells by either Triton/NH_4OH or urea treatment. Actin staining was detected on urea-generated matrices but not on those generated by Triton/NH_4OH treatment. A third approach to cell removal, use of EDTA, was completely ineffective in detaching CHO LA cells from cultured surfaces in our experiments. A comparison of the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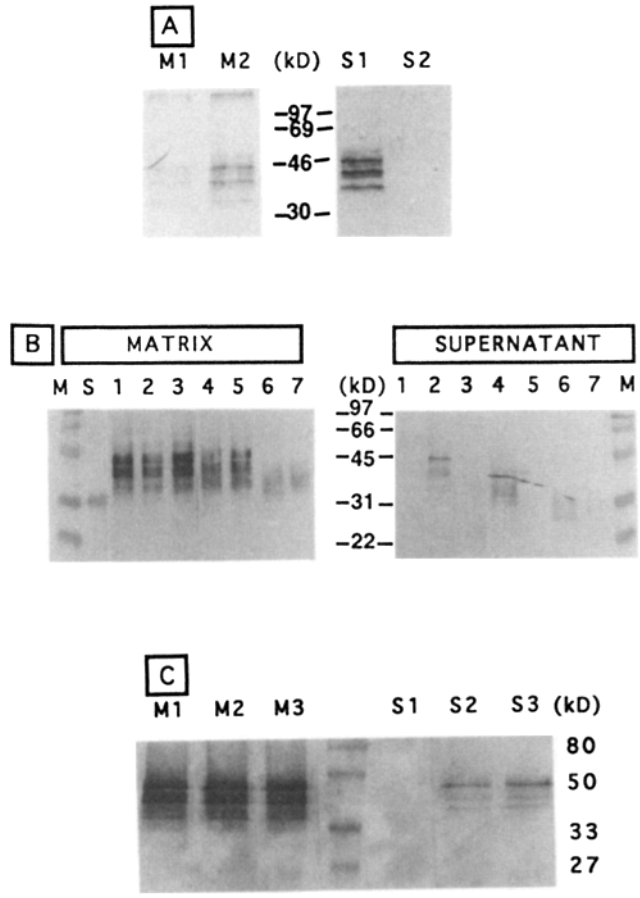

Figure 6. Release of PDGF A from the extracellular matrix prepared by Triton cell lysis. Matrix deposited by CHO LA cells was exposed by lysis of cells using the Triton procedure described in Materials and Methods. PDGF was detected by Western blotting. (*A*) Effects of heparin. Matrix was incubated (60 min, 37°C) in 2.5 ml PBS containing Ca^{2+} and Mg^{2+} with or without heparin (5 μg/ml). (Lane *Sl*) Immunoreactive PDGF A released into the supernatant by heparin. 8% of material released into the supernatant by 5 × 10^6 cells was loaded after concentration. (Lane *Ml*) Material remaining in the matrix after this treatment (5% of the total material deposited by 5 × 10^6 cells loaded). (Lanes *S2* and *M2*) PDGF A in supernatant and matrix after incubation with buffer alone. (*B*) Effect of enzymes with heparin. The left panel shows material remaining in matrix after treatment (1.7% of total from 5 × 10^6 cells loaded). The right panel shows supernatant samples (4% of total loaded). Samples shown are from two gels run in parallel. (*M*) Molecular weight markers (sizes shown in kD); (*S*) 10 ng standard PDGF AA; (*1*) no additives; (*2*) collagenase (C-0773, 100 μg/ml) + heparin (5 μg/ml); (*3*) collagenase (C-0773, 100 μg/ml); (*4*) collagenase (C-9407, 25 μg/ml) + heparin (5 μg/ml); (*5*) collagenase (C-9407, 25 μg/ml); (*6*) trypsin (50 μg/ml) + heparin (5 μg/ml); (*7*) trypsin (50 μg/ml). (*C*) Effect of chondroitin ABC lyase. Matrix was incubated (60 min, 37°C) in 2.5 ml of 0.3 M Tris HCl, pH 8.0, 18 mM Na acetate, aprotinin (100 Kallikrein inhibitor U/ml) and chondroitin ABC lyase (0, 0.03, 0.1 U/ml, shown as samples 1, 2, and 3, respectively). Material remaining in the matrix (*M*) or released into the supernatant (*S*) was measured by Western blotting. M samples represent 7% and S samples 8% of total material from a plate of 5 × 10^6 cells.

PDGF content of matrix exposed by the urea and Triton procedures indicated no significant differences in immunoreactive material (amount or sizes) detected by Western blotting. The Triton procedure was therefore used in all experiments for exposing extracellular matrix.

Mode of Attachment of PDGF LA in the Matrix

Studies on the differences of fate between PDGF SA and PDGF LA have suggested that the basic COOH-terminal extension in PDGF LA is responsible for cell attachment of this species (LaRochelle et al., 1990*a*,*b*; Ostman et al., 1991). Attachment to cellular heparan sulphate proteoglycan has been proposed for the COOH-terminal peptide, and it was implied that matrix attachment of the LA species might be mediated in the same way (Raines and Ross, 1992; Khachigian and Chesterman, 1992). The role of heparan sulphate binding in PDGF matrix attachment was tested by incubating PDGF LA–containing matrix exposed by Triton lysis either with an excess of heparin (Sigma Immunochemicals; 5 μg/ml) as a binding competitor, or heparitinase (0.1 U/ml; EC 4.2.2.8; Sigma Immunochemicals), which should digest heparan sulphate and liberate carbohydrate-bound material (Bashkin et al., 1989).

Heparin caused release of considerable amounts of immunoreactive material into solution (Fig. 6 *A*), with a corresponding decrease in the amount of material remaining at the plate surface. Released material was of similar size to that detected in the matrix, i.e., 34–45 kD. Heparitinase generally induced no release of PDGF (results not shown) although in some experiments, a very small amount of material, running at 40 kD and above, was detectable in the supernatant. In separate experiments (not shown) it was demonstrated that the heparitinase treatment used in Fig. 6 could release 20% of incorporated ^{35}S-labeled sulphate from matrix into the medium during the incubation period. Further experiments, in which the heparitinase concentration was increased up to 50-fold, did not result in any greater loss of PDGF A from the matrix (results not shown).

Previous studies examining attachment of bFGF in extracellular matrix have suggested that this growth factor is laid down during matrix deposition and that only surface material may be accessible to enzymes or other competitors of binding (Vlodavsky et al., 1991). We used bacterial collagenase to partially degrade the matrix in an attempt to in-

Figure 5. Immunolocalization of PDGF A and B in expressing CHO cells. The cell and matrix distribution of PDGF B, SA, and LA produced in transfected CHO cells was examined by laser confocal microscopy as described in Materials and Methods. The distribution of laminin was included as a control (*f*). For each panel, *a–g*, the top left box shows the phase contrast view of the cells, while top right, bottom left and bottom right show progressive scanning for fluorescence due to immunoreactive material from the level of cell–plate contact through to the top of the cells. (*a*) Untransfected CHO cells; (*b*) CHO-SA; (*c*) CHO-LA, all using anti-PDGF AA antibody. (*d*) Untransfected CHO cells; (*e*) CHO-B cells, both using first antibody directed against PDGF-BB; (*f*) CHO LA cells using anti-laminin antibody; (*g*) CHO LA cells tested in parallel, using anti-PDGF AA antibody. All cells pictured have undergone metal induction as described in Materials and Methods.

crease the accessibility of matrix-bound growth factor to other agents. Preliminary experiments (not shown) were carried out to measure the effects of matrix exposure to increasing amounts of collagenase (C-9407, tissue culture grade, 1.6 U/μg, containing 0.1% contaminating protease; Sigma Immunochemicals). Only at the highest levels (25–50 μg/ml) was immunoreactive PDGF A detectable in the supernatant in the absence of heparin, with no significant loss of total PDGF from the matrix under these conditions. The PDGF released appeared from this and other experiments to be smaller than bound forms, as identified on nonreducing gels, with 30–34 kD material predominating in the supernatant.

Collagenase treatment did not increase the amount of PDGF LA released from the matrix by heparin, but it did reduce the size of the material released, from 35–46 kD, to 30–35 kD (Fig. 6, compare *A*, lane *S1*, with *B*, lane S4). This cleavage of immunoreactive species appeared to be due to contaminating protease in the collagenase preparation. A more highly purified collagenase (C-0773; Sigma Immunochemicals) did not cause the same cleavage of heparin-released material (Fig. 6 *B*, lane *2*). As well, the degrading effects of crude collagenase were inhibited by 2 mM EDTA (an inhibitor of metalloproteinases including collagenase; Seifter and Harper, 1971) but not by the more general protease inhibitor PMSF, tested at 0.5 mM (results not shown). This suggests that the active component in the collagenase preparation may be a contaminating metalloproteinase. Treatment of PDGF LA–containing matrix with trypsin was able to directly liberate PDGF, as described previously for bFGF (Saksela and Rifkin, 1990). Cleavage products of similar size to those seen with collagenase plus heparin were produced early, but longer treatment with trypsin led to degradation and disappearance of immunoreactive PDGF from both matrix and supernatant in the presence or absence of heparin, as shown in Fig. 6 *B*. Taken together these results suggest that the release by proteases shown in Fig. 6 is likely to be due to degradation of the PDGF itself, with potential cleavage sites becoming available principally after dissociation of PDGF from the matrix by heparin.

The mode of attachment of PDGF LA was further tested by incubating Triton-generated matrix with chondroitin ABC lyase (EC 4.2.2.4; Sigma Immunochemicals). This treatment was effective in liberating a small proportion of attached PDGF LA. Little increase in release was seen with increased amounts of enzyme (Fig. 6 *C*). These results suggest a role for the glycosaminoglycan moieties of the chondroitin sulphate or dermatan types in attachment of PDGF LA. Competition studies with chondroitin sulphate A, B (dermatan sulphate), C, and keratan sulphate revealed that none of these glycosaminoglycans liberated detectable amounts of PDGF from the matrix (results not shown) under conditions where heparin (as shown in Fig. 6 *A*) released up to 50% of bound material.

Matrix Association of PDGF B

Immunofluorescence studies shown in Fig. 5 indicated that in CHO B cultures considerable amounts of PDGF were deposited into matrix material below cells. Material remaining on the plate after Triton lysis was examined using similar approaches to those outlined above for PDGF LA. Fig. 7, matrix lane *1* shows material specifically reactive with an antibody directed against PDGF B homodimer, following extraction of Triton-generated matrix deposited by CHO B cells. The major polypeptides observed were of 31, 35, and 38 kD. These can be compared with a major cell-retained species of 38 kD, as shown in Fig. 3 (minor species of smaller size were also observed here). Incubation of CHO B matrix with heparin (Fig. 7, lane *5*) released material of similar sizes, i.e., 38, 34, and 30 kD, with a minor species of 48 kD. When heparin and crude collagenase treatments were combined, released forms were reduced to 26 and 30 kD, with no overall increase in immunoreactive material released. This is likely to reflect cleavage of released PDGF by contaminating proteases in the collagenase, as discussed above. None of the treatments appeared to decrease significantly the total levels of PDGF B detected in the matrix, indicating incomplete extraction of the growth factor deposited by these cell cultures. A minimum of 80 ng of deposited material was detected per 10^6 cells over two days in these experiments, with the actual amount in the matrix almost certainly higher. This suggests that matrix-bound material represents at least 40% of the PDGF B made by CHO B cells, under the culture conditions described.

In these experiments, there was some leakage of material (38 kD) from Triton-prepared matrix incubated in PBS,

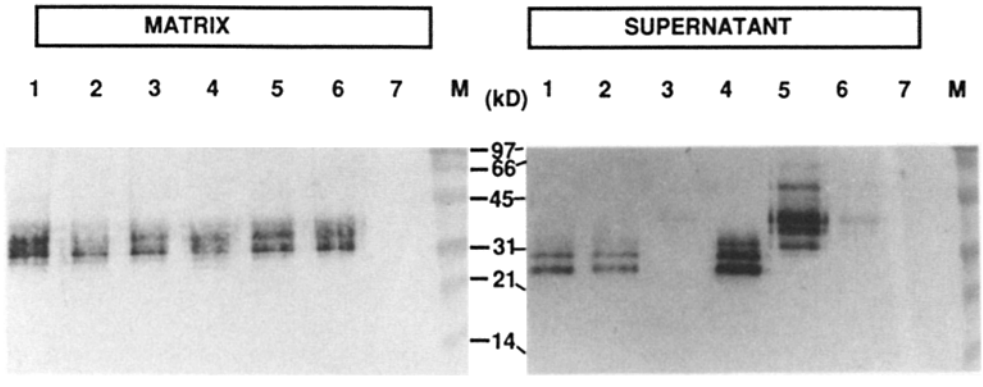

Figure 7. Release of PDGF B from extracellular matrix prepared by Triton lysis of cells. Matrix deposited by CHO-B cells was exposed by the Triton procedure and incubated as described for Fig. 6 except the following additions were made: (lane *1*) collagenase (C-9407, 50 μg/ml); (lane *2*) collagenase (C-9407, 50 μg/ml) + heparitinase (0.1 U/ml); (lane *3*) heparitinase (0.1 U/ml); (lane *4*) heparin (5 μg/ml) + collagenase (C-9407, 50 μg/ml); (lane *5*) heparin (5 μg/ml); (lane *6*) no additives; (lane *7*) matrix from CHO cells, no additives. (*M*) molecular weight markers, sizes in kD. Residual matrix samples (*left*) were 10 μl, representing 3.3% of the total matrix per 5×10^6 cells; supernatant samples (*right*) were 10 μl (10× concentrated) representing 4% of the total supernatant from the same plate.

without other additions (Fig. 7, lane 6). As for PDGF LA, it appears from these results that PDGF B deposited into the matrix by expressing cells can be partially released from its matrix attachment by addition of heparin, but not by heparitinase. Other glycosaminoglycans (chondroitin sulphate A, C, dermatan sulphate, and keratan sulphate) were unable to displace significant amounts of PDGF B from the matrix at concentrations where heparin was effective in releasing bound material (results not shown).

Discussion

Since the genes encoding PDGF B and the two forms of PDGF A were first isolated there has been considerable speculation on the relative biological roles of the three polypeptides they encode. The identification of two PDGF receptor subunits (PDGFR α and β) has revealed one level at which PDGF action may be controlled. Differential expression of the two receptor types may change the response of the cells to exogenous PDGF since the α receptor binds both A and B chains of PDGF, while the β receptor recognizes only the B form (Matsui et al., 1989; Hart et al., 1988; Heldin et al., 1988).

The expression of recombinant PDGF A and B homodimers has led to greater understanding of their relative biological properties, with PDGF B demonstrating greater transforming activity for most target cells and A being more readily processed for secretion (Beckmann et al., 1988; LaRochelle et al., 1990*b*). Initial work with the product of the exon 6-containing PDGF A mRNA–PDGF LA–suggested that it may be reduced in its capacity for processing and secretion from cells compared with SA. More recently, it has been shown that the additional basic COOH-terminal sequence present in this form of PDGF A is associated with retention of the polypeptide by the cell (LaRochelle et al., 1990*a*,*b*; Ostman et al., 1991). A peptide identical to this COOH-terminal region is able to compete with and displace attached forms of PDGF and other growth factors (Khachigian et al., 1992; Raines and Ross, 1992) although at least in the former study, very high concentrations of peptide were required.

PDGF LA appears to be produced in most or all PDGF A-synthesizing cells, and to make up no more than 5–10% of the total PDGF A produced (Matoskova et al., 1989; Young et al., 1990; Sánchez, A., C. Chesterman and M. Sleigh, manuscript in preparation). This would suggest that this moiety has little relative biological relevance, except in circumstances where it may accumulate to high levels. This could occur by retention and concentration of active material within cells, at the cell surface (LaRochelle et al., 1990*a*; Ostman et al., 1991) or in an extracellular depot such as the matrix. The results described in this paper exemplify this last possibility. As suggested (Raines and Ross, 1992; Khachigian and Chesterman, 1992), progressive deposition of PDGF LA into subcellular matrix (and also PDGF B, from results presented here) provides the potential for substantial amounts of growth factor to be held in preformed storage pools. This deposited growth factor is available for subsequent mobilization, possibly by the release of appropriate enzymes from damaged or otherwise activated cells.

The Triton/NH_4OH method used here to lyse cells and expose subcellular matrix may result in residual cell membrane material or material bridging cells and matrix remaining attached to the matrix through subsequent washing. Alternatively, material released from cells during lysis may become matrix-associated during the procedure. A further difficulty in clearly separating matrix and cellular material for study comes from the possible inclusion of some residual matrix or bridging material in the scraped cell samples. What evidence supports the idea that PDGF is deposited into matrix as it is synthesized?

Firstly, both the quantities (for PDGF LA) and size distributions (for both LA and B) of species detected in the matrix samples compared with cell extracts indicate that the procedures we have used have been largely successful in separating matrix and cellular material. Secondly, the laser confocal microscope studies on PDGF LA cells shown in Fig. 5 indicate a PDGF distribution spreading beyond the boundaries of the producing cells, to an even greater extent than that of the matrix protein laminin. The relative distributions of immunoreactive material between cells and underlying material seen by microscopy are consistent with the results from Western analysis. Since a similar localization was found for PDGF LA and the matrix protein laminin, it is highly likely that material examined in Triton cell culture extracts and visualized by fluorescence studies has matrix localization. Finally, we have examined release of PDGF from our matrix preparation by salt and have found that less than 20% of deposited PDGF B (and a similar amount of PDGF A) is released by NaCl concentrations as high as 2 M (Vandermark, S., C. Chesterman and M. Sleigh, unpublished results). Since PDGF and other growth factors are released from heparin–sepharose columns by much lower NaCl concentrations, our results indicate that PDGF is embedded within the matrix and possibly interacting with other matrix components rather than exposed at the surface (Vlodavsky et al., 1987).

Likely parallels to PDGF LA and B matrix attachment are retention of thrombin, bFGF, and TGFβ in matrix and at the cell surface through binding to proteoglycans (Bar-Shavit et al., 1989; Vlodavsky et al., 1991; Andres et al., 1992). The characteristics of binding are different in each case, with attachment primarily through the glycosaminoglycans in some cases (bFGF, thrombin) and through the proteoglycan protein cores in others (TGFβ) (Bar-Shavit et al., 1989; Wight et al., 1992).

It has been proposed previously that the 18 amino acid basic COOH-terminal peptide of PDGF LA was attached at cell surfaces (and perhaps also to matrix) through association with the heparan sulphate moieties of heparan sulphate proteoglycans (Raines and Ross, 1992; Khachigian et al., 1992). This was supported by release of the peptide with heparitinase and competition by heparin for its binding. In our studies with PDGF polypeptides synthesized by CHO cells, we have found heparitinase to be ineffective in releasing bound growth factor, indicating an association in the matrix that is more complex than attachment to heparan sulphate alone.

Some bound PDGF LA was released from matrix preparations by chondroitin ABC lyase. Chondroitin sulphates were ineffective although the more highly charged heparin was very effective in displacing PDGF from its attachment sites. Our results raise the possibility that while part of the attachment of PDGF LA and B involves chondroitin or dermatan sulphate residues, the binding to these moieties is stabilized

by other interactions which can be effectively blocked by the charge neutralizing effect of heparin (Kjellén and Lindahl, 1991). Thus, PDGF appears to share some features of cell surface/matrix attachment with thrombin and bFGF and perhaps also TGFβ.

For bFGF, low affinity binding to proteoglycans is required for growth factor activity, but this seems unlikely to be the case for PDGF. PDGF SA, with no apparent receptor-independent cell binding activity, has a somewhat higher affinity for the PDGF receptor than PDGF LA and has accordingly greater biological effects (Ostman et al., 1989). In addition Rapraeger et al. (1991) have shown that sulphated heparan is not essential for receptor-mediated activity of PDGF, unlike the situation for bFGF (Yayon et al., 1991). This suggests that at least for B-containing forms of PDGF (PDGF LA was not examined in these studies), surface attachment is not a prerequisite for high affinity binding in the same way that it is for bFGF. The significance of the occurrence of cell-binding forms of PDGF B and presumably also LA, may thus depend more on their ability to accumulate in the matrix than on their cell attachment properties. Zerwes and Risau (1987) have described preferential secretion of PDGF from the basal surface of endothelial cells. While the PDGF examined in their experiments was apparently of the soluble rather the cell-binding form, such directional secretion could assist in establishing extracellular stores of PDGF in vivo.

Previous studies (Khachigian et al., 1992; Khachigian and Chesterman, 1992; Raines and Ross, 1992) have pointed to overlapping mechanisms of attachment of PDGF species in the matrix and at the cell surface. In 3T3 and COS cells, a higher proportion of PDGF expressed from introduced genes was found to be cell associated (LaRochelle et al., 1991; Ostman et al., 1991) than has been detected for CHO cells (this work and Ostman et al., 1988). Since in all of these studies there is considerable overlap in the sizes of PDGF species found to be cell associated and reported to be matrix associated, it seems unlikely that differential processing is responsible for these localization effects. A possible explanation may be differences between cell types in the amounts and distribution of proteoglycans and other molecules involved in PDGF sequestration. Further detailed work on the identity of PDGF-binding matrix and cell surface molecules will be needed to clarify this point.

The CHO and other recombinant cells used to study PDGF distribution all express PDGF at higher levels than do normal producers, e.g., endothelial and vascular smooth muscle cells. Examination of the distribution of PDGF species made by normal producer cell types will be important to assess the true importance of matrix deposition of PDGF. Of particular interest will be to examine localization of PDGF in human arteries. Wren et al. (1986) reported that vascular smooth muscle cells responded to mitogenic and chemotactic factors deposited onto the culture dish by vascular endothelial and smooth muscle cells. In circumstances such as balloon angioplasty of arteries, damaged cells release enzymes such as proteases or heparanases (Godder et al., 1991; Saksela and Rifkin, 1990) which could cleave attached PDGF and other growth factors from the matrix. This may be an important step in the subsequent activation of vascular smooth muscle cells for migration and proliferation which contributes to restenosis at the site of damage.

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