Induction of Platelet-derived Growth Factor Receptor Expression in Smooth Muscle Cells and Fibroblasts Upon Tissue Culturing

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Abstract. The expression of platelet-derived growth factor (PDGF) receptors in porcine uterus and human skin in situ, was compared with that of cultured primary cells isolated from the same tissues. PDGF receptor expression was examined by monoclonal antibodies specific for the B type PDGF receptor and by RNA/RNA in situ hybridization with a probe constructed from a cDNA clone encoding the B type PDGF receptor. In porcine uterus tissue both mRNA and the protein product for the PDGF receptor were detected in the endometrium; the myometrium, in contrast, contained much lower amounts. Moreover, freshly isolated myometrial cells were devoid of PDGF receptors. However, after 1 d in culture receptors appeared, and after 2 wk of culturing essentially all of the myometrial cells stained positively with the anti-PDGF receptor antibodies and contained PDGF receptor mRNA. Similarly, B type PDGF receptors were not detected in normal human skin, but fibroblast-like cells from explant cultures of human skin possessed PDGF receptors. When determined by immunoblotting, porcine uterus myometrial membranes contained ~20% of the PDGF receptor antigen compared with the amount found in endometrial membranes. In addition, PDGF stimulated the phosphorylation of a 175-kD component, most likely representing autophosphorylation of the B type PDGF receptor in endometrial membranes, whereas only a marginal phosphorylation was seen in myometrial membranes. Taken together, these results demonstrate that PDGF receptor expression varies in normal tissues and that fibroblasts and smooth muscle cells do not uniformly express the receptor in situ. Furthermore, fibroblasts and smooth muscle cells that are released from tissues are induced to express PDGF receptors in response to cell culturing. The data suggest that, in addition to the availability of the ligand, PDGF-mediated cell growth in vivo is dependent on factors regulating expression of the receptor.

PLATELET-derived growth factor (PDGF)[1] is a major mitogen in serum for cultured mesenchymal cells. PDGF is a cationic protein of 30 kD which consists of two polypeptide chains, denoted A and B, interconnected by disulfide bridges (see references 18 and 34 for reviews). The two polypeptide chains are ~60% homologous to each other, and can be combined as homo- or heterodimers. Thus, three dimeric forms of PDGF exist, PDGF-AA, -AB, and -BB, with different functional activities. This is probably due to different specificities in binding to two distinct PDGF receptor classes, denoted type A and type B, that are present on the cell surface of responsive cells (14, 20, 30).

The A type PDGF receptor, which binds all dimeric forms of PDGF, has not been characterized. The B type receptor, which binds PDGF-BB and, with lower affinity, PDGF-AB, has been purified as a 170–185-kD component (1, 8, 32) with a protein tyrosine kinase activity associated with its cytoplasmic domain (reviewed in reference 15). The B type receptor seems to be responsible for a major part of the mitogenic effect of PDGF-like factors on human fibroblasts (20). The amino acid sequence of this receptor has been deduced from cDNA clones (5, 13, 38) and shows homology to other tyrosine kinases, especially those encoded by the protooncogene c-*kit* (39) and the receptor for colony stimulating factor-1 (7). Binding of PDGF to the receptor activates the tyrosine kinase domain and leads to enhanced phosphorylation of intracellular substrates (6, 9, 11, 27), as well as to autophosphorylation of the receptor (9–11, 29, 31).

The demonstration of high affinity receptors for PDGF, through use of ^{125}I-labeled PDGF have been limited to cultured cells of connective tissue origin (16). These include

1. *Abbreviations used in this paper*: PDGF, platelet-derived growth factor; PS, streptomycin.

dermal and tendon fibroblasts, vascular smooth muscle cells, glial cells, and chondrocytes (reviewed in reference 3). Studies on the expression of PDGF receptors in vivo have been hindered by the lack of an appropriate method to identify PDGF receptors in tissues. Consequently it is not known on which cell types, or under what conditions, PDGF receptors are expressed in the intact organism. Recently, we reported the development of mAbs to the B type PDGF receptor isolated from porcine uterus (33). These antibodies have been shown to recognize the external domain of the B type PDGF receptor in cultured cells (33), and to be useful for immunohistochemical detection of the receptor in rheumatoid arthritic synovium (35). In these initial studies it was observed that the B type PDGF receptor was not detectable in the synovium of healthy subjects, but was induced in conjunction with inflammation. In this report, we show, by use of immunohistochemical staining and in situ hybridization, that B type PDGF receptors are present at very low or undetectable levels in normal human dermis and porcine myometrium and that the level of functional receptor rises as cells isolated from these tissues are propagated in culture.

Materials and Methods

Tissues

Swine uterus was obtained from a meat packing plant immediately after the death of the animal. Human skin was obtained from healthy subjects undergoing elective mammary plastic surgery. Biopsies of both tissues were taken and immediately frozen in liquid isopentane at −150°C and stored at −70°C until cryosectioned (25). The remainder of the tissue was used for tissue culture experiments (see below).

Cell Isolation and Tissue Culture

Smooth Muscle Cells. Swine uterus was transported to the laboratory in PBS containing 100 U/ml penicillin, 100 μg/ml streptomycin (PS) on ice. The outer layer of smooth muscle was stripped from the uterus and minced into small pieces (1 mm^3). Similarly, endometrial folds were cut free with scissors and minced into small pieces. The minced tissues were digested with 800 U/ml collagenase type I and DNase type II (Sigma Chemical Co., St. Louis, MO), dissolved in DME containing 10% FBS and PS for 30 min at 37°C on a reciprocating shaker. After 30 min the dislodged cells were harvested, washed with DME-FBS-PS, resuspended in the same medium, and stored on ice for further use. Fresh enzyme was added to the undigested tissue and the digestion continued for 30 min. The cycle of digestion and harvesting was repeated at least five times. The isolated cells were filtered through 125-μm mesh nylon screen and plated on 22 × 22-mm fibronectin-coated coverslips for immunofluorescence, on 76 × 26-mm glass slides for in situ hybridization, or T-25 flasks (Falcon Labware, Oxnard, CA) for long term culture in DME-FBS-PS. The cultures were fed every other day with fresh DME-FBS-PS.

Human Skin Fibroblasts. Small pieces of dermis (1–2 mm^3) were explanted to 60-mm culture dishes (Corning Glass Works, Corning, NY) and fed with DME-FBS-PS. After 7–10 d in culture, outgrown fibroblast-like cells were harvested with 0.1% trypsin, 0.1% EDTA in PBS and plated onto 22 × 22-mm^2 coverslips, glass microscope slides, or into T-25 flasks and fed with DME-FBS-PS.

Antibodies

The characteristics and specificity of the two mouse mAbs against the B type PDGF receptor (PDGFR-B1, PDGFR-B2) used in this study have been described previously (33). PDGFR-B2 does not recognize the human A type PDGF receptor (20). The rabbit polyclonal antisera, PDGFR-1, was made against purified porcine uterus PDGF receptor (32), and PDGFR-3 was made against amino acids 981–984 of the mouse receptor sequence (38). PDGFR-3 specifically recognizes PDGF B type receptors, whereas PDGFR-1 recognizes both A and B type PDGF receptors (5a). The muscle specific actin mAb (HHF-35) was from Enzo Biochemical, Inc. (New York, NY) and a mAb specifically recognizing vimentin intermediate filaments from Labsystems (Helsinki, Finland).

Immunohistochemical Staining Procedure

Frozen sections from swine uterus and human skin biopsies were prepared as previously described (25). Briefly, the sections were fixed in acetone, exhausted of endogenous peroxidase and subsequently subjected to immunohistochemical staining using mAbs and the ABC-technique (22, 23). Control stainings were performed with an irrelevant parathyroid reactive mAb (24) of the same isotype and originating from the same mouse strain as the PDGFR-B1 antibody, or by omitting the respective primary antibodies. Control stainings were always negative.

Immunofluorescence Staining for B Type PDGF Receptors

Coverslips with cultured cells or freshly adhered cells were washed three times for 5 min each with DME and incubated for 60 min at 4°C with 50 μg/ml of receptor mAb PDGFR-B2 in DME. The coverslips were washed three times for 5 min each with DME and then incubated with 1:50 dilution of FITC-labeled goat-anti-mouse IgG in DME for 60 min at 4°C. After washing as above, the coverslips were mounted on slides in DME and viewed immediately in a Nikon microscope equipped with epifluorescence. The slides were kept at 4°C before viewing and photography, then warmed to 37°C for 30 min and examined and photographed again. Control experiments included the following: (*a*) substitution of unrelated mAbs for PDGF receptor antibody PDGFR-B2, (*b*) substitution of PBS for antibodies, and (*c*) down-regulation of PDGF receptors by preincubation of cells with 150 ng/ml of PDGF (purified from human platelets as described [19] and containing 70% PDGF-AB and 30% PDGF-BB) for 30 min at 37°C before incubation with PDGF receptor monoclonals. Previously we have demonstrated the efficiency of the absorption of the PDGF receptor monoclonal PDGFR-B2 with an excess of B type PDGF receptor before use (33).

In Situ Hybridization

Slide Preparation. Glass microscope slides were washed in water and then in 96% ethanol, dried, and autoclaved two times before use for culturing of cells. The cultured cells were washed three times with PBS before fixation. Cryostat sections (6 μm) were mounted on slides previously coated with 100 μg/ml of poly-L-lysine (Sigma Chemical Co.) and allowed to air dry for 5 min. The cultured cells and sections were then fixed in 4% paraformaldehyde in PBS for 5 min and stored until used in 70% ethanol.

Construction and Labeling of Probe for In Situ Hybridization. A Pst I fragment of a cDNA corresponding to the aminoterminal part of the B type human PDGF receptor (5) was subcloned into p GEM blue (Promega Biotec, Madison, WI) linearized, and used as a template for RNA probe. In a control experiment on endothelial cells, a 2.7-kb Bam HI fragment of a human PDGF B-chain cDNA originating from a glioma cell line and covering the whole translated sequence, the whole 3′ untranslated sequence, and part of the 5′ untranslated sequence. The ^{35}S-labeled probe was transcribed in vitro using the T7 promotor, as previously described (12).

Hybridization. In situ hybridization was performed essentially as described before (12). 20 μl of the probe mixture (0.5–1 ng labeled RNA probe in 50% formamide, 10% dextran, 10 mM dithiotreitol [DTT], 1 mg/ml *E. coli* tRNA, 1 mg/ml sheared herring sperm DNA, 2 mg/ml nuclease-free BSA in 2× SSC) (1× SSC is 0.15 M NaCl/7.5 mM sodium citrate) was used to hybridize the cells or cryostat tissue sections. Hybridization was carried out for 3 h at 52°C.

The hybridization signals were detected by autoradiography using liquid emulsion (NTB-2, Eastman Kodak Co., Rochester, NY), as described (12). The slides of cultured cells were either stained with May-Grünewald-Giemsa or viewed with phase-contrast objectives. The tissue sections were stained with hematoxylin and eosin.

Controls included cells positive and negative for PDGF receptor (human foreskin fibroblasts type AG1523; American Type Culture Collection, Rockville, MD) and porcine aortic endothelial cells, respectively, pretreatment of the slides with RNase before hybridization, the use of a ^{35}S-labeled sense-strand RNA probe transcribed from a human B-chain cDNA containing plasmid, and competition with an excess of nonradioactive antisense PDGF-receptor probe.

Quantitation of Silver Grains. The density of silver grains in the autoradiograms was determined by an Image-1/AT Digital Image Processing System (Universal Image Corp., Ithaca, NY) interfaced with a MTI SIT 66

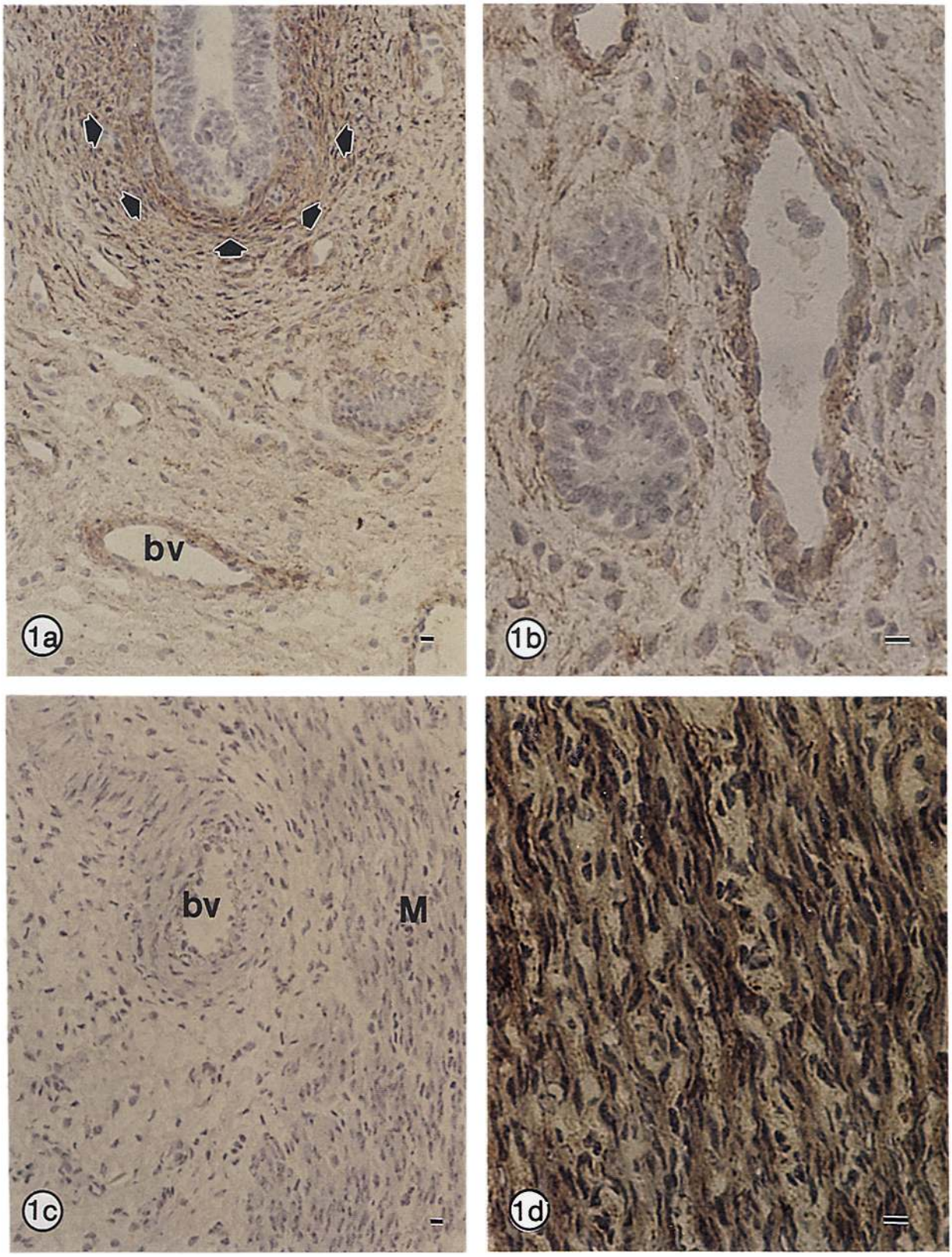

Figure 1. Distribution of B type PDGF receptor in various parts of porcine uterus as detected by immunohistochemical staining. (*a*) A section of endometrium stained with PDGFR-B2 antibodies (50 μg/mL) showing the golden brown reaction product indicative of B type PDGF receptor on the stromal cells surrounding an endometrial gland (*arrows*). The stromal cells of the loose connective tissue also contain some reaction product as do the walls of small blood vessels (*bv*). (*b*) A higher magnification of an endometrial blood vessel (analogous to the coiled arteries in the humans) showing reaction product in the wall of the blood vessel. Scattered stromal cells also contain reaction product of the B type PDGF receptor. (*c*) A section of myometrium and larger blood vessel (*bv*) showing the lack of B type PDGF receptor staining in the wall of the blood vessel and in the myometrial smooth muscle. (*d*) Myometrium from an actively growing pig (8 wk of age) showing reaction product on what appears to be myometrial smooth muscle cells. Bars, 10 μm.

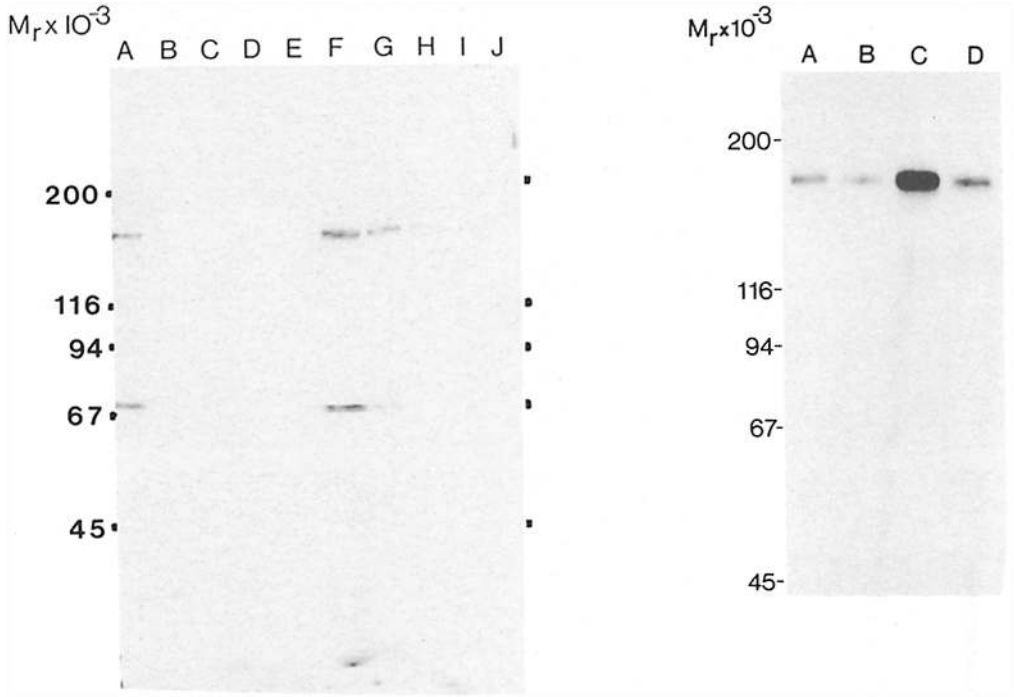

Figure 3. Autophosphorylation of B type PDGF receptors in membrane preparations. Membranes prepared from porcine uterus myometrium (lanes *A* and *B*) and endometrium (lanes *C* and *D*) were incubated with [^{32}P]ATP in the absence (lanes *B* and *D*) or presence (lanes *A* and *C*) of PDGF. Samples were analyzed by SDS–gel electrophoresis and autoradiography.

Figure 2. Quantitative determination of PDGF receptor amounts in porcine uterus myometrial and endometrial membranes. Crude membrane proteins in serial dilutions from the two regions of the uterus were subjected to immunoblotting using a rabbit PDGF receptor polyclonal antiserum (PDGFR-1). Bound rabbit IgG was detected by incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase. (Lanes *A–E*) Myometrial membranes; and (lanes *F–J*) endometrial membranes. (Lanes *A* and *F*) 100 μg protein; (*B* and *G*) 30; (*C* and *H*) 10; (*D* and *I*) 3; and (*E* and *J*) 1 μg.

Video Camera (DAGE-MTI, Inc. Michigan City, IN) and a photomicroscope (model IM-35; Carl Zeiss, Inc., Thornwood, NY) using the procedure recommended by the manufacturer. Briefly, digital images of the autoradiograms are captured with the SIT camera and stored in the computer. Intensity levels were set so that the size and density of the silver grains could be detected and the number of silver grains per unit area was determined. Background levels of silver grains on control slides (nonsense probe or RNase pretreatment) were subtracted from the density found on the experimental slides. Data was expressed as silver grains/100 μm^2 ± SEM.

Preparation of Membranes

Preparation of membranes from swine endometrial and myometrial tissues were done essentially as described (32). Membranes were stored at −20°C in 20 mM Hepes, 1 mM EGTA, 1 mM DTT, pH 7.4, before use.

Autophosphorylation Assay

Membranes (1 mg) from each source were solubilized in 2.5% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 20 mM Hepes, 1 mM EGTA, 1 mM DTT, pH 7.4, in a total volume of 500 μl, on ice for 30 min. The solubilizate was then spun in an Eppendorf Microfuge at 14,000 rpm for 10 min. The supernatant was incubated in 50-μl aliquots with 3 mM $MnCl_2$, 25 μM Na_3VO_4, in the presence or absence of 2.5 μg/ml of PDGF for 10 min on ice. The kinase reaction was initiated by addition of 15 μM [^{32}P]ATP (5 × 10^6 dpm). After 10 min on ice, the kinase reaction was stopped by adding 15 mM ATP and 40 mM phenylphosphate. The samples were given 3 μl of rabbit anti-PDGF receptor antiserum (PDGFR-1) and incubated for 2 h on ice. Subsequently 50 μl of a 1:1 slurry of Protein A–Sepharose was added and the samples were incubated for 30 min on ice with occasional shaking of the tubes. At the end of the incubation, the beads were spun down and washed five times with 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 0.15 M NaCl, 20 mM Hepes, 10 mM NaF, 10 mM EDTA, 30 mM $Na_4P_2O_7$, pH 7.4, once with 0.2% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 1 mM EGTA, 0.5 M NaCl, 20 mM Hepes, pH 7.4, and finally with 20 mM Hepes, pH 7.4. The beads were then heated at 95°C for 3 min in the presence of 50 μl of reducing SDS-sample buffer, alkylated, and subjected to PAGE in SDS using a 5–10% gradient gel and the buffer system described by Blobel and Dobberstein (2).

Immunoblotting

Membranes from the tissues and cultured cells were subjected to SDS-gel electrophoresis, using a 5–10% gradient gel (2). After electrophoresis, proteins were electrophoretically transferred to nitrocellulose sheets (membrane filters, BA 83, 0.2 μm, Schleicher & Schuell, Dassel, BRD), as described (4). After transfer, the nitrocellulose papers were blocked by incubation in 5% BSA, 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, overnight at 4°C. The sheets were incubated with PDGF receptor antisera (PDGFR-1 or PDGFR-3; diluted 1:50 in the blocking buffer) for 1 h at room temperature. The blots were washed twice for 5 min in 0.15 M NaCl, 0.01 M Tris, pH 7.4, twice in the same buffer supplemented with 0.05% Triton X-100, and finally three times for 5 min with Triton-free wash buffer. The blots were incubated for 30 min with a 1:1,000 dilution of alkaline phosphatase–conjugated swine anti-rabbit immunoglobulins (DAKOPATS, Glostrup, Denmark) and a 1:1,000 dilution of alkaline phosphatase conjugated to avidin (DAKOPATS) in 0.1% BSA, 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4. The blots were washed as above and developed using a 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.) as substrate (22). Biotinylated molecular weight standards were visualized after incubation with alkaline phosphatase conjugated to avidin and developed as above.

Results

Immunohistochemical Staining of Frozen Sections

Porcine uterus has been identified as a rich source of PDGF receptor (32). Staining of sections of adult porcine uterus with PDGFR-B1 and PDGFR-B2 antibodies demonstrated that the immunoreactivity was principally located on stromal cells adjacent to the endometrial glands (Fig. 1, *a* and *b*). Small blood vessels (analogous to coiled arteries in human) located deeper in the endometrium were also positive with these antibodies (Fig. 1, *a* and *b*). The smooth muscle layers and surrounding connective tissue of the myometrium did

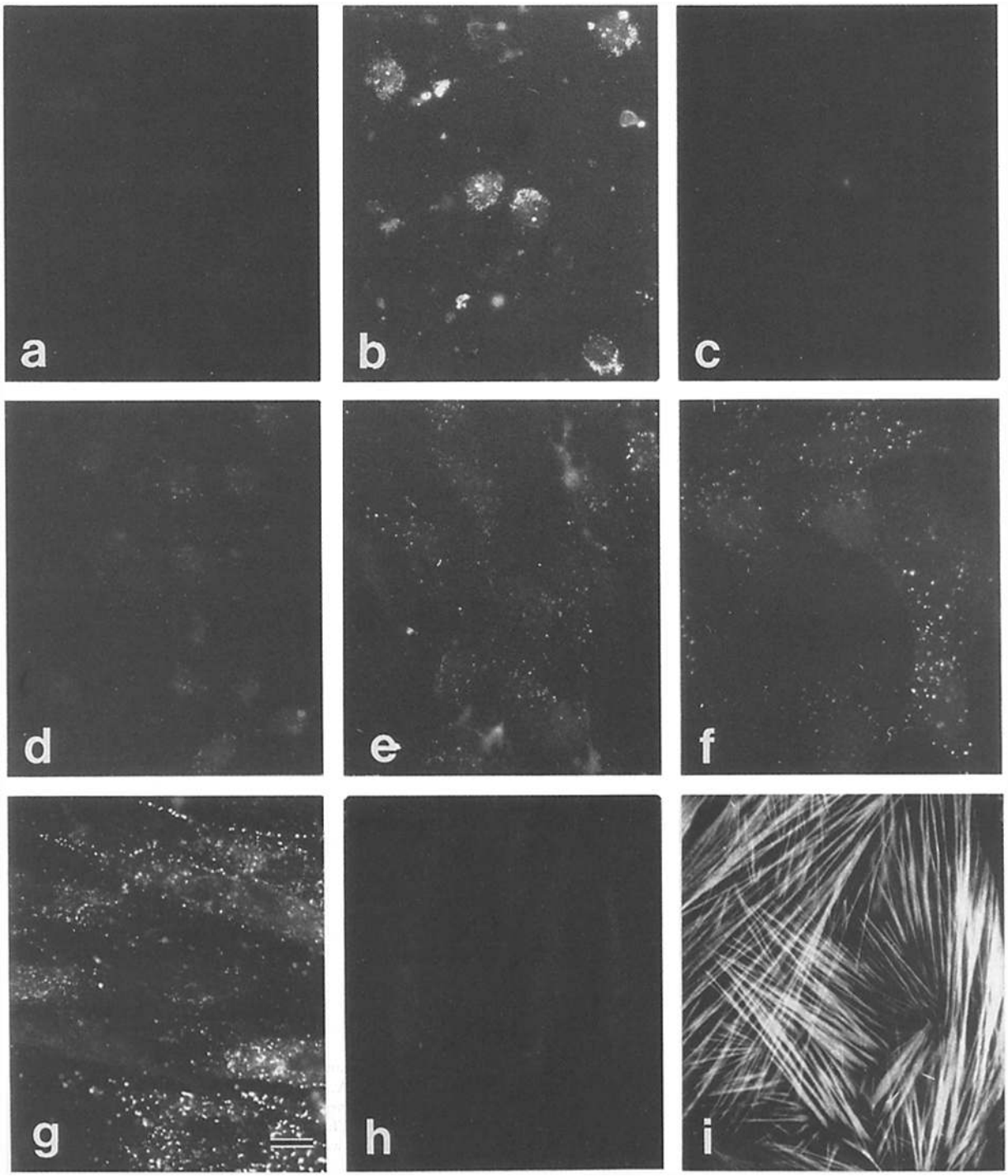

Figure 4. Immunofluorescence staining with PDGFR-B2 antibodies on myometrial and endometrial cells in culture. Cells were isolated by collagenase treatment from porcine myometrial (*a*, *c–i*) and endometrial (*b*) tissue and stained with PDGFR-B2 antibodies directly after isolation (*a* and *b*), after 10 (*c*), 18 (*d*), 24 (*e*), 48 (*f*), and 72 h in culture (*g*). Myometrial cells cultured for 72 h and 98 h, were also treated with 150 ng/mL of PDGF-AB before staining with PDGFR-B2 (*h*), and stained with muscle-specific anti-actin antibody (*i*), respectively. Bar, 10 μm.

not stain for B type PDGF receptor (Fig. 1 *c*), neither did smooth muscle in the tunica media of larger blood vessels (Fig. 1 *c*). This is a notable finding since smooth muscle cells grown in vitro have been found to possess PDGF receptors (3, 16). Similarly, sections of dermis from normal human skin (see below, Fig. 5 *a*) did not stain with anti-PDGF receptor mAbs in spite of the fact that fibroblasts in vitro have PDGF receptors. However, uterus from an actively growing pig (8 wk of age) demonstrated staining on both endometrial stromal cells (not shown) and myometrial smooth muscle (Fig. 1 *d*).

Analysis by Immunoblotting

Immunoblotting of membranes isolated from the outer mus-

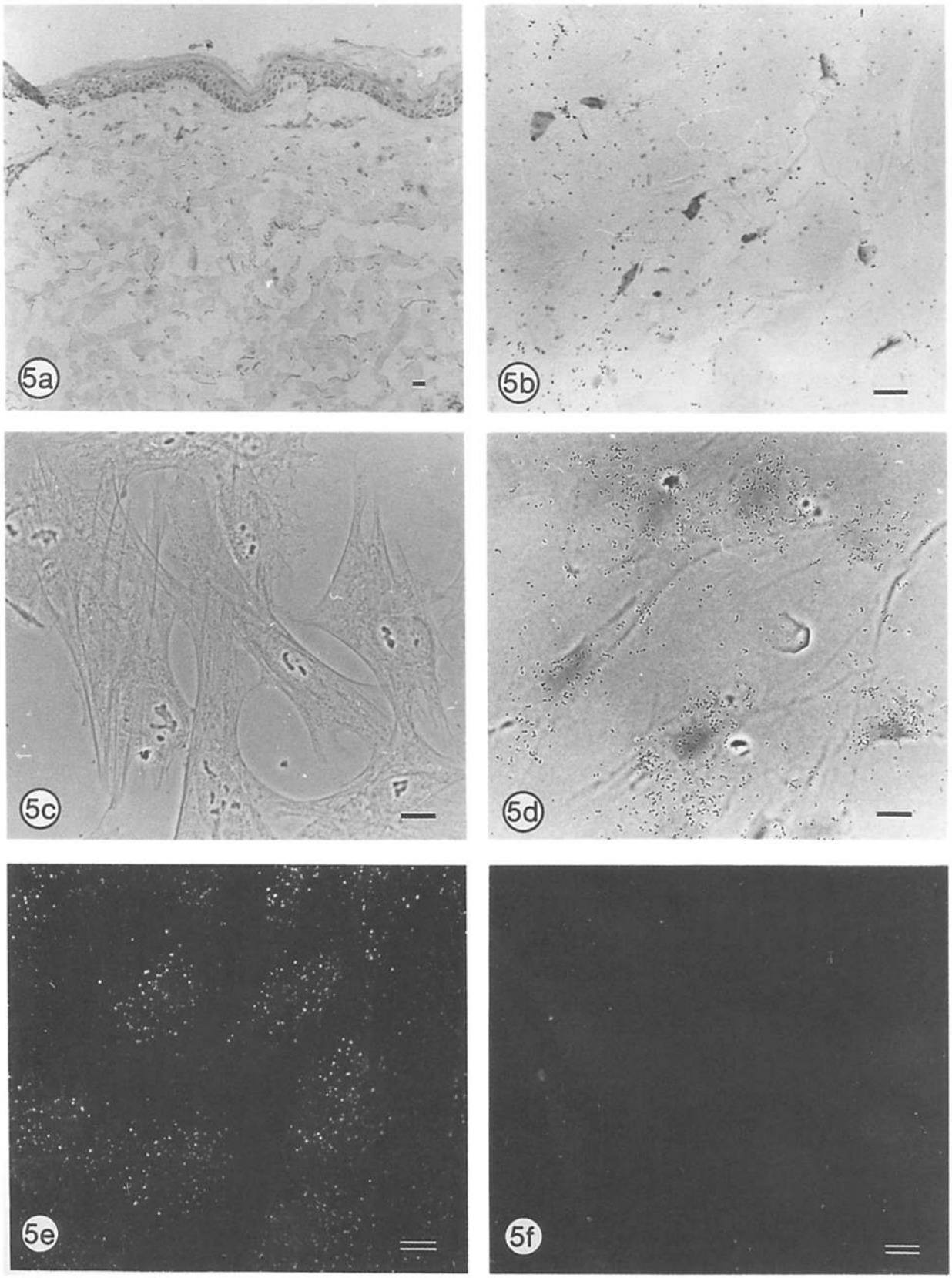

Figure 5. Expression of B type PDGF receptors in human skin and skin fibroblasts as determined by staining with the PDGFR-B2 antibody and in situ hybridization. (*a*) Immunohistochemical staining of human dermis; note that no PDGF receptor staining is seen anywhere in the tissue. (*b*) In situ hybridization with the PDGF receptor probe on human dermis; no B type PDGF receptor mRNA is seen. (*c*) Phase contrast micrograph of cells grown from skin explants; cells have a typical fibroblast-like morphology. (*d*) In situ hybridization on human skin fibroblast culture (same as in *c*) with an mRNA probe for the B type PDGF receptor—positive hybridization is seen on the cells.

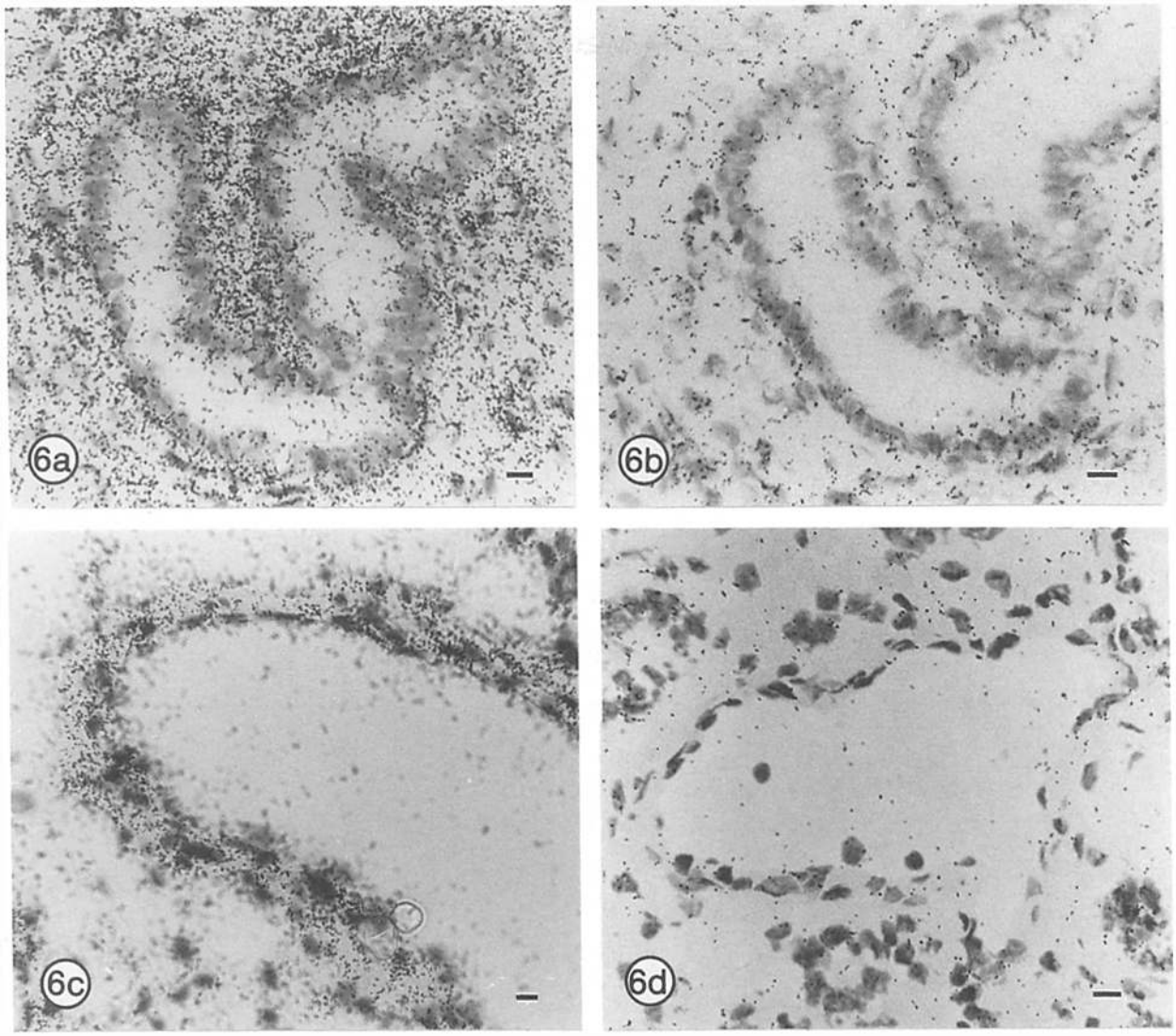

Figure 6. In situ hybridization of B type PDGF receptor mRNA in sections of porcine uterus endometrium. (*a*) Endometrium showing a heavy deposition of silver grains indicative of B type PDGF receptor mRNA over the stromal cells surrounding an endometrial gland. (*b*) Serial section hybridized with a "nonsense" probe showing the background levels of silver grains. (*c*) A high magnification of a blood vessel (similar to Fig. 1 *b*) hybridized with the B type PDGF receptor mRNA probe. Numerous silver grains can be seen over the wall of the blood vessel indicating the presence of receptor mRNA. (*d*) A similar section to that seen in *c* of a blood vessel hybridized with a "nonsense" probe showing background level of silver grains. Bars, 10 μm.

cle layer and endometrium of swine uterus and human dermis supported the histochemical stainings. A rabbit anti–PDGF receptor serum, PDGFR-1, recognized a 175-kD PDGF receptor in endometrial membranes (Fig. 2), as well as in human dermis (not shown). The concentration was about five times higher in endometrial than in myometrial membranes when normalized per mg of membrane proteins (Fig. 2). A very similar result was obtained when antiserum PDGFR-3 recognizing only B-type PDGF receptors, was used (not shown). Immunoblots of membranes from cultured myometrial smooth muscle cells and fibroblasts indicated that PDGF receptors are present on these cells (not shown).

Autophosphorylation Assay

The presence of functional B type PDGF receptors in membranes prepared from porcine myometrium and endometrium and human dermis, as well as from cultured porcine smooth muscle cells and dermal fibroblasts, were examined by use of a phosphorylation assay. In membranes from porcine endometrium (Fig. 3), as well as from cultured fibroblasts and smooth muscle cells (not shown), PDGF stimulated the phosphorylation of a 175-kD band, most likely representing autophosphorylation of the PDGF receptor (10). In contrast, only a limited PDGF-stimulated phosphorylation was noticed in membrane from porcine myo-

(*e*) Immunofluorescence staining of a replicate culture of human skin fibroblasts with PDGFR-B2 antibodies; PDGF receptors are demonstrated as bright fluorescent punctate areas. (*f*) A replicate culture of human skin fibroblasts incubated with 150 ng/ml of PDGF to down-regulate PDGF receptors, before staining with PDGFR-B2 antibodies; no staining is seen. Bars, 10 μm.

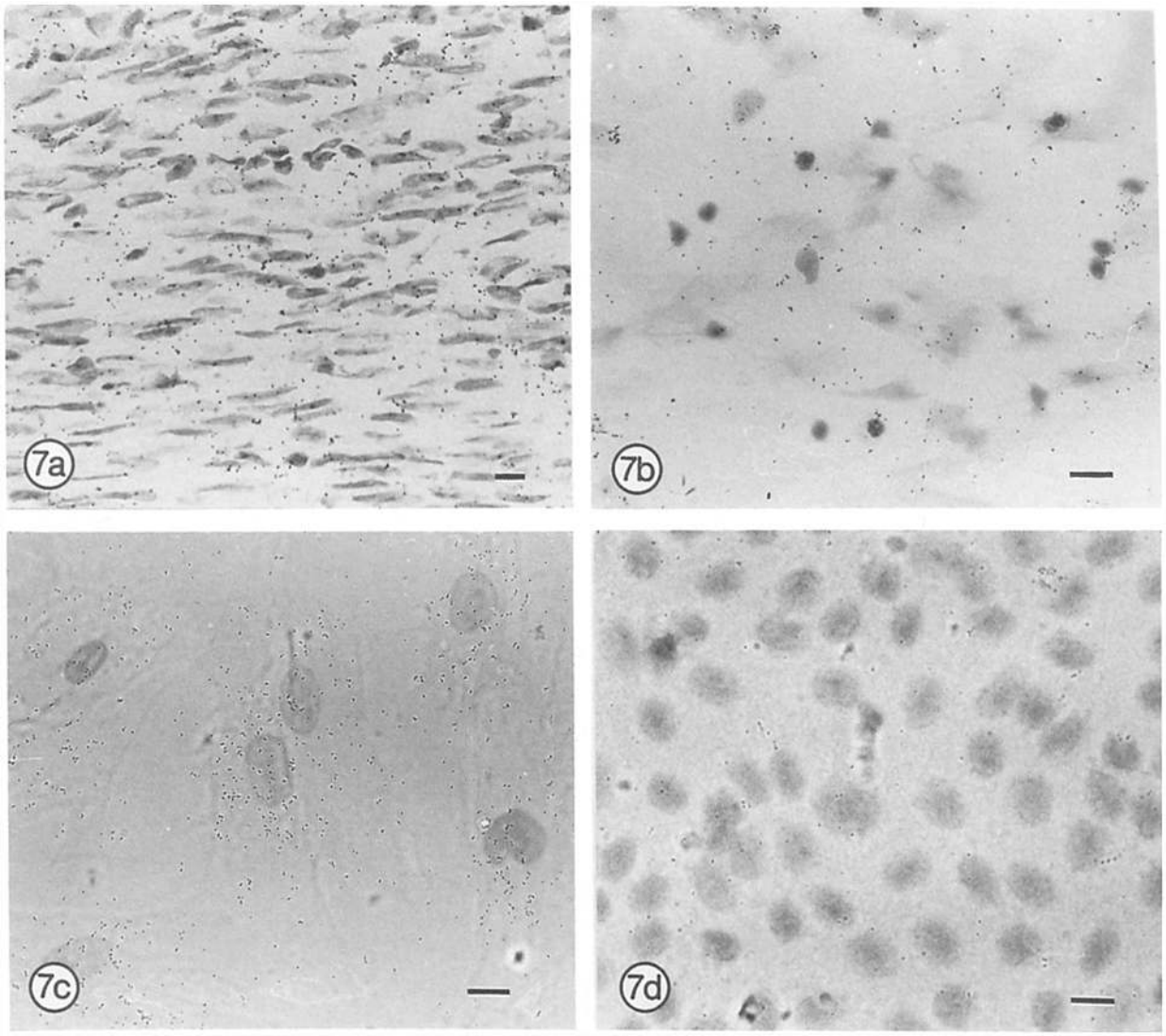

Figure 7. In situ hybridization of B type PDGF receptor mRNA in myometrium and cultured cells. (*a*) Myometrium, showing a low but uniform level of hybridization. (*b*) Myometrial cells directly after isolation, showing background levels of labeling. (*c*) Myometrial cells after 7 days in culture, demonstrating heavy labeling. (*d*) Cultures of endothelial cells known to be devoided of PDGF-B receptors, few or no silver grains are seen. Bars, 10 μm.

metrium (Fig. 3) and human dermis (not shown). These data support the results of immunohistochemistry and immunoblotting, and indicate that the PDGF receptors recognized by the antibodies are functionally active.

Immunofluorescence of Cells Progressing into Culture

Since smooth muscle cells of uterus myometrium appeared not to contain detectable levels of the B type PDGF receptors, while smooth muscle cells in tissue culture have PDGF receptors, experiments were designed to follow the kinetics of induction of PDGF receptors as cells progress into culture. Cells freshly isolated by collagenase digestion of swine uterus myometrium appeared to be essentially devoid of B type PDGF receptor as demonstrated by staining with the receptor-specific mAb PDGFR-B2 (Fig. 4 *a*). The receptor appeared to be absent or at low levels also after 10 h in vitro (Fig. 4 *c*), but after 18 h it started to appear on a few cells (Fig. 4 *d*). By 24–48 h a majority of cells possessed the receptor (Figs. 4, *e* and *f*), and by 3 d essentially all cells in culture possessed B type PDGF receptors (Fig. 4 *g*). The receptors on these cells could be down-regulated by prior exposure to PDGF (Fig. 4 *h*). The isolated cells were identified as smooth muscle cells by staining with a muscle-specific actin mAb (HHF-35). More than 90% of the freshly isolated cells were positive (not shown) and essentially all the cells in culture at 3 d possessed HHF-35 positive stress fibers (Fig. 4 *i*).

Cells isolated from porcine endometrium were composed of fibroblast-like stromal cells and glandular epithelial cells. The stromal cells were positive for B type PDGF receptors immediately after isolation and attachment (Fig. 4 *b*). The epithelial cells were negative immediately after isolation and remained negative even in long term culture (21 d, data not shown). The patched receptor stain seen on endometrial stromal cells directly after their isolation could be eliminated by incubation with PDGF which down-regulates the receptor

(not shown). These cells retained the B type PDGF receptor in culture and were distinctly fibroblastoid in appearance. The cells were negative with the muscle-specific actin antibody HHF-35 but possessed vimentin intermediate filaments (not shown).

Cells established from human skin by explant culture appeared to be fibroblasts, since they displayed fibroblast-like growth patterns (Fig. 5 *c*) and vimentin intermediate filaments, but not muscle-specific actin (not shown). These cells exhibited patched receptors when stained with PDGFR-B2 (Fig. 5 *e*), which were eliminated by down-regulation with PDGF before staining with PDGFR-B2 (Fig. 5 *f*).

In Situ Hybridization

This distribution of B type PDGF receptor mRNA in tissue sections and in cultured cells was determined by in situ hybridization techniques using a human B type PDGF receptor probe. The results were fully compatible with those obtained by immunohistochemistry. Silver grains, indicating hybridization of the probe with mRNA, were preferentially located over stromal cells surrounding glands (Fig. 6 *a*), and frequently over small blood vessels in the endometrium (Fig. 6 *c*). The degree of labeling was much higher than the background hybridization with the irrelevant probe (Fig. 6, *b* and *d*). In contrast, there was a weak uniform distribution of silver grains over the myometrium (Fig. 7 *a*), which was only slightly heavier than the background seen with the irrelevant probe (not shown). Quantitation of silver grain density with background levels subtracted (Fig. 8) indicates that there is a 25-fold greater number of silver grains over labeled areas in the endometrium than in the myometrium. Although the levels seen in myometrium are low they are above background levels. Similarly, the B type PDGF receptor probe only marginally labeled normal human dermis, in agreement with the immunohistochemical results using the PDGFR-B2 antibodies (Fig. 5, *a* and *b*). RNAse pretreatment of the sections or competition with nonradioactive probe eliminated the specific hybridization signal (not shown).

Freshly isolated cells from porcine uterus myometrium did not show any significant labeling (Fig. 7 *b*). However, cells cultured for 7 d demonstrated significant labeling (Fig. 7 *c*). Cultured endothelial cells, which do not bind [^{125}I]PDGF-AB (not shown), were not stained by silver grains indicating that they did not express the B type PDGF receptor mRNA (Fig. 7 *d*). Endothelial cells prepared and handled at the same time did hybridize with a probe to the B chain of PDGF (not shown), indicating that the processing procedures did preserve mRNA detectable by in situ hybridization.

Discussion

The results of this study indicate that cells in nonproliferating adult tissues, exemplified by porcine myometrial uterus and human dermis, possess low levels of B type PDGF receptors. This is based on the observed lack of histochemically detectable B type PDGF receptors on vascular and myometrial smooth muscle cells in uterus, as well as on dermal fibroblasts in skin. Similar results were obtained with in situ hybridization for B type PDGF receptor mRNA. By contrast, using the same techniques, high levels of B type PDGF receptors were detected on stromal cells in the endometrium. The results obtained using the cytochemical techniques were

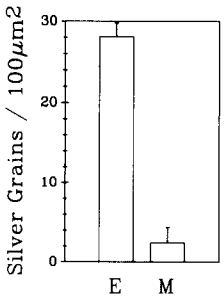

Figure 8. A graphic representation of silver grain density over labeled areas in the swine uterus endometrium (*E*) and myometrium (*M*). The in situ hybridization with the PDGF-B type receptor probe and the Digital Image Analysis were performed as described in Materials and Methods. The background levels of silver grains present using an irrelevant probe have been subtracted. Data shown is ± SEM.

corroborated by a functional assay for the B type PDGF receptor; PDGF stimulated the autophosphorylation of a 175-kD receptor protein in solubilized membranes isolated from the endometrium but to a much lesser extent in membranes from the myometrium. In addition, immunoblot analysis revealed about a fivefold difference in B type PDGF receptor amounts in membranes isolated from the two regions of uterus. Whether the presence of a low amount of receptors in the myometrial membranes is due to a small contamination of endometrial membranes (it is technically difficult to dissect myometrium and endometrium from each other during preparation), or indicates a low expression of B type PDGF receptor also in cells of the myometrium, remains to be elucidated. Of particular interest was the staining of stromal cells adjacent to the proliferating endometrial glands. As expected, there was no B type PDGF receptor staining on the epithelial cells in the glands. However, the dense accumulation of staining on fibroblast-like cells surrounding the growing end of the glands suggests the possibility of stimulation or direction of glandular growth by the PDGF receptor–bearing cells. The mechanism of regulation of PDGF receptor expression on cells of the endometrium is not known. It is an interesting possibility, which remains to be investigated, that the PDGF receptor levels are steroid hormone–dependent.

The presence of receptor staining on the proliferating (coiled-like) arteries of the endometrium, but not on larger blood vessels in the myometrium, is in keeping with our previous observations in rheumatoid arthritic synovium, where B type PDGF receptor staining was seen on proliferating blood vessels in conjunction with the intense neovascularization seen in some forms of rheumatoid arthritis, but not in blood vessels from normal tissue (35). In addition, expression of B type PDGF receptor mRNA was found in proliferating endothelium of a human glioblastoma (21). These observations suggest that PDGF receptor induction may play a role in neovascularization.

A possible explanation for the lack of staining in the myometrium could be that the cells were so tightly packed that the antibody did not have access to the receptor. Evidence against this possibility includes the fact that the myometrium from a young animal, handled in the same way, did stain for the receptor. The finding of PDGF receptor expression in the myometrium of a young animal is intriguing. Together with the finding that other smooth muscle cell types, under certain conditions, produce PDGF-like factors (28, 36), it may suggest a role for PDGF-like factors in autocrine or paracrine stimulation of cell growth in the growing uterus.

Interestingly, B type PDGF receptors appeared on certain

cell types as they progressed and adapted to tissue culture conditions. This was best observed in the porcine uterus smooth muscle where the cells could be seen to acquire the receptors over a 3-d period, but was also evident in human dermal fibroblasts. Once the receptors could be demonstrated by immunofluorescence, they could be down-regulated by PDGF. Cells maintained and passaged in culture had receptors that would autophosphorylate in membrane preparation. The lack of staining on freshly isolated cells appears to reflect the lack of receptor and not to be an artifact caused by the collagenase digestion, since endometrial cells isolated in the same way still possess the receptors (Fig. 4 *b*), and exposing cultured smooth muscle cells to the collagenase digestion solution for up to 9 h did not eliminate receptor staining in these cells (data not shown).

An alternative explanation for the lack of receptors on freshly isolated smooth muscles cells is that local high concentrations of PDGF in the tissue had caused the receptors to down-regulate. However, this possibility is unlikely, since only very high PDGF concentrations would produce such an efficient down-regulation, and since it takes only ~4 h for receptors to reappear on the cell surface of cultured cells after PDGF has been removed from the cell culture medium (16), whereas the receptor does not appear on the cultured smooth muscle cells until after 18 h in culture. Thus, it appears that tissue culturing induces an up-regulation of the receptor on these cells. The mechanism behind the receptor induction remains to be elucidated. It may involve humoral factors, e.g., present in the serum added to the culture medium. In addition, the interruption of cell–cell and cell–matrix interactions may be important.

Recent studies have revealed that human PDGF occurs in three isoforms, PDGF-AA, PDGF-AB and PDGF-BB, that bind with different affinities to two separate receptor classes, denoted type A and type B (14, 20). PDGF purified from porcine platelets was identified as PDGF-BB (37). However, porcine endothelial cells contain both PDGF A and B chain mRNA (Hammacher, A., K. Miyazono, B. Westermark, and C.-H. Heldin, unpublished observations), suggesting that all three isoforms of PDGF occur also in pigs. Our present studies indicate that porcine cells contain B type PDGF receptors; whether A type receptors also occur in pigs, remains to be determined.

The data from the present study indicates that the response of normal connective tissue cells to PDGF may be regulated, not only by the availability of the ligand, but also by the expression of PDGF receptors at the cell surface.

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