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Platelet-Derived Growth Factor Induces Proliferation of Hyperplastic Human Prostatic Stromal Cells

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Abstract Prostatic hyperplasia (BPH) is a very common disease in elderly men and is characterized by abnormal proliferation of the stromal and epithelial cells of the prostate. The observation that BPH often occurs in association with chronic inflammation has led to the examination of the possibility that platelet-derived growth factor (PDGF), which is released in response to inflammation, may be an etiological factor in the genesis of the disease. It has been shown that cultured cells derived from human prostatic tissue express high affinity PDGF-β receptors based on receptor binding and cross-linking studies with [^{125}I]-PDGF-BB. The experiments presented below demonstrate that PDGF receptors are activated in response to the growth factor and that mitogenesis is induced. PDGF-BB treatment of cultured human prostate cells derived from patients with BPH activates the signal transduction pathway of the PDGF receptor as shown by the presence of several phosphoproteins in antiphosphotyrosine immunoprecipitates, including autophosphorylation of the PDGF receptor. Phosphatidylinositol (PI) 3-kinase activity is also increased in cells stimulated with PDGF The addition of PDGF-BB to the medium causes a variable but dose-dependent increase in [^{3}H]-thymidine incorporation This paper describes the first demonstration that PDGF is a potent mitogen for human cells derived from patients exhibiting prostatic hyperplasia, and also demonstrates that the cellular response to PDGF-BB is heterogeneous in a manner that is consistent with the varying degree of hyperplasia and inflammation clinically and histologically in the tissue specimens.

Key words: inflammation, mitogen, protein phosphorylation, signal transduction, tyrosine kinase

Hyperplasia of prostatic glands and stroma is virtually ubiquitous among aging men, causing urinary obstruction and necessitating more than 400,000 surgical operations per year in the U.S. [Eble and Epstein, 1990; McNeal, 1990]. Normal growth and function of the prostate is dependent upon androgens. However, the exact role of androgens in BPH has not been elucidated. Although testicular steroids are important for prostate development, androgens alone have not been shown to induce the proliferation of prostate cells in culture [McKeehan et al., 1984]. In contrast, growth factors such as EGF, bFGF, and TGF-α have been shown to stimulate prostate cell proliferation in culture. Thus, it has been proposed that androgens may act indirectly by regulating the expression of growth factors and/or growth factor receptors [Jinno et al., 1986; Kyprianou and Isaacs, 1988; Eaton et al., 1988; Mydlo et al., 1988].

In addition to cellular hyperplasia, histologic examination of BPH tissue has revealed the presence of chronic inflammation [Kohnen and Drach, 1979]. This observation has led to the hypothesis that inflammation may be a significant etiological factor in the development of BPH. Fibroblasts, platelets, and inflammatory cells release a number of chemoattractant and growth promoting substances [De Gaetano et al., 1989; Shimokado et al., 1985; Takemura and

Abbreviations used BPH, prostatic hyperplasia, EGF, epidermal growth factor, bFGF, basic fibroblast growth factor, TGF-α, transforming growth factor alpha, PDGF, platelet-derived growth factor, FBS, fetal bovine serum, PBS, phosphate buffered saline, BSA, bovine serum albumin, PMSF, phenylmethylsulfonyl fluoride, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, TLC, thin layer chromatography, HPLC, high performance liquid chromatography, PtdIns, phosphatidylinositol, gPtdIns, glycerophosphatidylinositol, SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Werb, 1984], including PDGF [Ross et al., 1986]. It is possible that the inflammation often found in association with BPH may be responsible for the release of growth factors that act to induce cellular proliferation. Furthermore, androgens may be involved in modulating this process by affecting the capacity of prostatic elements to respond to mitogenic factors.

Not only does PDGF play a central role in the regulation of normal cell growth, but it also is involved in mediating cell proliferation in atherosclerosis, fibrosis, and other benign proliferative disorders [Antoniades, 1991]. PDGF is released by platelets and other cells involved in the inflammatory response, where it functions to stimulate cellular proliferation, chemotaxis, and extracellular matrix production. Cellular responses to PDGF include stimulation of tyrosine kinase activity, hydrolysis of PtdIns, stimulation of PtdIns 3-kinase activity, an increase in the level of cytosolic calcium, and an increase in DNA synthesis [Williams et al., 1988]. Since BPH is frequently associated with inflammation and since PDGF is released during inflammation, experiments were performed to determine whether primary cultures of human prostatic stromal cells derived from patients being treated for BPH were capable of responding to PDGF. The results presented below demonstrate that primary cultures of human prostatic stromal cells have functional PDGF receptors as shown by the activation of the PDGF receptor signal transduction cascade following exposure of the cells to PDGF-BB. Further, the results of these studies indicate that PDGF-BB is a potent mitogen for prostatic stromal cells, and that the growth response is correlated to the presence and degree of inflammation. These results suggest a link between inflammation, PDGF, and the development of BPH.

MATERIALS AND METHODS

Materials

PDGF-BB was purchased from BioSource International (Camarillo, CA). Antiphosphotyrosine monoclonal antibodies were obtained from Upstate Biologicals, Inc. (Lake Placid, NY). Protein A Sepharose, aprotinin, sodium orthovanadate, phosphatidylinositol, and PMSF were products of the Sigma Chemical Co., Inc. (St. Louis, MO). [γ-^{32}P]-ATP (6,000 PtdIns-4-phosphate (inositol-2-^{3}H(N)) (10 Ci/mmol) were products of New England Nuclear (Boston, MA), while [methyl-^{3}H] thymidine (75–80 Ci/mmol) was procured from Amersham Corp. (Arlington Heights, IL). Flo-Scint IV liquid scintillator was obtained from Radiomatic Instruments and Chemical Co., Inc. (Meriden, CT). Milli-Q water (Millipore Corp., Bedford, MA) was used for all aqueous solutions. Tissue culture media and reagents were purchased from Gibco BRL (Gaithersburg, MD). All other reagents were of the highest quality commercially available.

Tissue Procurement

Prostate tissue was obtained by transurethral resection of the prostate from patients with clinical diagnoses of BPH, and was assessed histologically with hematoxylin and eosin staining to confirm the diagnosis. The quantity, chronicity, distribution, and cellular response of the inflammation in each sample were characterized by light microscopy (Table I). Samples of the tissue were stabilized in pH-balanced Hanks' solution containing 8.5% sucrose for subsequent cell culture. This use of surplus tissue was approved by the Institutional Review Boards of the Indiana University Medical Center, The Richard L. Roudebush Veterans Affairs Medical Center, and the Methodist Hospital of Indianapolis. Tissue specimens were coded in order to maintain the anonymity of the patients.

Cell Culture

Fresh prostatic tissue was minced and then chemically dissociated with collagenase (200 unit/ml) and DNAse (0.1 mg/ml) at 37°C for 1 h. Cell aggregates were sequentially passed through 2 mm^2 and 1 mm^2 stainless steel screens followed by passage through a 253 μm nylon mesh. The cell suspension was centrifuged and resuspended in RPMI 1640 media supplemented with 10% FBS, 6.9 nM testosterone, amphotericin B (2.5 μg/ml), and gentamicin (2.5 μg/ml). The cells were plated in 75 cm^2 flasks and maintained in a humidified atmosphere of 95% air-5% CO_2. After 3 days in culture, the cells were placed on Mixed Media (1:1 mixture of RPMI-1640:WAJC-404, Kyokuto Pharmaceutical Industrial Co., Tokyo) supplemented with 5% FBS, 0.5 mM dexamethasone, 6.9 nM testosterone, prolactin (1.5 ng/ml), cholera toxin (5 ng/ml), EGF (5 ng/ml), bFGF (5 ng/ml), bovine insulin (2.5 μg/ml), heparin (12.5 μg/ml), transferrin (2.5 ng/ml), selenous acid (2.5 ng/ml), gentamicin (2.5 μg/ml), and amphotericin B (2.5 μg/ml). The medium was changed every 3–4 days. The cells were passaged by exposure to trypsin-

TABLE I. Clinical Evaluation of BPH Specimens*

Cell line (patient)	Age (yr)	Pathology	Inflammation score (0–2)	Basal doubling time (h)	Specimen size (g)	Sites/cell
1	57	Hyperplasia with a single focus of carcinoma in one chip	1	ND	7	411,000
2	67	Hyperplasia with moderate acute and chronic inflammation	2	23	16	308,000
3	75	Hyperplasia with foci of carcinoma in two chips	0	319	22	288,000
4	66	Hyperplasia with chronic inflammation	2	36	22	180,000
5	70	Hyperplasia with chronic inflammation	2	48	32	208,000
6	66	Hyperplasia with chronic inflammation	2	107	17	211,000
7	65	Hyperplasia with mild chronic inflammation, foci of carcinoma in two chips, moderate atrophy	1	39	10	521,000
8	69	Hyperplasia with mild chronic inflammation	1	128	8	244,000
9	67	Hyperplasia with chronic inflammation, atrophy, foci of dysplasia	2	111	10	272,000

*Each of the patients underwent transuretheral resection of the prostate with the resected specimens ranging from 7 to 32 g. Pathologic examination including gross inspection as well as hematoxylin and eosin light microscopy of all prostatic tissues revealed the heterogeneity of the disease. The tissue was characterized by varying degrees of both stromal and epithelial hyperplasia consistent with the diagnosis of BPH. Inflammation was commonly seen, including predominant chronic inflammatory changes of diffuse and peri-ductal lymphocytic infiltrates. ND, not determined.

EDTA solution (0.025% trypsin, 0.265 mM EDTA). Cell lines were originally a mix of stromal and epithelial cells, but after a few passages consisted predominantly of stromal cells.

Swiss-3T3 cells (CCL 92) were originally purchased from the American Type Culture Collection. The cells were routinely grown in Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% fetal bovine serum along with fungizone (2.5 μg/ml) and gentamycin (25 μg/ml) (GIBCO, Grand Island, NY). Cells were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2, and passaged every 3–4 days. Cultures were maintained at low cell density in order to guard against spontaneous transformation. Changes in cellular morphology were accompanied with a loss of contact inhibition, an activation of the elements of the PDGF signal transduction pathway and an increase in the rate of cellular proliferation. Any culture that exhibited such changes was discarded and new cultures established.

Thymidine Incorporation Assay

Human prostate cells were seeded into Falcon 24-well culture plates and grown to near confluence in Mixed Media supplemented with 2.5% FBS (Thymidine Assay Media). This medium was selected based on its capacity to support only minimal cellular proliferation. The cells were serum starved for a minimum of 72 h and then exposed to fresh media containing PDGF-BB (0–50 ng/ml). Following a 24-h incubation at 37°C, the cells were incubated with fresh media containing 0.5 mCi [^{3}H]-thymidine for 4 h at 37°C. At the end of the incubation period, cells were washed once with ice-cold PBS, twice with ice-cold 10% trichloroacetic acid, and finally with ice-cold methanol. The cells were then solubilized with 3% SDS for counting by liquid scintillation spectrometry using Beckman Ready-Solv HP Scintillation Fluid. Data were expressed as incorporation of [^{3}H]-thymidine as a percent of unstimulated controls.

Cell Proliferation Assay

Human prostate cells were plated in Falcon 6-well culture plates at a density of 50,000 cells/well. After 72 h in culture, baseline cell counts were measured using a Coulter Counter Model ZM (Coulter Electronic, Ltd., Luton, Bedfordshire, England). The remaining cells were then exposed to Thymidine Assay Media supplemented with PDGF-BB (0–50 ng/ml). Cell counts of representative wells were performed on days 2, 4, and 7. At the same time the remaining cells were exposed to fresh media. Data generated from unstimulated cells were

used to determine basal doubling times. Data were expressed as cell number as a percent of the unstimulated controls.

PDGF Receptor Binding Assay

Human prostate cells were grown to near confluence in Falcon 24-well plates. Cells were washed with 1.0 ml PBS containing 0.5% BSA, followed by 1.0 ml of 20 mM acetic acid in 0.5 M NaCl containing 0.5% BSA (pH 3.8) to strip any PDGF from receptors. Following a wash with PBS/0.5% BSA, cells were incubated in RPMI medium containing 0.5% BSA (without serum) for 2 h at 37°C. After the incubation period, the media was aspirated and the cells were washed twice with PBS containing 0.5% BSA. The wash was aspirated and replaced with 0.5 ml aliquots of media containing [^{125}I]-PDGF-BB (Amersham Corp.) at concentrations ranging from 0.04–2.0 nM (labeled PDGF diluted with unlabeled PDGF to a final specific activity of 450 Ci/mmol). The 24-well plates were incubated 2 h at 22°C. At the end of the incubation period, the cells were washed three times with 1.0 ml of 10 mM Tris/0.15 M NaCl containing 0.5% BSA, and the cells were then solubilized by addition of 3% SDS. Binding was measured using a gamma counter. Saturation curves were determined using Lundon 1 software (Lundon Software Inc., Cleveland, OH).

Antiphosphotyrosine Immunoprecipitations

Swiss 3T3 cells or human prostatic cells derived from patients being treated for BPH were grown to near confluence in 75 cm^2 flasks. The cells were washed with 1.0 ml PBS containing 0.5% BSA followed by exposure to 10 ml of medium containing 0.5% BSA. The plates were then returned to the incubator for 30 min. The medium was withdrawn and the cells exposed to fresh medium containing 0.5% BSA in the absence or presence of 10 ng/ml PDGF-BB. The cells were returned to the incubator for 5 min, after which time the cells were washed with ice-cold PBS. The cells were then lysed in 1 ml of 1% NP-40 lysis buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 10% glycerol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin at 4°C for 20 min. Insoluble material was removed by centrifugation at 10,000*g* for 10 min at 4°C. Antiphosphotyrosine antibody (5 μg) was incubated with the lysate supernatants for 3 h at 4°C. Immobilized protein A Sepharose beads were used to precipitate the antibody-antigen complexes. Immunoprecipitates were washed once with PBS and then twice with 0.1 M Tris-0.5 M LiCl (pH 7.5). During the second wash, 300 μl of the resuspended immunoprecipitate was removed for use in the in vitro autophosphorylation assay. The remaining material (700 μl) was assayed for PtdIns 3-kinase activity.

Phosphatidylinositol 3-Kinase Activity

The antiphosphotyrosine immunopellet was assayed for PtdIns 3-kinase activity as described previously [Whitman et al., 1985; Kaplan et al., 1987]. The immunoprecipitate was washed with 20 mM HEPES, pH 7.4, containing 10 mM $MgCl_2$ and 25 μM ATP, and was then resuspended in 40 μl of the above buffer containing 32 μCi [γ-^{32}P]-ATP. Reaction was initiated by the addition of 10 μl PtdIns sonicated in 20 mM HEPES buffer, pH 7.4 (0.2 mg/ml final concentration). The samples were incubated at room temperature for 10 min with frequent mixing, after which time the reaction was quenched by addition of 40 μl 1 N HCl. Lipids were extracted with addition of 80 μl chloroform:methanol (1:1, v:v). The samples were centrifuged and the lower organic phase was applied to a silica gel TLC plate, which was developed in $CHCl_3$:MeOH:H_2O:NH_4OH (45:35:8.5:1.5, v:v). Plates were dried, and the kinase reaction visualized by autoradiography.

Products of the PtdIns kinase reaction were confirmed by HPLC [Whitman et al., 1988]. Phospholipids were deacylated in methylamine reagent and separated using a Whatman Partisphere SAX anion exchange column as previously described [Auger et al., 1989]. A Radiomatic Model A-100 Flo-One/Beta on-line radioactivity detector with Flo-Scint IV liquid scintillator was used to simultaneously monitor the [^{32}P]-gPtdIns enzyme products and standard [^{3}H]-gPtdIns-4-P.

In Vitro Autophosphorylation

The antiphosphotyrosine immunopellet was washed with 0.2 M Tris·HCl buffer, pH 7.5, containing 5 mM $MnCl_2$. The reaction was initiated by the addition of 40 μl of the above buffer containing [γ-^{32}P]-ATP (16 μCi), and the samples were allowed to stand 25 min at room temperature. Immunoprecipitates were then washed with 1.0 ml of the Tris/$MnCl_2$ buffer (without ATP) and following centrifugation, the

reaction was quenched by boiling the sample for 5 min with 30 μl 4× SDS-PAGE sample buffer (0.2 M Tris·HCl, pH 6.8, containing 40% glycerol, 4% SDS, 4% β-mercaptoethanol, and 0.025% bromphenyl blue). The [^{32}P]-labeled proteins were separated by SDS-PAGE on a 7.5% gel. Gels were dried in vacuo onto Whatman #3 paper and were analyzed by autoradiography.

Cross-Linking of [^{125}I]-PDGF to PDGF-Receptor

Prostatic stromal cells were plated in Falcon 6-well plates at a density of 80,000–100,000 cells/well. After washing with PBS containing 0.5% BSA, cells were treated with RPMI medium containing [^{125}I]-PDGF-BB (30 ng/ml, 0.84 μCi/ml) in the presence or absence of nonlabeled PDGFBB (1 μg/ml) for 2 h at 4°C. Cells were washed with ice-cold PBS and then incubated for 90 min at room temperature with cross-link buffer (0.1 mM disuccinimidyl suberate in 0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, and 8 mM glucose, pH 8.0). Following removal of the buffer, cells were washed with 10 mM Tris·HCl, pH 7.4, containing 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cells were then solubilized in 60 mM Tris·HCl, pH 6.8, containing 0.3% SDS, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The cross-linked receptor-PDGF complex was subjected to SDS-PAGE using 4–20% gradient gels (Novex Inc., San Diego, CA). Gels were dried in vacuo onto Whatman #3 paper and were analyzed by autoradiography.

RESULTS

Antiphosphotyrosine Immunoprecipitation: In Vitro Autophosphorylation

Primary cultures of human prostatic stromal cells derived from surgical specimens of patients exhibiting prostatic hyperplasia were stimulated with 10 ng PDGF-BB. Following cell lysis, a monoclonal antibody against phosphotyrosine was used in immunoprecipitation experiments to measure differences in protein phosphorylation between cells stimulated with PDGF and unstimulated cells. Several proteins were phosphorylated on tyrosine in response to PDGF stimulation of human prostatic stromal cells; the phosphoprotein pattern was similar to that seen in PDGF-stimulated Swiss 3T3 cells (Fig. 1). Tyrosine phosphorylation was not observed in unstimulated cells. Among the labeled bands

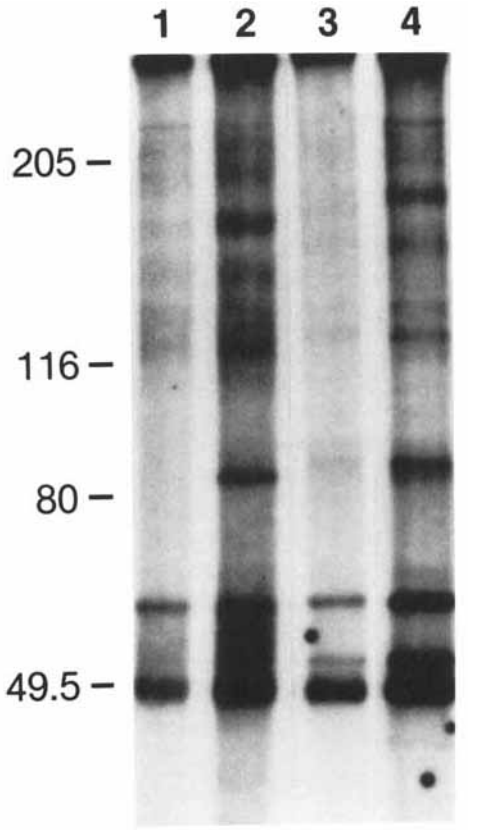

Fig. 1. Human prostate cells were grown to near confluence in 75 cm^2 flasks in Mixed Medium supplemented with 2.5% FBS. The cells were serum starved for a minimum of 48 h and washed with 1.0 ml PBS containing 0.5% BSA followed by 10 ml of medium containing 0.5% BSA. After 30 min at 37°C, the medium was withdrawn and the cells exposed to fresh medium ± 10 ng/ml PDGF-BB for 5 min at 37°C. Cells were washed with ice cold PBS and lysed 20 min at 4°C as described under Materials and Methods. Cell lysates were immunoprecipitated using an antiphosphotyrosine monoclonal antibody. Following labeling with [γ-^{32}P]-ATP, phosphoproteins were analyzed by SDS-PAGE on a 7.5% gel. **Lanes 1** and **2:** Untreated and PDGF-stimulated Swiss 3T3 cell lysates. **Lanes 3** and **4:** Untreated and PDGF-stimulated cell lysates from BPH stromal cells. Molecular weight markers: 205 kDa, myosin; 116 kDa, β-galactosidase; 80 kDa, bovine serum albumin; 49.5 kDa, ovalbumin.

present following SDS-PAGE were those corresponding in molecular weight to the autophosphorylated receptor (185 kDa), the regulatory subunit of the PtdIns 3-kinase (85 kDa), as well as two bands around 120 and 140 kDa that may correspond to phospholipase Cγ and GTPase activating protein, respectively. These data demonstrate that the PDGF receptors in the hyperplastic human prostatic stromal cells contain functional tyrosine kinase domains responsive to PDGF and which phosphorylate the same substrates as do the PDGF receptors character-

ized in long-term cultures of 3T3 cells and smooth muscle cells.

Phosphatidylinositol 3-Kinase Activity in Antiphosphotyrosine Immunoprecipitates

The presence of an 85 kDa tyrosine phosphoprotein in the antiphosphotyrosine immunoprecipitates from PDGF-treated cells suggests that PtdIns 3-kinase activity is present in these cells following stimulation by PDGF [Cohen et al., 1990]. Antiphosphotyrosine immunoprecipitates of PDGF-stimulated human prostatic stromal cells were washed and assayed for PtdIns 3-kinase activity (Fig. 2); Swiss 3T3 cells were used as a positive control as they have previously been shown to respond to PDGF. Two representative primary cultures are depicted. Stimulation of either human prostate stromal cells or Swiss 3T3 cells with PDGF caused an increase in PtdIns kinase activity compared to unstimulated cells, demonstrating a functional linkage between the PDGF receptor and PtdIns kinase activation in prostatic stromal cells. Furthermore, HPLC analysis of the deacylated lipid products of the PtdIns kinase assay showed that the increased PtdIns kinase activity in the PDGF-stimulated cells was due to stimulation of PtdIns 3-kinase rather than due to PtdIns 4-kinase (data not shown).

PDGF-Induced Cell Proliferation

Following exposure to PDGF-BB (0–50 ng/ml), all primary cultures tested showed dose-dependent increases in [^{3}H]-thymidine incorporation when compared to unstimulated control (data not shown). A highly variable response was seen, with maximal increases in [^{3}H]-thymidine incorporation ranging from 2-fold to 10-fold over that observed in unstimulated cells (Fig. 3). A similarly heterogeneous response was observed with respect to the extent to which PDGF induced cellular proliferation (Fig. 4). For example, cells derived from Patients 2 and 7 both exhibited dose-dependent increases in cell number in response to PDGF (Fig. 5). In the case of Patient 2, the maximum proliferative response of 2-fold was observed at 50 ng/ml. In contrast, cells derived from Patient 7 exhibited an 8-fold increase in cell number with the maximal response being observed at 15 ng/ml. Basal doubling times determined from these studies range from 23 h to 319 h (Table I), which again is consistent with the heterogeneous nature of the disease and differences in progression to symptoms.

PDGF-Receptor Characterization

The tissues used in these experiments were derived from patients being treated for BPH and had varying degrees of chronic inflammation. It has recently been demonstrated that these cells in primary culture express high affinity (Kd = 0.19–9.12 nM) PDGF receptors [Hirsch et al., 1991]. Cross-linking experiments (Fig. 6) further established the expression of specific PDGF receptors on human prostatic cells. Not

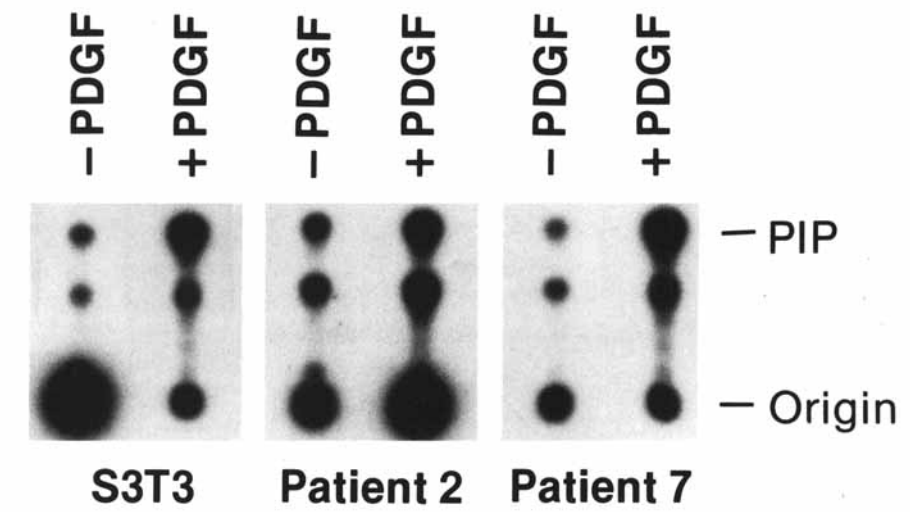

Fig. 2. Immunoprecipitates derived from human prostate cells exposed to media ± 10 ng/ml PDGF-BB were assayed for PtdIns kinase activity as described under Materials and Methods. Thin layer chromatography was used to determine PtdIns kinase activity.

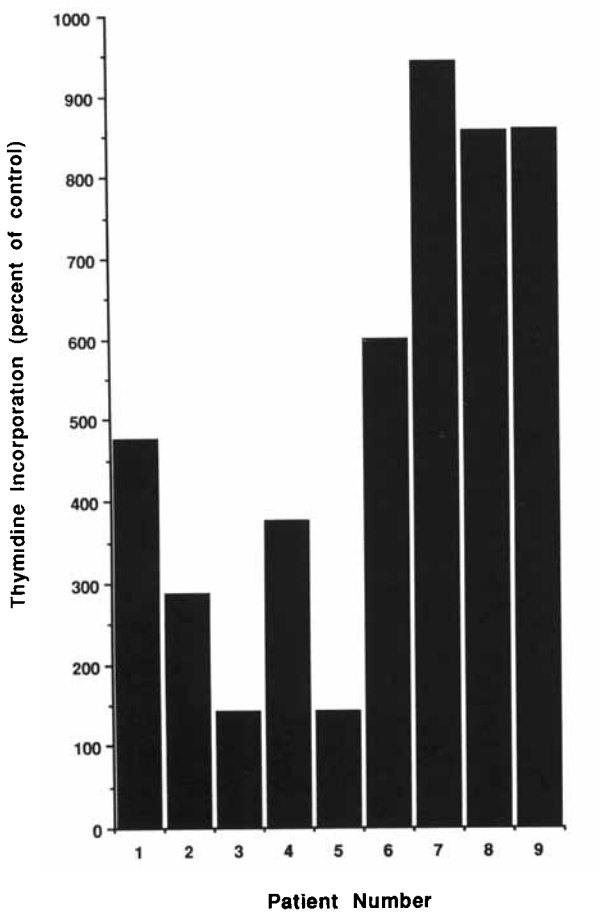

Fig. 3. Human prostate cells were exposed to PDGF-BB (0–50 ng/ml) for 24 h, and then exposed to fresh media containing 0 5 mCi [^{3}H]-thymidine as described under Materials and Methods The bars represent the maximal response observed when the cells were exposed to 50 ng/ml PDGF and are an average of two duplicate samples

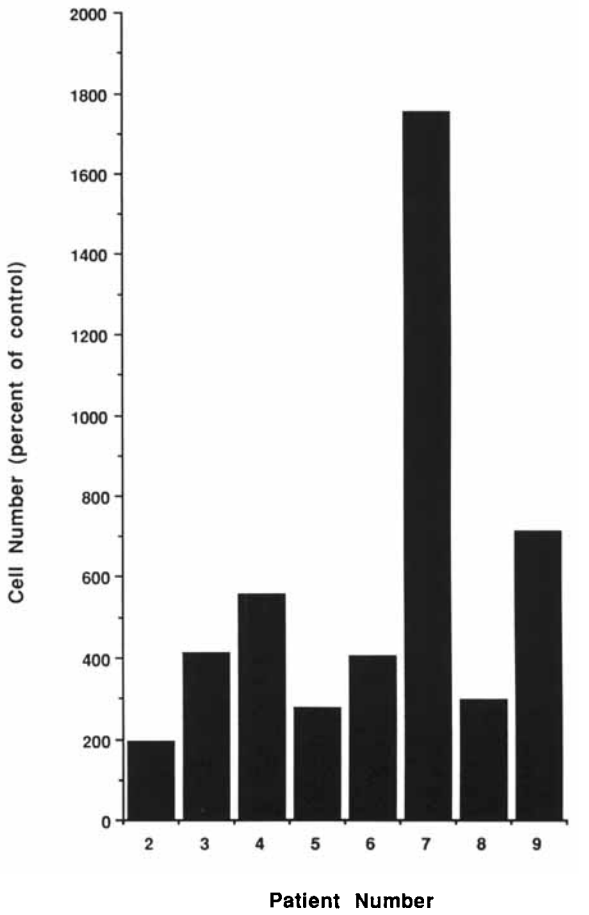

Fig. 4. Cultured human prostate cells in Mixed Medium supplemented with 2 5% FBS were plated into 6-well culture plates at a density of 5 × 10^4 cells/well After 72 h in culture, baseline cell counts were measured and the cells were then exposed to Mixed Medium supplemented with 2 5% FBS and PDGF-BB (0–50 ng/ml) and incubated at 37°C Cell counts of representative wells were performed on days 2, 4, and 7 The effects of PDGF on proliferation were expressed as a percent of unstimulated controls The bars represent the maximal response observed when the cells were exposed to 50 ng/ml PDGF as measured on day 7 and are an average of two duplicate samples

only was [^{125}I]-PDGF covalently bound to cellular receptors in the presence of disuccinimidyl suberate (lanes 2–4), but nonlabeled PDGF competed for the receptor sites and inhibited the binding of the [^{125}I]-labeled material (lanes 5–7). Saturation curves revealed that the number of PDGF-β receptors on the various human prostatic stromal cells ranged from 1.80 to 5.21 × 10^5 sites/cell (Table I).

DISCUSSION

Platelet-derived growth factor is released during inflammation and is a potent mitogen for stromal cells [Ross et al., 1986]. In order to examine the potential role of PDGF in the development of prostatic hyperplasia, cultures of human prostatic cells were established and were examined for their capacity to bind and respond to PDGF. The results of these studies indicate that cultured human prostatic cells express high affinity PDGF receptors and that PDGF is capable of activating the PDGF signal transduction pathway and subsequently stimulating cellular proliferation, supporting the concept that inflammation may be an etiological factor in the development of prostatic hyperplasia.

Treatment of cells derived from hyperplastic human prostatic tissue with PDGF-BB caused

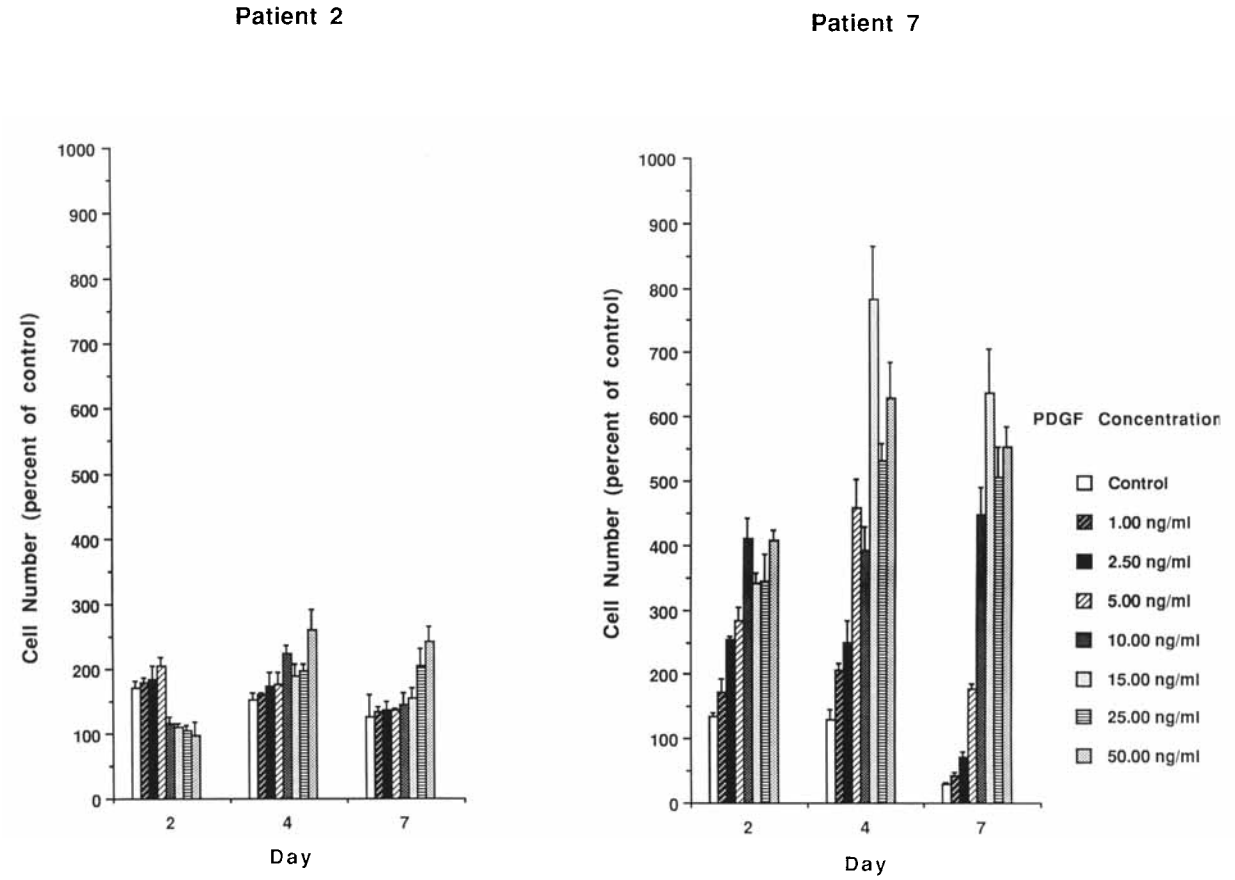

Fig. 5. A comparison of the PDGF-induced cellular proliferation in two human prostate cell lines. Note the difference in capacity of PDGF to induce the proliferation of the two cell lines. Mean ± SEM from six replicate samples.

an activation of the PDGF-receptor signal transduction cascade as determined using antiphosphotyrosine immunoprecipitations. Stimulation of the PDGF-receptor tyrosine kinase causes autophosphorylation of the receptor [Kazlauskas and Cooper, 1989] as well as tyrosine phosphorylation of a number of proteins including phospholipase Cγ, [Kumjian et al., 1989; Morrison et al., 1990], GTPase activating protein [Kaplan et al., 1990], the 85 kDa regulatory subunit of PtdIns 3-kinase [Kazlauskas and Cooper, 1990; Escobido et al., 1991a], and the serine/threonine kinase raf [Morrison et al., 1991]. Several of these phosphoproteins were detected in antiphosphotyrosine immunoprecipitations of PDGF-stimulated primary human prostatic stromal cells that were absent in unstimulated cells, including labeled bands corresponding in molecular weight to the autophosphorylated receptor (185 kDa), the regulatory subunit of the PtdIns 3-kinase (85 kDa), as well two bands around 120 and 140 kDa that may correspond to phospholipase Cγ and GTPase activating protein, respectively. In addition to the presence of the 85 kDa phosphoprotein, the antiphosphotyrosine immunoprecipitates from PDGF-stimulated cells contained elevated levels of PtdIns 3-kinase activity that was not present in unstimulated cells. PtdIns 3-kinase associates with a number of proteins having intrinsic or associated tyrosine kinase activities, including the PDGF-receptor [Kazlauskas and Cooper, 1990; Escobido et al., 1991]. Mutants of the PDGF-receptor that lack the PtdIns 3-kinase binding site still bind PDGF, stimulate tyrosine kinase activity, and phosphorylate GTPase activating protein and phospholipase Cγ; however, PtdIns 3-kinase activity is missing and cells expressing these mutant receptors fail to respond to mitogens with increased DNA synthesis and cell division, suggesting an important role for PtdIns 3-kinase in mitogenesis [Williams, 1989]. To date, however, the physiological role of PtdIns 3-kinase or its products has not been elucidated, although there is some

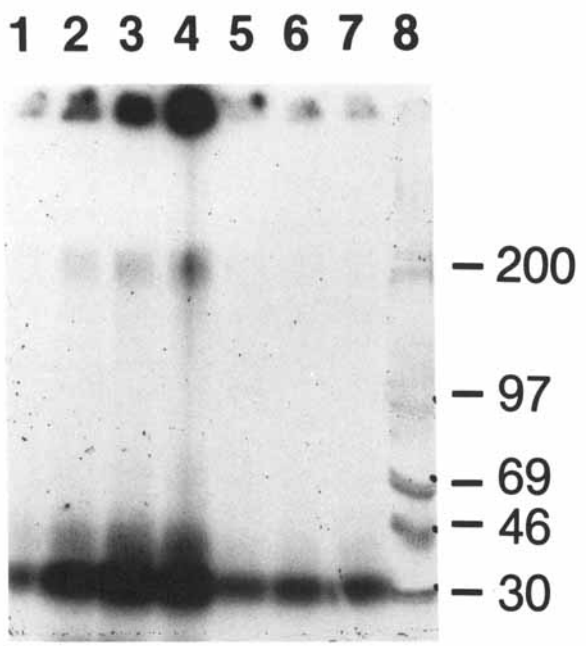

Fig. 6. Cross-linking of [^{125}I]-PDGF-BB to PDGF-β receptors from stromal prostatic cells on 4–20% gradient SDS-PAGE **Lane 1:** [^{125}I]-PDGF control **Lanes 2–4:** Cells treated with [^{125}I]-PDGF (30 ng/ml) **Lanes 5–7:** Cells treated with [^{125}I]-PDGF plus nonlabeled PDGF (1 mg/ml) **Lane 8:** [^{14}C]-molecular weight markers myosin (200 kDa), phosphorylase b (97 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) Receptor-PDGF monomer complex migrates to 200 kDa, while the radioactivity associated with the top of the gel may represent the dimeric complex Unbound [^{125}I]-PDGF migrates with the gel front

suggestion that the PtdIns-3-phosphates are involved in the regulation of cytoskeletal structure [Eberle et al., 1990].

Tissues used in these experiments were derived from patients being treated for prostatic hyperplasia. Histological evaluation of the surgical specimens revealed the heterogeneous nature of the disease particularly with respect to the degree of both hyperplasia and inflammation (Table I).

An examination of the data by analysis of variance has revealed a number of potentially interesting relationships. The expression of PDGF receptors by prostatic cells and the capacity of PDGF to activate the signal transduction pathway were relatively constant. Basal doubling times tended to increase with age. Since the cells used in these experiments were derived from patients being surgically treated for BPH, it is not unreasonable to suspect that individuals being treated at an older age would have a slower growing gland. The presence and degree of inflammation observed in histologic sections was also associated with more rapidly growing cells in culture. The size of the resected tissue was inversely related to the capacity of PDGF to stimulate thymidine incorporation and cell proliferation. This observation may be indicitive of a gland which is undergoing relatively rapid growth and the capacity of PDGF to induce further stimulation may be limited. The ability of PDGF to stimulate thymidine incorporation was, however, related to the number of cellular PDGF receptors.

In these experiments, human prostatic stromal cells were characterized for their response to only the BB form of PDGF. Preliminary in situ hybridization experiments revealed that these cells expressed the message for PDGF-β receptors but not the message for PDGF-α receptors [Hirsch et al., 1991]. In addition, it has previously been shown that PDGF-BB, but not PDGF-AA, is a potent mitogen of human fibroblasts [Nister et al., 1988]. By the activation of the tyrosine kinase response and subsequent stimulation of PtdIns 3-kinase activity, these results demonstrate the functionality of the receptor. A dose-dependent increase in thymidine incorporation and cellular proliferation was also observed. The expression of PDGF receptors by prostatic cells was confirmed in binding studies and through the specific cross-linking of [^{125}I]-PDGF. These findings represent the first observation that primary prostatic stromal cells derived from patients exhibiting BPH respond to PDGF-BB, and are consistent with the role of growth factors and inflammation in other chronic inflammatory diseases.

Since a relatively small number of patients was evaluated in this study, the significance of the data should be viewed with caution. These data do, however, provide the basis for further research. Additional experiments will need to be conducted in order to characterize the nature of the cellular interactions which occur between epithelial and stromal cells, as well as the role of other growth factors (including other forms of PDGF) and other mediators of inflammation in the development of hyperplasia. Most importantly, experiments should be conducted to critically evaluate the potential importance of inflammation in the development of prostatic hyperplasia. If a role for prostatic inflammation in the development of BPH can be confirmed, it may be advisable to incorporate the treatment of inflammation in the clinical management of the disease.

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REFERENCES

Antoniades HN (1991) PDGF A multifunctional growth factor Balliere's Clin Endocrin Metab 5 595–613

Auger KR, Serunian LA, Soltoff SP, Libby P, Cantley LC (1989) PDGF dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells Cell 57 167–175

Cohen B, Liu Y, Drucker B, Roberts TM, Schaffhausen BS (1990) Characterization of pp85, a target of oncogenes and growth factor receptors Mol Cell Biol 10 2909–2915

De Gaetano G, Cerletti C, Nanni-Costa MP, Poggi A (1989) The blood platelet as an inflammatory cell Eur Respir J Suppl 6 441s

Eaton CL, Davies P, Phillips MEA (1988) Growth factor involvement and oncogene expression in prostatic tumors J Steroid Biochem 30 341–345

Eberle M, Traynor-Kaplan AE, Sklar LA, Norgauer J (1990) Is there a relationship between phosphatidylinositol trisphosphate and F-actin polymerization in human neutrophils? J Biol Chem 265 16725–16728

Eble JN, Epstein JI (1990) Stage A carcinoma of the prostate In Bostwick DG (ed) "Pathology of the Prostate" New York Churchill-Livingstone, pp 61–82

Escobido JA, Kaplan DR, Kavanaugh WM, Turck CW, Williams LT (1991) A phosphatidylinositol-3 kinase binds to platelet-derived growth factor receptors through a specific receptor sequence containing phosphotyrosine Mol Cell Biol 11 1125–1132

Hirsch KS, Jones JA, Regan JR, Eble JN, Mayne NG, Falcone JF, Gehlert DR (1991) Identification and characterization of the PDGF receptors in cells derived from human benign prostatic hyperplasia J Cell Biochem Suppl 15B 105

Jinno H, Udea K, Otaguro K, Kato T, Ito J, Tanaka R (1986) Prostate growth factors in the extracts of benign prostatic hypertrophy Eur Urol 12 41–48

Kaplan DR, Whitman M, Schaffhausen BS, Pallas DC, White M, Cantley LC, Roberts TM (1987) Common elements in growth factor stimulation and oncogenic transformation 85 Kd phosphoprotein and phosphatidylinositol kinase activity Cell 50 1021–1029

Kaplan DR, Morrison DT, Wong G, McCormick F, Williams LT (1990) PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex Cell 61 125–133

Kazlauskas A, Cooper JA (1989) Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins Cell 58 1121–1133

Kazlauskas A, Cooper JA (1990) Phosphorylation of the PDGF receptor β subunit creates a tight binding site for phosphatidylinositol 3 kinase EMBO J 9 3279–3286

Kohnen PW, Drach GW (1979) Patterns of inflammation in prostatic hyperplasia a histologic and bacteriologic study J Urol 121 755–760

Kumjian DA, Wahl MI, Rhee SG, Daniel TO (1989) Platelet-derived growth factor (PDGF) binding promotes physical association of PDGF receptor with phospholipase C Proc Natl Acad Sci USA 86 8232–8236

Kyprianou N, Isaacs JT (1988) Identification of a cellular receptor for transforming growth factor-β in rat ventral prostate and its negative regulation by androgens Endocrinology 123 2124–2131

McKeehan WL, Adams PS, Rosser MP (1984) Direct mitogenic effect of insulin, epidermal growth factor, glucocorticoids, cholera toxin, unknown pituitary factors, and possibly prolactin, but not androgens on normal rat prostate epithelial cells in serum-free primary cultures Cancer Res 44 1998–2010

McNeal JE (1990) The pathobiology of nodular hyperplasia In Bostwick DG (ed) "Pathology of the Prostate" New York Churchill-Livingstone, pp 31–36

Morrison DK, Kaplan DR, Rhee SG, Williams LT (1990) Platelet-derived growth factor (PDGF)-dependent association of phospholipase C-γ with the PDGF receptor signaling complex Mol Cell Biol 10 2359–2366

Morrison DK, Kaplan DR, Escobido JA, Rapp UR, Roberts TM, Williams LT (1991) Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF β-receptor Cell 58 649–657

Mydlo JH, Michaeli J, Heston WDW, Fair WR (1988) Expression of basic fibroblast growth factor mRNA in benign prostatic hyperplasia and prostatic carcinoma Prostate 13 241–247

Nister M, Hammacher A, Mellstrom K, Siegbahn A, Ronnstrand L, Westermark B, Helden C-H (1988) A glioma-derived PDGF A chain homodimer has different functional activities than a PDGF AB heterodimer purified from human platelets Cell 52 791–799

Ross RA, Raines EW, Bowen-Pope DF (1986) The biology of platelet-derived growth factor Cell 46 155–169

Shimokado K, Raines EW, Madtes DK, Barrett TB, Benditt EP, Ross RA (1985) A significant part of macrophage-derived growth factor consists of at least two forms of PDGF Cell 43 277–286

Takemura R, Werb Z (1984) Secretory products of macrophages and their physiological functions Am J Physiol 246 C1–C9

Whitman M, Kaplan DR, Schaffhausen B, Cantley L, Roberts TM (1985) Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation Nature 315 239–242

Whitman M, Downes CP, Keeler M, Keller T, Cantley L (1988) Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate Nature 332 644–646

Williams LT, Escobedo JA, Keating MT, Coughlin SR (1988) Signal transduction by the platelet-derived growth factor receptor Cold Spring Harbor Symposium on Quantitative Biology LIII 455–465