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Antiproliferative Effect in Human Prostatic Smooth Muscle Cells by Nitric Oxide Donor

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ABSTRACT

We obtained a primary culture of prostatic cells through explantation from patients with benign prostatic hyperplasia. Structural morphology, immunohistochemical staining, and growth characteristics of these cells demonstrate that they are consistent with the population of smooth muscle cells (SMCs). We examined the influence of a nitric oxide donor, sodium nitroprusside (SNP), on the regulation of human prostatic SMC proliferation. SNP exhibited a concentration-dependent (0.1–10 μM) inhibition of fetal calf serum-induced proliferation in human prostatic SMCs. In addition, growth-inhibitory responses to 8-bromo-cGMP (1–30 μM) were observed. However, the responses to SNP were significantly diminished by the presence of 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (3 μM; a selective guanylate cyclase inhibitor). Furthermore, SNP induced an increased concentration-dependent accumulation of intracellular cGMP in human prostatic SMCs. After 48-hr period of deprivation of serum, cells were restimulated with serum to permit cell cycle progression. The addition of SNP (10 μM) at various times after the addition of serum to serum-deprived cells showed maximal inhibition of cell proliferation even when added 6 hr after the serum. This blocking effect of cell cycle progression was lost gradually as the delay from serum to SNP application increased from 6 to 18 hr. The membrane-associated protein kinase C (PKC) activity was studied in human prostatic SMCs; results showed that fetal calf serum (10%, v/v) significantly increased membrane-associated PKC activity. SNP (10 μM), which had little effect on basal kinase activity, completely abolished serum-induced augmentation of PKC activity. Therefore, we suggest that SNP mediates its antiproliferative effect by the inhibition of PKC activity on human prostatic SMCs; furthermore, its antiproliferative effect occurs at the early G_1 phase of the cell cycle.

BPH is one of the most common diseases in elderly men and is characterized by abnormal proliferation of the stromal and epithelial cells of the prostate. Despite the high frequency of its occurrence, we still have little insight into the fundamental causes of BPH at the cellular level. SMCs constitute a major cellular component of prostatic stroma (Shapiro *et al*., 1992). In addition, SMC proliferation and tension play important roles in bladder outflow obstruction secondary to BPH (Tenniswood *et al*., 1992; Moriyama *et al*., 1994; Guh *et al*., 1995). Therefore, it is important to know the physiological and pathological effects and their mechanisms of action in prostatic SMCs.

Recently, Burnett *et al*. (1995) used biochemical and immunohistochemical analyses to characterize the localization of NOS in the human prostate. Their results revealed NOS activity in this tissue, and it is proposed that NO may modulate smooth muscle tone in the human prostate. In addition, Takeda *et al*. (1995) conducted functional pharmacological experiments and used electrical field stimulation to investigate the effects of NO on human prostates. They provided evidence that NO plays a role in mediation of the contractile function of human prostate. In addition to the roles on contractile activity, NO and NO donor have roles in growth regulation of several types of cells. They exhibit antiproliferative effects on rat vascular SMCs (Nakaki *et al*., 1990; Shimizu *et al*., 1991; Etienne *et al*., 1996), bovine pulmonary arterial SMCs (Lee *et al*., 1996), and rabbit aortic SMCs (Kariya *et al*., 1989). Nevertheless, there has been no study to investigate whether NO, endogenously synthesized in human prostate, plays a role in the regulation of human prostatic SMC proliferation.

Recently, we successfully used the explant culture method for the selective cultivation of human prostatic SMCs. These cultures are used to provide a useful model with which to investigate their roles in the development of BPH. The purpose of the current study was to investigate the effect of SNP,

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ABBREVIATIONS: BPH, benign prostatic hyperplasia; SMC, smooth muscle cell; NO, nitric oxide; SNP, sodium nitroprusside; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; FCS, fetal calf serum; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; PKC, protein kinase C; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

a NO donor, on the growth regulation of human prostatic SMCs. The mechanism of action and signal transduction occurring after the challenge to SNP also were examined.

Experimental Procedures

Materials. RPMI-1640 medium and all other tissue culture reagents were obtained from GIBCO (Grand Island, NY). Testosterone; mouse anti-vimentin, anti-cytokeratin, anti-smooth muscle α-actin, anti-desmin, and fluorescein-conjugated goat anti-mouse IgG; MTT; β-isopropanol; 8-bromo-cGMP; ODQ; EDTA (disodium salt); EGTA; and SNP were purchased from Sigma Chemical (St. Louis, MO). cGMP enzyme immunoassay kits were obtained from Cayman Chemical (Ann Arbor, MI). [γ-^{32}P]ATP (3000 Ci/mmol) and PKC enzyme assay kits were purchased from Amersham International (Buckinghamshire, UK). Apoptosis detection kits were from Upstate Biotechnology (Lake Placid, NY).

Human prostate tissues. Human hyperplastic prostates were obtained during surgery from four men through transurethral resection of the prostate. All these patients had histories of prostatism and were diagnosed as having BPH on the basis of the combination of rectal digital examinations, transrectal sonography of the prostate, and urodynamic studies (including uroflowmetry, urethral pressure profile, and cystometry).

Tissue explants and subcultures. Tissue specimens were immediately placed into a sterile Petri dish and minced into small pieces of ~2 × 2 × 2 mm under a laminar flow hood. The minced tissue pieces then were transferred into a 15-ml polypropylene centrifuge tube containing 3 ml of 0.1% collagenase type 1 in HBSS, pH 7.4, and bubbled with a mixture of 5% CO_2/95% O_2 at 37° for 40 min. The tube was subjected to a rotator at a speed of 300 rpm for 3 min. After a brief settlement, the supernatant fraction was discarded, and the tube was filled with 3 ml of 0.1% trypsin in PBS in the above condition for 10 min. The tissue pieces were centrifuged at a speed of 1000 rpm for 3 min, and then the pieces were washed twice with HBSS and transferred to sterile flasks, which were precoated with 10 μg/ml collagen type 1, containing RPMI-1640 medium supplemented with 10% FCS (v/v), penicillin (100 units/ml)/streptomycin (100 μg/ml), amphotericin B (2.5 mg/ml), and testosterone (10 nM). Cultures were maintained in a humidified incubator at 37° in 5% CO_2/air. Usually with 5–7 days, successfully attached explants would have cells emanating around the tissue. After 1 week, the medium was changed and thereafter changed every 3 days. Once the cells in the flask reached confluence (~2–3 weeks), the explant was transferred to another flask and cultured to reach confluence. After this cultivation procedure was followed for five times, the cells that grew out of the explant were trypsinized from the fifth culture flask for subculture and split into a 1:3 ratio as soon as monolayers became confluent.

Immunofluorescence. Isolated human prostatic cells were plated onto a chamber slide (Falcon, Cowley, UK), cultured for 48 hr, and fixed with 100% methanol for 5 min. After washing (three times) with PBS, fixed cells were incubated (37°) with anti-vimentin, anti-cytokeratin, anti-smooth muscle α-actin, or anti-desmin IgG (dilution, 1:50–100) for 40 min. Cells were again washed (three times) with PBS and incubated with fluorescein-conjugated goat anti-mouse IgG (dilution, 1:100) for 40 min. Green fluorescence was evaluated using a fluorescence microscope (Nikon).

Cell proliferation assay. The cell proliferation assay was carried out using the MTT assay described by Mosmann (1983). MTT was dissolved in PBS at a concentration of 5 mg/ml and filtered (Millipore, Bedford, MA). From this stock solution, 10 μl/100 μl of medium was added to each well, and plates was gently shaken and incubated at 37° for 4 hr. The treatment of living cells with MTT produces a dark-blue formazan product, whereas no staining is observed in dead cells. After the loading of MTT, the medium was replaced with 100 μl of acidified (0.04 M KCl) β-isopropanol and maintained for 20–30 min at room temperature for color development; then, the 96-well plate was read with an enzyme-linked immunosorbent assay reader (570 nm) to obtain the absorbance values.

For measurement of the effects of SNP and 8-bromo-cGMP, quiescent cells were stimulated by 10% FCS in the absence or presence of indicated agents for 48 hr. After the incubation period, cells were washed twice with PBS, and cell proliferation was assayed by MTT method. Results are expressed as a percentage increase over control, which represents the pretreatment cell density.

Assay of cGMP contents. Confluent and quiescent cultures were washed three times with PBS (37°) and then incubated in 1 ml of RPMI-1640 medium in the absence or presence of the indicated agents for 5 min. At the end of the specified incubation period, the medium was removed rapidly, and 0.5 ml of ice-cold trichloroacetic acid [10% (w/v)]/4 mM EDTA was added to the wells. After incubation for 30 min at 4°, cells were scraped, and the resulting suspensions were centrifuged at 10,000 × g for 5 min. The supernatants were removed and extracted four times with 3 volumes of ether. Next, the cGMP contents were assayed using enzyme immunoassay kits. Results are expressed as fmol/5 × 10^4 cells.

Assay of PKC activity. Human prostatic SMCs were incubated in RPMI-1640 medium in the absence or presence of SNP (10 μM) for 15 min at 37°. FCS [10% (v/v)] was added, and the cells were incubated for an additional 10 min. The cells were washed twice with ice-cold PBS, scraped into 0.5 ml of buffer A (0.25 M sucrose, 20 mM HEPES, 2 mM EDTA, 5 mM EGTA, 10 mM 2-mercaptoethanol, 0.21 mM leupetin, and 2 mM phenylmethylsulfonyl fluoride, pH 7.5), and ultrasonically disrupted (20 sec at setting 5; Vibra Cell, Sonics and Materials, Danbury, CT) at 4°. The homogenate was centrifuged at 100,000 × g for 20 min at 4°. The pellet (particulate fraction) was resuspended in 0.5 ml of buffer A containing 0.2% Triton X-100, incubated at 4° for 60 min, and centrifuged for 30 min at 50,000 × g at 4°. The supernatant was used as a crude membrane extract. The crude extract was loaded onto a DEAE-cellulose DE-52 column (Whatman, Maidstone, UK) in a volume of ~0.5 ml. The column was washed with 1.5 ml of equilibration buffer (20 mM Tris·HCl, 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.5) and 12.5 ml of equilibration buffer containing 20 mM NaCl. PKC was eluted with 1.5 ml of equilibration buffer containing 120 mM NaCl, and PKC activity was measured using a commercially available kit (Amersham). Protein kinase assay mixture contained the enzyme preparation (25 μl), reaction mixture (25 μl of 3 mM calcium acetate, 75 μg/ml phosphatidylserine, 6 μg/ml phorbol-12-myristate-13-acetate, 225 μM peptide, 7.5 mM dithiothreitol, 50 mM Tris·HCl), and 25 μl of [γ-^{32}P]ATP (10 μCi/ml) in a final volume of 75 μl. After a 15-min incubation at 37°, reactions were terminated by the addition of 10 ml of 300 mM orthophosphoric acid. Terminated reaction mixture (35 μl) was transferred onto the paper disc and dried for 5 min. The paper disc was washed twice with 10 ml of orthophosphoric acid (75 mM). The PKC activity is calculated as P = T × 1000/IR pmol/min, where T is total phosphate transferring to peptide, I is the incubation time (min), and R is the specific radioactivity/nmol of ATP. PKC activity was expressed as pmol of ^{32}P incorporated into peptide/min/10^6 cells.

***In situ* labeling of apoptotic cells.** A commercial kit for detection of apoptotic cells was used in this study. Cells were cultured on a chamber slide for 24 hr and then made quiescent by serum deprivation for 48 hr. The cells were switched to FCS-containing medium, with or without SNP (10 μM) for 48 hr. After removal of the medium, the cells were washed twice with PBS, fixed for 1 min with ice-cold ethanol/acetic acid (1:1) solution, and then washed three times with PBS. The fixed cells were permeabilized in ice-cold 0.2% Triton X-100 detergent for 5 min and then washed three times with PBS. Staining was carried out according to the protocol provided by the supplier (Upstate Technology). The TUNEL method identifies apoptotic cells *in situ* by using terminal deoxynucleotidyl transferase to transfer biotin-dUTP to the free 3′-OH of cleaved DNA. The biotin-labeled cleavage sites then are visualized reaction with fluorescein

conjugated avidin (avidin-fluorescein isothiocyanate). Photomicrographs were obtained with a fluorescence microscope (Nikon).

Statistical analysis. Data are presented as the mean ± standard error for the indicated number of separate experiments. Statistical significance between drug-treated and untreated groups was evaluated by unpaired two-tailed Student's t test, and values of $p < 0.05$ were considered significant.

Results

Identification of cultured prostatic cells. It has been well reported that the human prostatic epithelial cells can successfully proliferate in WAJC 404 medium rather than in RPMI-1640 medium (Chaproniere and McKeehan, 1986; Fong *et al*., 1991). By taking advantage of the different nutritional requirements for stromal cells compared with that of epithelial cells, stromal cells were successfully separated and cultured in RPMI-1640 medium containing 10% FCS in this study. The purity of cultured prostatic stromal cells was assessed by light microscopy and immunofluorescence staining of vimentin, cytokeratin, smooth muscle α-actin, and desmin. Cultured prostatic stromal cells exhibited a spindle-shaped morphology (Fig. 1A, C, E, and G) and demonstrated positive staining for vimentin (Fig. 1B), an intermediate filament polypeptide that is specific for mesenchymal cells. Prostatic epithelial cell contamination was ruled out by negative immunostaining with epithelial cytokeratins (Fig. 1D). Furthermore, the stromal cells exhibited immunoreactivity with smooth muscle α-actin (Fig. 1F) and desmin (Fig. 1H); this staining pattern is consistent with smooth muscle differentiation. The cells did not exhibit contact inhibition of growth because they proliferated at a steady rate. In addition, culture morphology was characterized by the formation of nodules of cells, that is, "hills and valleys" (data not shown), similar to the configuration demonstrated by vascular SMCs *in vitro* (Ross and Kariya, 1980). It reveals that these cells are the population of prostatic SMCs.

Correlation between cell number and absorbance value for MTT assay. We measured the correlation between cell number and absorbance value for MTT assay of prostatic SMCs. As shown in Fig. 2, there was a proportional increase in the absorbance values parallel to the increase in cell numbers with an r value of 0.997. These results indicate a very good correlation between the absorbance value and cell number and demonstrate that this MTT assay is suitable for the measurement of cell population.

Inhibition of cell proliferation by SNP and 8-bromo-cGMP. We examined the effect of SNP on the modulation of cell proliferation. Prostatic SMCs were made quiescent in serum-free RPMI-1640 medium for 48 hr before restimulation by the addition of serum. As shown in Fig. 3, FCS [10% (v/v)] significantly induced cell proliferation by 92.4%, 103.4%, 131.3%, and 112.5% above the pretreatment cell density for patient A, B, C, and D, respectively. SNP exhibited a concentration-dependent (0.1–10 μM) inhibitory action on FCS-induced cell proliferation. However, SNP had various degrees of antiproliferative potency in cultured cells from different patients with BPH; the maximal reduction in proliferation induced by SNP was 66.2%, 39.3%, 21.3%, and 33.3% for patient A, B, C, and D, respectively (Fig. 3). The effect of ODQ, a selective guanylate cyclase inhibitor, on SNP action also was examined. ODQ (3 μM), which alone did not modify the cell growth, significantly shifted upward the SNP-induced inhibition of cell proliferation (Fig. 4).

Because it is generally thought that the effects of SNP are mediated through cGMP production as the second messenger, we tested the effect of 8-bromo-cGMP on FCS-induced cell proliferation in human prostatic SMCs. In this study, 8-bromo-cGMP had little effect on cell viability at a high concentration (30 μM) (97.0 ± 6.0% survival compared with pretreatment control) but concentration-dependently inhibited (1–30 μM) the FCS-induced cell proliferation with a maximal inhibition of 46.9%, 46.5%, 32.2%, and 35.0% for patient A, B, C, and D, respectively (Fig. 5).

Effect of SNP on cellular cGMP accumulation. We measured the cellular cGMP content in RPMI-1640 medium, which is essentially the same medium used for the experiments on cell growth inhibition. SNP induced a concentration-dependent (0.1–3 μM) increase in intracellular cGMP content (Table 1). It showed that the concentration ranges for the stimulation of cGMP accumulation roughly corresponded with those for the inhibitory effects on cell proliferation.

Effect of SNP on serum-stimulated cell cycle. To determine when in the cell cycle SNP exerted its inhibitory effect on proliferation, subcultured SMCs were made quiescent by serum deprivation for 48 hr before restimulation with serum. SNP (10 μM) was added at various times after the addition of FCS [10% (v/v)] to serum-deprived cells. The data showed that the maximal inhibitory effect of cell proliferation occurred only when SNP was present within the first 6 hr after the readdition of serum and there was no significant effect on cell proliferation when SNP was added 18 hr after the serum refeeding (Fig. 6).

Effect of SNP on membrane-associated PKC activity. To elucidate whether the antiproliferative action of SNP involved a PKC-dependent pathway, quiescent human prostatic SMCs were stimulated by FCS [10% (v/v)], and protein kinase activity associated with the particulate fraction was measured. As a result, serum significantly increased the membrane-associated PKC activity (6.35 ± 1.02 pmol/min/10^6 cells, four experiments, $p < 0.01$ compared with basal activity of 1.38 ± 0.27 pmol/min/10^6 cells) in human prostatic SMCs. SNP (10 μM), which alone did not affect basal PKC activity (1.34 ± 0.28 pmol/min/10^6 cells), completely abolished the serum-induced augmentation of membrane-associated PKC activity (1.36 ± 0.04 pmol/min/10^6 cells, four experiments) (Fig. 7) This reveals that the SNP action likely is due mainly to the inhibition of a PKC-dependent pathway to cause the cell growth inhibition.

Effect of SNP on cell viability and apoptosis. The MTT assay method, trypan blue exclusion test, and TUNEL technique were used to examine the effect of SNP on cell viability and apoptosis. The results showed that the addition of SNP (10 μM) to human prostatic SMCs for 48 hr neither influenced the cell viability (98.8 ± 2.1% and 97.5 ± 1.2% survival compared with respective control by MTT assay and trypan blue exclusion test) nor induced the apoptosis (data not shown). These data suggest that SNP does not mediate its antiproliferative action via the cytotoxic effect and the induction of apoptosis.

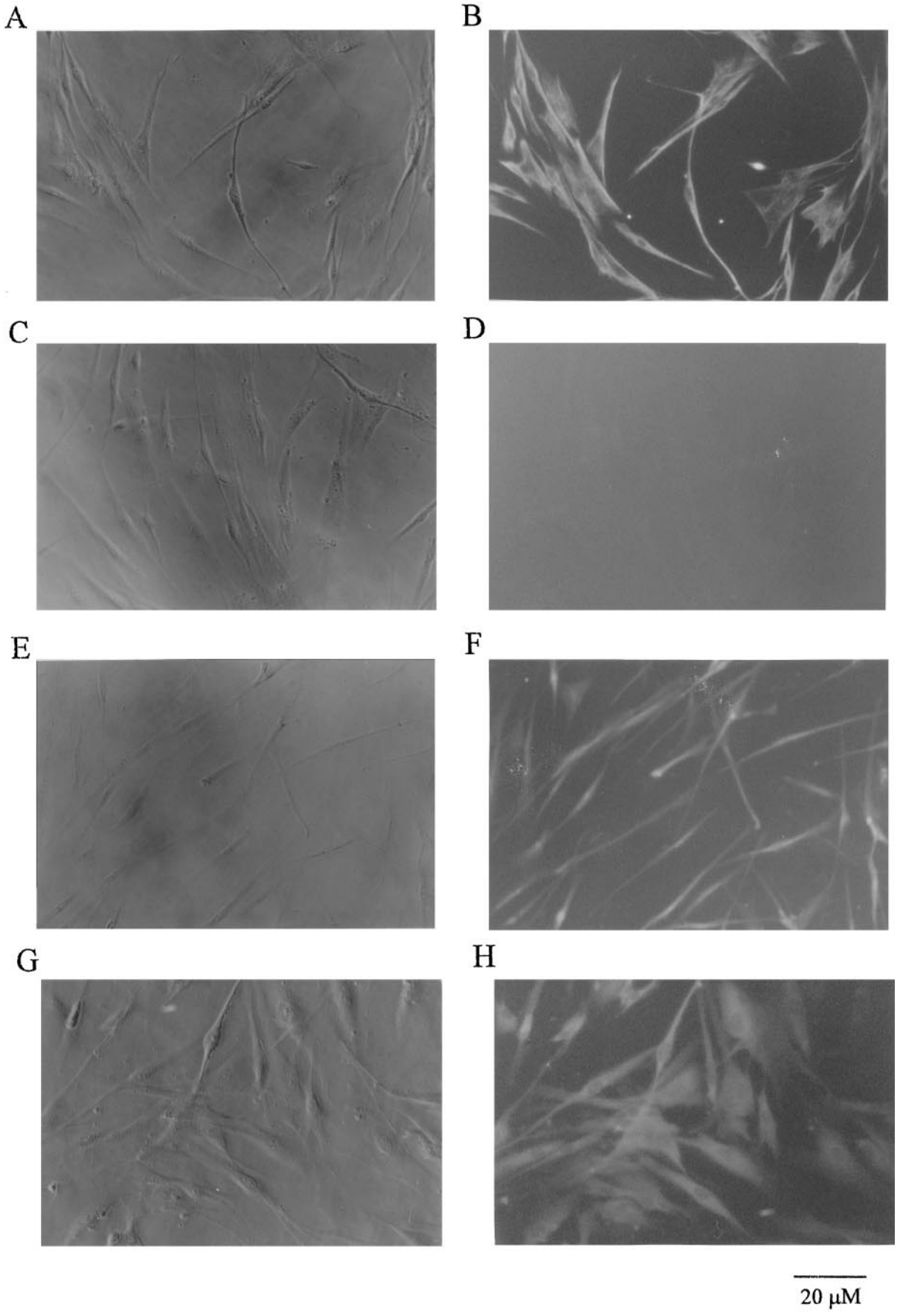

Fig. 1. Characterization of cultured human prostatic cells. Cells were plated onto a chamber slide and cultured for 48 hr. Cultured cells were stained with antibodies to vimentin, cytokeratine, smooth muscle α-actin, and desmin, as described in Experimental Procedures. Prostatic cells (A, C, E, and G) were immunostained with anti-vimentin (B), anti-cytokeratin (D), anti-smooth muscle α-actin (F), and anti-desmin (H), respectively.

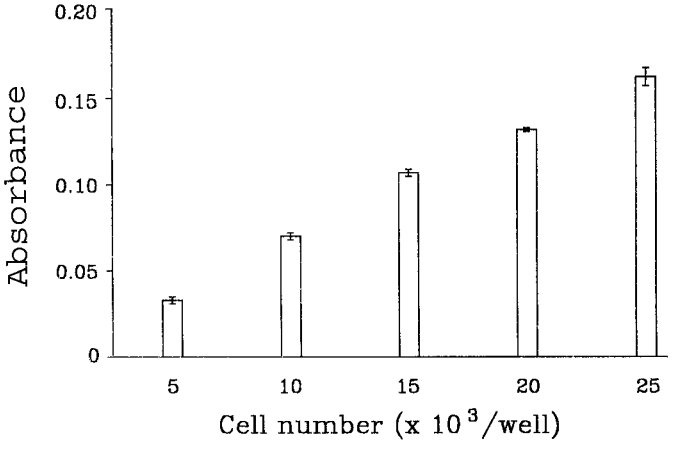

Fig. 2. Correlation between cell number and absorbance of prostatic SMCs; the absorbance of cells were measured by the MTT assay method. The coefficient of correlation value for these two variables was 0.997. Data are mean ± standard error of six determinations (each in triplicate).

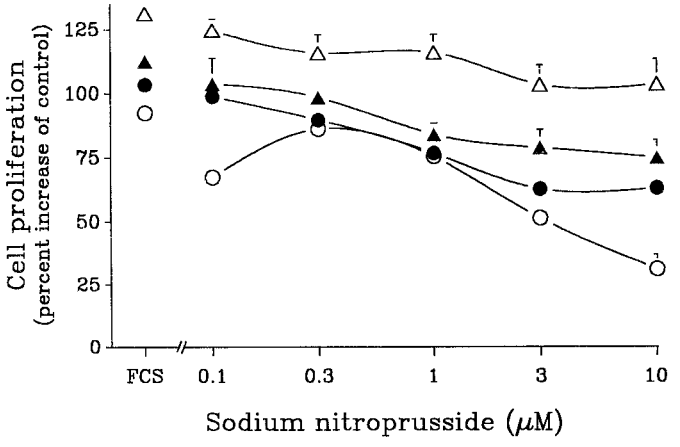

Fig. 3. Effect of SNP on FCS-induced cell proliferation in human prostatic SMCs. Quiescent cells were stimulated by 10% FCS in the absence or presence of SNP for 48 hr. Cell proliferation was assayed by the MTT assay method as described in Experimental Procedures. Results are expressed as percentage increase over control, which represents the pretreatment cell density. Data are mean ± standard error of three determinations. *Symbols*, four different patients with BPH.

Discussion

Initially, in this study we describe the isolation and characterization of human prostatic stromal cells. This subculture monolayers exhibited the convex and spindle-shaped morphology, no contact inhibition of growth, and a hill-and-valley growth pattern, which were distinctive from the culture morphology and growth characteristics of fibroblasts. As determined by immunocytochemical analysis of vimentin and cytokeratin in subcultures, the prostatic stromal cell outgrows without contamination of epithelial cells throughout the subcultivation. In addition, the stromal cells were stained positive for smooth muscle α-actin and desmin; these results are indicative of the smooth muscle differentiation. However, the prostatic stromal cells cultured by Roberson *et al*. (1995) stained positive for vimentin and fibronectin, lightly positive for smooth muscle α-actin, and negative for desmin. Roberson *et al*. concluded that their stromal cells were myofibroblasts. Nevertheless, the prostatic stromal cells cultured in our laboratories not only showed the staining pattern of smooth muscle cells (positive staining with smooth muscle α-actin and desmin) but also exhibited some characteristics different from fibroblasts (Guh J-H, Hwang

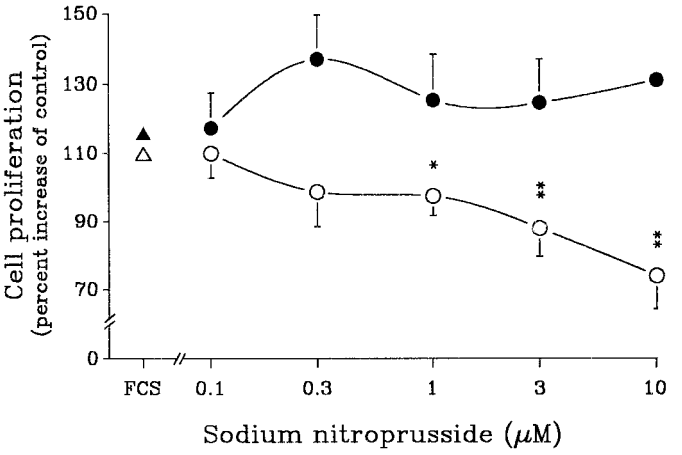

Fig. 4. Effect of ODQ on SNP-induced inhibition of prostatic SMC proliferation. ODQ (3 μM, ▲) exhibited little effect on 10% FCS-induced cell proliferation (FCS alone, △). However, the SNP-induced antiproliferative effect (○) was completely abolished in the presence of ODQ (3 μM, ●). Results are expressed as percentage increase over control, which represents the pretreatment cell density. Data are mean ± standard error of four determinations. *, $p < 0.05$; **, $p < 0.01$ compared with FCS alone (△).

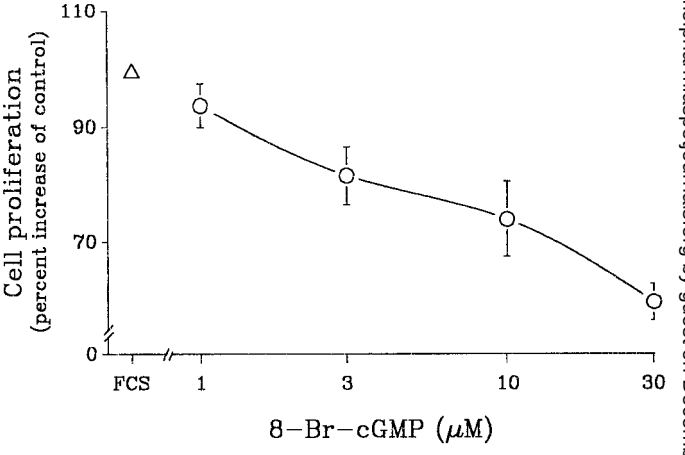

Fig. 5. Effect of 8-bromo-cGMP on FCS-induced cell proliferation in human prostatic SMCs. Quiescent cells were stimulated by 10% FCS in the absence (△) or presence (○) of 8-bromo-cGMP for 48 hr. Cell proliferation was assayed by the MTT assay method as described in Experimental Procedures. Results are expressed as percentage increase over control, which represents the pretreatment cell density. Data are mean ± standard error of four determinations.

TABLE 1

Effect of SNP on cGMP content in human prostatic SMCs.

Cells were seeded onto wells (5×10^4 cells/p well). After a 24 hr incubation, cells were made quiescent for 48 hr by deprivation of serum. SNP was added to the sychronized cells for 5 min; then, cGMP was extracted and quantified by enzyme immunoassay.

	cGMP accumulation
	fmol/5 × 10⁴ cells
Control	4.3 ± 1.4
SNP	
0.1 μM	28.1 ± 10.2
0.3 μM	43.8 ± 12.0
1 μM	57.4 ± 10.3
3 μM	58.1 ± 12.4

Values represent mean ± standard error of four determinations.

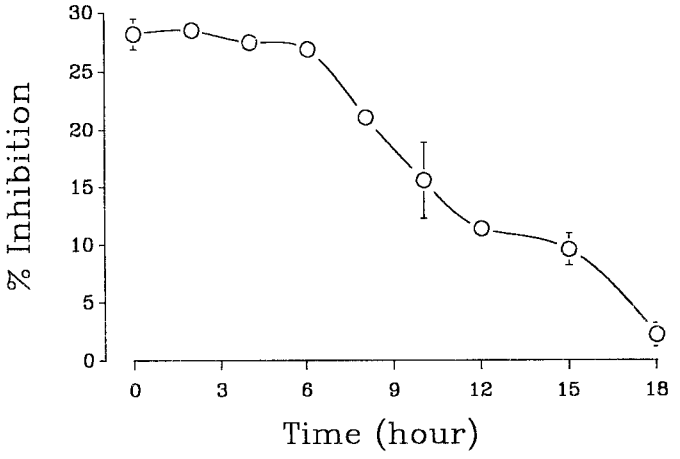

Fig. 6. Time-dependent effect on FCS-induced cell proliferation of the addition of SNP. Subcultured prostatic SMCs were made quiescent by serum deprivation for 48 hr. Cells were exposed to SNP (10 mM) at the indicated times after the addition of 10% FCS. Cell proliferation was assayed by the MTT assay method. Results are expressed as percentage inhibition of FCS-induced cell proliferation. Data are mean ± standard error of three determinations.

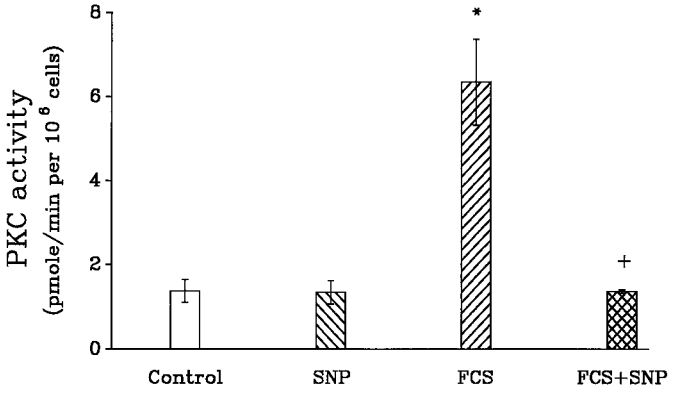

Fig. 7. Membrane-associated PKC activity of human prostatic SMCs. Quiescent human prostatic SMCs were preincubated in RPMI-1640 medium in the absence or presence of 10 μM SNP for 15 min at 37°. FCS [10% (v/v)] was added for an additional 10 min. The membrane-associated PKC activity was determined as described in Experimental Procedures. Data are mean ± standard error of four determinations. *, $p < 0.01$, compared with control. +, $p < 0.001$, compared with FCS alone.

T-L, Teng C-M, unpublished observations), such as (1) basic fibroblast growth factor was a potent stimulator of human prostatic fibroblasts (Sherwood *et al.*, 1992; Story *et al.*, 1993) but exhibited a weak mitogenic effect on cultured prostatic stromal cells in our laboratories, and (2) the addition of an α_1-adrenoceptor agonist (phenylephrine) to our cultured stromal cells induced a significant increase in intracellular Ca^{2+} mobilization, a pharmacological second messenger in human prostatic SMCs. These data combined with the results obtained from light microscopy and immunostaining verification and characteristics of culture morphology revealed that our cultured stromal cells resembled the prostatic SMC population. The use of these cultures allowed us to study the effect of NO, endogenously synthesized in the prostate, on growth regulation of this cell type.

The MTT assay method was developed by Mosmann (1983) in the early 1980s. During the past 14 years, this method has been used for the measurement of both cell proliferation and cytotoxic effect on several types of cells and provides reproducible and accurate measurements of cell killing and proliferation compared with the [^{3}H]thymidine incorporation assay and trypan blue exclusion test. The loading time of MTT and the time for color development were examined to establish the optimal conditions. The results showed that the optimal conditions for these variables were 4 hr and 20–30 min, respectively (data not shown). Further investigations were performed to measure the correlation between cell number and absorbance value to examine whether this MTT assay method provided an accurate determination of human prostatic SMC proliferation. As shown in Fig. 2, a direct correlation was found between these two variables with an r value of 0.997, indicating that the MTT assay could be used for the measurement of prostatic SMC proliferation.

The effect of SNP on cell proliferation was examined. The current results clearly indicate that SNP can effectively inhibit FCS-induced cell proliferation in human prostatic SMCs. In addition, the antiproliferative action of SNP was mimicked by 8-bromo-cGMP, a membrane-permeable cGMP analogue. Furthermore, SNP concentration-dependently induced the cGMP formation. The concentration ranges for the stimulation of cGMP accumulation by SNP were similar to those necessary for the antiproliferative action. These results, combined with the evidence that the antiproliferative action of SNP was completely abolished by ODQ (3 μM, a selective guanylate cyclase inhibitor) (Garthwaite *et al.*, 1995), revealed that the antiproliferative action of SNP was mediated by the formation of cGMP. Although there are findings that the relaxation of vascular muscle in response to NO is mediated by cGMP-dependent and -independent pathways (Moncada *et al.*, 1991; Bolotina *et al.*, 1994), the SNP-induced inhibition of prostatic SMC proliferation in this study apparently occurs via a cGMP-dependent mechanism. However, the antiproliferative potencies of SNP varied among individual prostatic SMCs, which were cultured from different patients with BPH. It was likely due to the varying susceptibility to SNP in the cultured cells of different patients.

It has been suggested that prostaglandin E_1, heparin, interferon-α, and cyclic nucleotide generating agent exert their antiproliferative action through inhibition of the progression from the G_1 into the S phase of the cell cycle in vascular SMCs, because they still inhibit the mitogen-induced DNA synthesis and cell proliferation when added 6–12 hr after the initial exposure of the cells to mitogens (Hoover *et al.*, 1980; Nilsson and Olsson, 1984; Fukumoto *et al.*, 1988; Guh *et al.*, 1996). To define the inhibition point of the cell cycle by SNP, human prostatic SMCs were synchronized to quiescent state by serum deprivation and then stimulated to enter the cell cycle by serum refeeding. The time of SNP (10 μM) exposure to cultured cells was varied relative to the time of serum stimulation. The results indicated that the maximal antiproliferative action occurred only when SNP was present within the first 6 hr after the initial exposure of serum. These data imply that SNP inhibits early events in the G_1 phase of the cell cycle.

PKC has been shown to be a key regulatory enzyme in the signal transduction pathway leading to cell proliferation. It was found that PKC acts as a positive regulator in the transition from the quiescent to a proliferative state of the aortic SMCs (Ohmi *et al.*, 1990) and plays a pivotal role in the signal transduction of platelet-derived growth factor (Ber-

ridge, 1987), epidermal growth factor (Hunter *et al*., 1984), and fibroblast growth factor (Feige and Baird, 1989). Inhibitors of PKC activity such as H7 and staurosporine suppressed cell proliferation induced by phorbol-12-myristate-13-acetate or serum in vascular SMCs (Takagi *et al*., 1988; Matsumoto and Sasaki, 1989; Ohmi *et al*., 1990). We examined the effect of serum on the membrane-associated PKC activity as well as the inhibitory action by SNP in human prostatic SMCs. The data showed that FCS [10% (v/v)] caused a remarkable increase in membrane-associated PKC activity; however, this stimulatory action by FCS was completely abrogated in the presence of SNP (10 μM). In parallel experiments, we examined the effect of PKC down-regulation on serum-induced prostatic SMC proliferation and its influence on antiproliferative effect of SNP. As a result, PKC down-regulation by the treatment of synchronized cells with phorbol-12,13-dibutyrate (1 μM) for 24 hr significantly reduced FCS-induced cell proliferation (~30%). Moreover, SNP was tested and found to be ineffective on the remaining cell proliferation induced by FCS via the PKC-independent pathway (data not shown); that is, the inhibition of FCS-induced cell proliferation by SNP was completely abolished by the inhibition of PKC activity. Therefore, SNP may inhibit cell proliferation through inhibition of PKC-dependent pathway in human prostatic SMCs.

It has been reported that NO exerts cytotoxic activity and causes cell death in several cell types (Nathan, 1992). Recently, a link was proposed between NO formation and apoptosis (Albina *et al*., 1993; Sarih *et al*., 1993; Member *et al*., 1995). To clarify whether SNP induced cell cytotoxic effect and apoptosis in human prostatic SMCs, three independent assay techniques were used. The trypan blue exclusion test and MTT assay were used to measure the effect of SNP on cell cytotoxicity. The results showed that SNP had no cytotoxic effect on prostatic SMCs at a concentration of <10 μM. Furthermore, with the use of TUNEL techniques, SNP (10 μM) also was found to not induce apoptosis in these cultured cells.

Overall, our results suggest that the inhibition of a PKC-dependent reaction is the major mechanism by which SNP suppresses FCS-induced prostatic SMC proliferation. Furthermore, the antiproliferative effect of SNP occurs at the early G_1 phase of the cell cycle. Moreover, there is increasing evidence that the small reduction in prostate size (~30%) significantly improves symptoms in men with BPH (Gormley *et al*., 1992; Stoner, 1992; Nacey, 1994). Although the average antiproliferative effect of SNP was only 41.4%, this could be effective in the relief of obstructive and irritating symptoms in patients with BPH. To date, no differences have been found between growth factor or growth factor receptor levels in human BPH and normal prostatic tissue (Bonnet *et al*., 1983; Glynne-Jones *et al*., 1994). In addition, no endogenous inhibitory modulator in prostatic SMC growth has been found. In the current study, NO is shown to exert an antiproliferative action on human prostatic SMC growth. We suggest it is a endogenous modulator of prostate growth that could play a beneficial role in the restriction of prostate size.

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