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Human Bladder as a Novel Target for Vitamin D Receptor Ligands

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Human prostate is now considered a target for vitamin D receptor (VDR) ligands, such as BXL-628. Because BXL-628 inhibited prostate growth without interfering with androgen signaling, it represents a new option for benign prostate hyperplasia (BPH) therapy. However, BPH symptoms are related not only to prostate size, but also to compensatory bladder hypertrophy and eventual overactivity. We now report that human bladder expresses VDR (determined by real-time PCR immunohistochemistry and Western blot) and responds to VDR agonists, such as the natural ligand, calcitriol, and its synthetic and less hypercalcemic derivative, BXL-628. Experiments were conducted with stromal cells derived from human bladder neck obtained at surgery from BPH patients. BXL-628 counteracted keratinocyte growth factor (KGF) and androgen-induced cell proliferation and stimulated apoptosis with a parallel reduced expression of the survival oncoprotein Bcl-2. Prolonged serum starvation time-dependently pushed bladder stromal cells to express activated myofibroblast markers, such as desmin and smoothelin, without changing other contractile-related proteins and intermediate filaments, such as vimentin. Chronic exposure to BXL-628 prevented starvation-induced cell phenotype modification. Because hypertrophy and starvation-induced bladder remodeling are supposed to underlie bladder overactivity, it is possible that BXL-628 might be helpful in reducing not only cumbersome symptoms related to prostate overgrowth, but also those related to bladder irritation. (*J Clin Endocrinol Metab* 90: 962–972, 2005)

THE AGE OF the world's population is progressively increasing, and today, the average life expectancy for the male population definitively surpasses the seventh decade. Hence, health-related quality of life of elderly men is receiving increasingly more attention. An important medical problem affecting the well-being of the aging male is defined by lower urinary tract symptoms (LUTS), usually caused by an androgen-dependent hyperplastic growth of the prostate gland, *i.e.* benign prostatic hyperplasia (BPH). BPH is an extremely common condition, affecting more than half of the male population over 60 yr of age (1). Essentially, the hyperplastic prostate, by compressing the urethra, decreases bladder outlet and, therefore, urine outflow. In the earliest stages this urethral obstruction is compensated by bladder wall hypertrophy and increased contractile activity. Later, the hypertrophic bladder becomes dysfunctional, preventing complete voiding, and eventually it decompensates, as shown by more frequent nonvoiding contractions and reduced ability to generate efficient voiding pressure. Symptoms such as urinary urgency and frequency, which often impair quality of life, are related to this overactive behavior of the bladder muscular system. Although the pathophysiological mechanisms underlying this end-stage decompensation are not yet clear, a state of relative hypoxia of the overgrown muscle layer might be involved (2).

A major advance in the medical treatment of BPH has been the introduction of finasteride (F), an inhibitor of 5α-reductase and, therefore, of dihydrotestosterone (DHT) formation, which can partially (15–30%) decrease prostate overgrowth (3). However, it is now clear that some of the bothersome bladder voiding symptoms are only partially related to prostate enlargement (4). Indeed, although the F-induced decrease in prostate size reduces the risk of acute urinary retention (AUR) and the related need for surgery, it only partially ameliorates LUTS (3, 5). In addition, overall patient satisfaction is partially hampered by the relatively frequent adverse sexual side-effects of F, which are often bothersome in elderly men who already complain of an age-related decline in sexual function (6). Conversely, LUTS are substantially decreased by α_1-adrenergic antagonists, which reduce bladder neck and prostate smooth muscle tone and therefore

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Abbreviations: AR, Androgen receptor; AUR, acute urinary retention; BPH, benign prostate hyperplasia; Cyp, cyproterone acetate; DHT, dihydrotestosterone; EC_{50}, half-maximal stimulatory concentration; E_{max}, maximal stimulatory concentration; F, finasteride; GAPDH, glyceraldheyde-3-phosphate dehydrogenase; hBC, human bladder smooth muscle cell; IC_{50}, half-maximal inhibitory concentration; I_{max}, maximal inhibitory concentration; ISEL, *in situ* end labeling; KGF, keratinocyte growth factor; KGFR, keratinocyte growth factor receptor; LUTS, lower urinary tract symptom; MHC, myosin heavy chain; MLCK, myosin light chain kinase; α-SMA, α-smooth muscle actin; T, testosterone; VDR, vitamin D receptor.

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greatly increase the urinary flow rate. However, α_1 blockers fail to affect prostate size and thus cannot prevent AUR. Recently, a clear-cut advantage of a combination therapy with the α_1 blocker doxazosin and F has been demonstrated on BPH-related LUTS and AUR (7).

We have recently shown that the nonhypercalcemic vitamin D receptor (VDR) ligand, BXL-628, can decrease *in vitro* and *in vivo* prostate cell growth, similarly to F, postulating its use for the medical treatment of BPH (8). In this study we demonstrate that the human bladder represents an additional target for VDR ligands, notably for BXL-628, suggesting that this analog may be able to control the mechanistic processes that eventually lead to LUTS.

Materials and Methods

Materials

MEM, DMEM-Ham's F-12 (1:1 mixture), Ham's F-12 medium, PBS, BSA (fraction V), glutamine, streptomycin, penicillin, collagenase type IV, calcitriol, testosterone (T), DHT, cyproterone acetate (Cyp), dithiothreitol, phenylmethylsulfonylfluoride, and peroxidase-conjugated secondary antibodies together with reagents for SDS-PAGE were obtained from Sigma-Aldrich Corp. (St. Louis, MO). The protein measurement kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Fetal bovine serum was purchased from Unipath (Bedford, UK). The ApopTag kit for *in situ* end labeling (ISEL) was obtained from Oncor (Gaithersburg, MD). Finasteride [pure substance; 17β-(*N*,*t*-butyl)carbamoyl-4-aza-5α-androst-1-en-3-one] was a gift from Merck, Sharp, & Dohme Research Laboratories (Rahway, NJ). The vitamin D analog 1α-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol (BXL-628) was provided by BioXell (Milan, Italy). Keratinocyte growth factor (KGF) was obtained from PeproTech EC (London, UK). Plasticware for cell cultures was purchased from Falcon (Oxnard, CA). Disposable filtration units for growth medium preparation were purchased from PBI International (Milan, Italy). Mouse antihuman monoclonal antibody against Bcl-2 and antibodies against desmin, vimentin (clone V9), α-smooth muscle actin (α-SMA), cytokeratin, androgen receptor (AR), VDR, anti-KGF receptor (anti-KGFR; or fibroblast growth factor receptor-2) and factor VIII were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The BM enhanced chemiluminescence system was purchased from Roche (Milan, Italy).

Human tissue collection

Human tissues from male genital tract (testis, vas deferens, epididymis, and corpora cavernosa) were collected during surgery for benign diseases as previously reported (9). In detail, corpora cavernosa samples were obtained from patients undergoing penile prosthesis implantation (n = 5; age range, 40–67 yr), correction of Peyronie's disease (n = 1; 52 yr), or congenital curvature of the penis (n = 1; 24 yr). Prostate and bladder samples were derived from patients undergoing suprapubic prostatectomy for BPH and partial rebuilding of bladder neck area. The normal counterpart of kidney specimens was obtained from three subjects suffering from renal carcinoma who underwent surgical excision. Stomach (corpus), duodenum, and colon biopsies were obtained during routine upper gastrointestinal endoscopy and colonoscopy in control subjects.

Human fetal bone tissues were prepared from two jaws, one femur, and one rachis of fetuses (11–12 wk gestation) obtained after spontaneous or therapeutic abortion. Legal abortions were performed in authorized hospitals, and certificates of consent were obtained. All tissue samples were collected after approval of the hospital committee for investigation in humans and with the patient's informed consent. The local ethical committee gave approval for the use of all human material.

Immediately after removal, tissue samples were snap-frozen in liquid nitrogen and stored at −80 C until RNA/protein preparation. Bladder tissues for immunohistochemistry were placed in a 4% paraformaldehyde solution in PBS.

Cell cultures

Primary human bladder smooth muscle cells (hBC) were obtained from bladder neck tissues of five patients undergoing suprapubic prostatectomy for BPH, after obtaining informed consent and approval by the local ethical committee. The bladder was carefully cleaned from fat, if present, and connective tissue and gently scraped with a scalpel to remove mucosa and serosa. Bladders were then washed at least three times in PBS with 250 U/ml penicillin and 0.250 mg/ml streptomycin, cut into small fragments, and incubated with sterile collagenase type IV (2 mg/ml) for 45–60 min at 37 C. The dissociated cells were harvested and centrifuged for 10 min at 1500 rpm. The supernatant was discarded, and the cell pellet was plated onto tissue culture dishes (10-cm diameter) in DMEM/Ham's F-12 (Sigma-Aldrich Corp.) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 C in humidified air with 5% CO_2. The cells were continuously subcultured when confluent. All experiments were performed using different primary cell cultures after three to six passages.

Specific antibodies were used to characterize hBC cells. They were positively stained for α-SMA, vimentin, and desmin and negatively stained for epithelial and endothelial markers, such as cytokeratin and factor VIII.

The established androgen-independent human prostate adenocarcinoma DU145 cell line was obtained from American Type Culture Collection-LGC Promochem (Middlesex, UK).

Gene expression analysis

Isolation of RNA and cDNA synthesis. Total RNA was extracted from frozen tissues using TRIzol (Invitrogen Life Technologies, Inc., Carlsbad, CA) and from cell cultures with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentration and quality were measured by spectrophotometric analysis at 260 and 280 nm. RNA integrity was assessed by electrophoresis in agarose gel. For each sample, 400 ng total RNA were reverse transcribed to cDNA in a final volume of 80 μl using the TaqMan RT kit (Applied Biosystems, Foster City, CA) under the following conditions: 10 min at 25 C, 30 min at 48 C, and 5 min at 95 C.

Real-time RT-PCR. The quantitative assay was performed according to the fluorescent TaqMan methodology as previously reported (9). Primers and probe for AR, VDR, vimentin, desmin, smoothelin, myosin light chain kinase (MLCK), myosin heavy chain (MHC) 11 isoform SM1-like, and α-smooth muscle actin (α-SMA) mRNAs were Assay-On-Demand gene expression products (assay no. Hs00171172, Hs00172113, Hs00185584, Hs00157258, Hs00199489, Hs001364926, Hs00293680, and Hs00426835, respectively) purchased from Applied Biosystems. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was chosen as the reference gene and was quantitated using a predeveloped control assay provided by Applied Biosystems. PCR mixture (25 μl final volume) consisted of 1× final concentration of Assay-On-Demand mix, 1× final concentration of Universal PCR Master Mix (Applied Biosystems), and 25 ng cDNA. Amplification and detection were performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with the following thermal cycler conditions: 2 min at 50 C, 10 min at 95 C, and 40 cycles at 95 C for 30 sec and 60 C for 1 min. Each measurement was carried out in duplicate.

The mRNA quantitation was based on the comparative Ct method according to the manufacturer's instructions (Applied Biosystems), where Ct represents the cycle number at which the fluorescent signal, associated with an exponential increase in PCR products, crossed a given threshold. The maximum change in Ct values of the sample (ΔCt_{sample}) was determined by subtracting the average of duplicate Ct values of the reference gene from the average of duplicate Ct values of the target gene. For each time-course experiment, data were also normalized to an internal calibrator ($\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$), consisting of mRNA level from cells harvested after 24-h starvation (time zero).

RT-PCR. Bladder tissues, bladder cells, and prostate tissue were analyzed for KGFR and the GAPDH housekeeping gene by RT-PCR, performed using the Superscript One Step RT-PCR kit (Invitrogen Life Technologies, Inc., Milan, Italy).

Total RNA (500 ng) was reverse transcribed for 30 min at 50 C, denatured for 2 min at 95 C, and amplified for 35 cycles with the following parameters: denaturation for 30 sec at 94 C, annealing for 30

sec at 56 C, and extension for 30 sec at 72 C, for both KGFR and GAPDH. The specific primers for KGFR were chosen from GenBank (accession no. BC039243) and were as follows: KGFR sense primer, 5′-TTT ACA GTG ATG CCC AGC CC-3′; and antisense primer, 5′-ACC ACC ATA CAG GCG ATT AA-3′. The quality of total RNA used was assessed by performing additional RT-PCR using primers specific for the GAPDH: GAPDH sense primer, 5′-CCA TGG AGA AGG CTG GGG-3′; and GAPDH antisense primer, 5′-CAA AGT TGT CAT GGA TGA CC-3′ (10). The negative control was performed without template.

Cell culture assay

Cell proliferation. For cell proliferation assay, 2.5×10^4 hBC were seeded onto 12-well plates in their growth medium. After 24 h, the cells were washed in PBS and incubated overnight in phenol red- and serum-free medium containing 0.1% BSA. Increasing concentrations of T, DHT (10^{-13}–10^{-7} M), and KGF (0.5×10^{-12} to 0.5×10^{-8} M) or increasing concentrations (10^{-18}–10^{-7} M) of calcitriol or BXL-628 were added for 48 h. Cells in phenol red- and serum-free medium containing 0.1% BSA were used as controls. Growth assays were also carried out using a fixed concentration of androgens (10^{-8} M) or KGF (0.5×10^{-9} M) with the same scalar concentrations of BXL-628, or with T (10^{-8} M) and DHT (10^{-8} M) in the presence and absence of F (10^{-9} M), Cyp (10^{-7} M), or BXL-628 (10^{-9} M) for 48 h. Antiandrogens alone were also tested. Cells in phenol red- and serum-free medium containing 0.1% BSA were used as controls. After 48 h, cells were trypsinized, and each experimental point was derived from hemocytometer counting and then averaging six to nine different fields for each well. In the same experiment each experimental point was repeated in duplicate or triplicate; experiments were performed two or three times using separate hBC preparations, each derived from different patients. Results are expressed as the percent variation (mean ± SEM) over control or over the maximal T- or KGF-induced stimulation, as appropriate.

Desmin, vimentin, and KGFR. To detect desmin, vimentin, and KGFR at both gene and protein levels, hBC cells were seeded in their growth medium onto 10-cm diameter culture dishes or onto sterile glass slides (~10^4 cells/ml), for mRNA, Western blot, or immunocytochemical analysis. For KGFR analysis, hBC cells were harvested when they were near confluence. For desmin and vimentin analysis, hBC cells at about 30% confluence, after overnight starvation in phenol red- and serum-free medium, were incubated in phenol red- and serum-free medium containing 0.1% BSA with or without BXL-628 (10^{-8} M) for 2, 4, 8, and 12 d, and the medium was changed every 2 d.

Cells were harvested for mRNA or protein analysis by TaqMan, RT-PCR, or Western blot analysis, respectively, and the slides were processed for specific protein immunocytochemical detection, as described. Results were obtained from three to five different experiments derived from three distinct hBC cell preparations (each derived from a different patient).

Apoptosis and Bcl-2. To evaluate apoptosis along with Bcl-2 expression, cells (10^4 cells/ml) were seeded on sterile glass slides in their growth medium on 150-mm diameter culture dishes and starved overnight in serum-free medium. Cells were then washed in PBS and incubated for 48 h in phenol red- and serum-free medium containing 0.1% BSA with T (10^{-8} M) and KGF (0.5×10^{-9} M), either combined or not with BXL-628 (10^{-8} M). Data were obtained from three different experiments derived from three distinct hBC cell preparations (each from a different patient).

Protein expression analysis

SDS-PAGE and Western blot analysis were performed. Frozen tissue samples were directly suspended in lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 0.25% Nonidet P-40, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonylfluoride] and homogenized for protein analysis as previously described (9). The homogenates were centrifuged at 2000 rpm for 10 min at 4 C, and the protein content of the supernatants was evaluated according to Bradford's method (11) using Coomassie reagent (Bio-Rad Laboratories, Inc.). Cultured cells scraped in PBS were pelleted and extracted in lysis buffer. Aliquots containing 30 μg proteins were diluted in reducing 2× SB [SB = 62.5 mM Tris (pH 6.8), 10% glycerol, 20% SDS, 2.5% pyronin, and 100 mM dithiothreitol] and loaded onto 10% SDS-PAGE. Equivalent protein loading for Western blot shown was verified by staining with Coomassie R a parallel gel loaded with the same amount of proteins (not shown). After separation by SDS-PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked for 2 h at room temperature in 10% BM blocking buffer-TTBS (0.1% Tween 20, 20 mM Tris, and 150 mM NaCl), washed in TTBS, and incubated overnight with primary antibodies (1:1000 antidesmin; 1:3000 antivimentin; 1:100 anti-AR; 1:1000 anti-VDR), followed by peroxidase-conjugated secondary IgG (1:3000). Finally, reacted proteins were revealed by BM-enhanced chemiluminescence system.

Immunohistochemistry

AR, VDR, KGFR, desmin, and vimentin. Immunohistochemical studies were carried out on deparaffinized and rehydrated tissue sections or cultured cells fixed in 3.7% paraformaldehyde for 15 min, as previously described (12). Tissue specimens were rinsed in tap water, then immersed in EDTA (pH 8.0), microwaved for 20 min at 350 W to enhance antigen exposure, and subsequently exposed to 0.3% hydrogen peroxidase-methanol solution to quench endogenous peroxidase activity. The primary antibodies diluted in PBS, AR (1:50), VDR (1:500), or desmin or vimentin (1:100) were added to the specimens and incubated overnight at 4 C. Specimens were rinsed in PBS, incubated with biotinylated secondary antibodies (1:1000), and finally incubated with streptavidin-biotin peroxidase complex (LSAB kit, DakoCytomation, Carpinteria, CA). The reaction product was developed with deaminobenzidine tetrahydrochloride or 3-amino-9-ethyl-carbazole as chromogen. Slides were washed in running tap water and counterstained with hematoxylin, followed by dehydration and coverslip mounting. Immunostaining for KGFR was performed using the primary antibody anti-KGFR (1:100), followed by A-11001 Alexa Fluor488 rabbit antigoat conjugate (1:200) antibody (Molecular Probes, Eugene, OR). Controls were performed by processing slides lacking the primary antibodies or by staining with the corresponding nonimmune serum. The slides were evaluated and photographed with a Microphone-FX microscope (Nikon, Tokyo, Japan). The percentage of desmin- or vimentin-positive hBC was calculated by counting the number of stained cells over the total number of cells in at least five separate fields per slide. Three distinct hBC cell preparations (each from a different patient) were analyzed.

Bcl-2. Bcl-2 protein expression was evaluated by immunocytochemistry using an anti-Bcl-2 mAb and the procedure previously described (13). The slides were examined with a phase contrast microscope (Nikon Microphot-FX microscope). Slides lacking the primary antibody or stained with the corresponding nonimmune serum served as controls. The percentage of Bcl-2 expression was calculated by counting the number of stained cells over total cells in at least five separate fields per slide. Three distinct hBC cell preparations (each from a different patient) were analyzed.

ISEL. Apoptosis was evaluated for hBC by ISEL using ApopTag *in situ* apoptosis detection kit peroxidase following the manufacturer's instructions, as previously described (8). The percentage of apoptotic cells (the number of stained cells divided by the total number of cells) was calculated in at least five separate fields per slide. Three distinct hBC cell preparations (each from a different patient) were analyzed.

Statistical analysis

Statistical analysis was performed by one-way ANOVA and paired or unpaired *t* tests, when appropriate. Whenever appropriate, data were logarithmic transformed. The computer program ALLFIT (14) was used for the analysis of sigmoid dose-response curves to obtain estimates of half-maximal inhibition values (IC_{50}) and half-maximal stimulatory values (EC_{50}), as well as maximal inhibitory (I_{max}) and stimulatory (E_{max}) effects. Data were expressed as the mean ± SEM. $P < 0.05$ was used to define statistical significance.

Results

AR expression in human bladder neck

We first compared gene and protein distribution of the AR in the human male reproductive tract, in particular in pros-

tate and bladder neck. Quantification of mRNA distribution by real-time RT-PCR (Fig. 1A) indicated that AR transcripts are less expressed (~1 log unit lower) in bladder than in prostate or epididymis, although they are comparable to those in vas deferens and corpus cavernosum, considered to be classic androgen-dependent tissues. AR mRNA amplification was undetectable in DU 145, which was used as a negative control (15, 16). We found a similar AR gene expression in stromal cells derived from human bladder (hBC) and prostate (hBPHC) tissues obtained from the same subjects undergoing prostatectomy for BPH. Western blot analysis (Fig. 1B, *left blot*) indicates that AR protein is expressed, at the expected molecular weight, in all tissues investigated, except DU145 cells (Fig. 1B, *right blot*). Interestingly, specific bands were present in both hBC and hBPHC protein lysates (n = 3). To localize the AR in human bladder neck samples, immunohistochemical studies, using the same antibody employed for Western blot analysis, were performed (n = 3). Figure 1C shows that nuclei from both epithelial and stromal cells were strongly immunopositive. According to previous observations (17, 18), endothelial cells lining the blood vessels were also positive.

VDR expression in human bladder neck

Similar studies were carried out to detect VDR expression. VDR gene expression (Fig. 2A) was several-fold higher in gastrointestinal tract than in human bladder samples. Interestingly, bladder showed a similar amount of VDR transcripts as prostate, a well defined target organ for VDR ligands (8, 12, 13, 16, 19). Accordingly, stromal cells derived from human prostate and bladder showed virtually identical expression of VDR mRNA (Fig. 2A). VDR protein of the expected molecular weight (20) was evident not only in the gastrointestinal tract, but also in prostate and bladder tissues and cells (Fig. 2B). In bladder neck sections, the immunodistribution of VDR labeling, detected with the same antibody as that used for Western blot analysis, was similar to that observed for the AR (Fig. 2C), with positive nuclei in bladder epithelium and stromal cells along with vascular endothelial cells. VDR expression in endothelial cells has been previously reported (21).

Opposite effects of AR and VDR ligands on human bladder cell growth

Having demonstrated AR and VDR expression in bladder cells, we next investigated the functions of these receptors. Our previous studies indicated that in prostate stroma-derived cells these receptors regulate cell survival and mitogenesis in opposite directions (8, 12). Therefore, we first tested the effects of different AR and VDR ligands on bladder cell proliferation. Both T and its 5α-reduced metabolite, DHT, stimulated hBC proliferation, without apparent differences

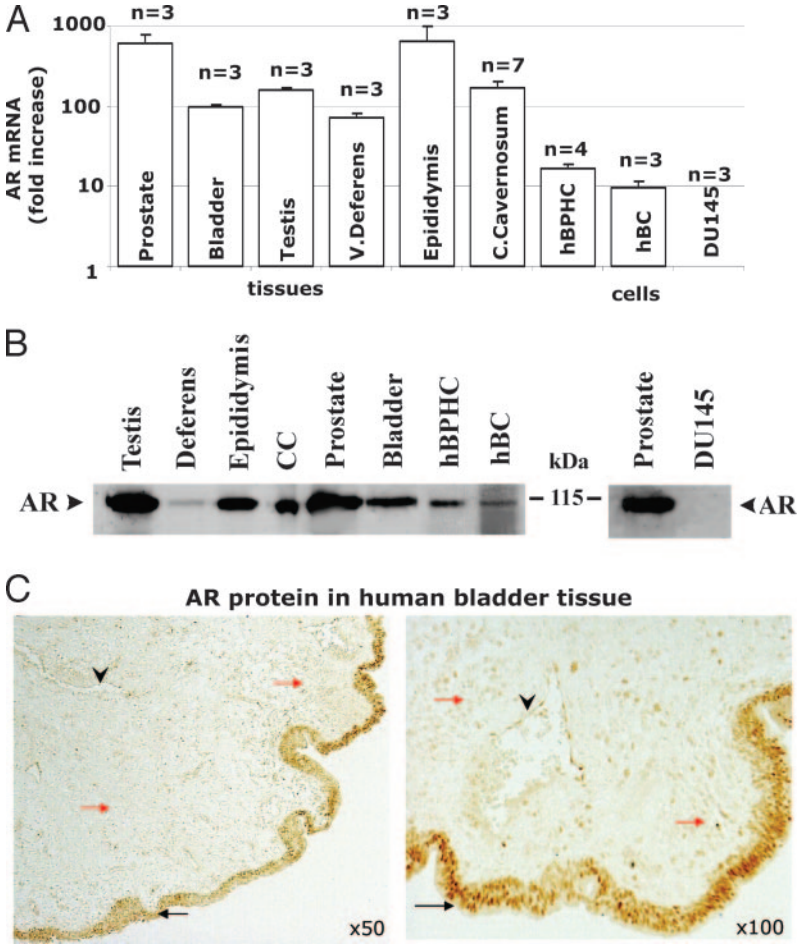

FIG. 1. Expression of AR in the human bladder neck. A, Quantitative analysis using real-time RT-PCR of AR mRNA expression in human bladder neck homogenates and cells (hBC, n = 3, from three independent preparations) compared with androgen target tissues and prostate cells (hBPHC, n = 4, from three independent preparations). Data were first normalized to GAPDH mRNA expression. Results are expressed as fold mRNA variations compared with bladder neck (taken as 100). DU145 cells were used as negative controls of AR mRNA expression. B, Western blot detection of AR in human bladder neck tissue and cells. Thirty micrograms of proteins from each tissue or cell type were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed for AR expression with anti-AR antibody (1:100). A band of about 110 kDa (*arrowhead*) was present in bladder tissue and cells (hBC) as well as in several androgen target tissues and prostate cells (hBPHC), which were used as a positive control. DU145 cells were used as a negative control of AR protein expression. The blot is representative of three different experiments, each performed with samples derived from a different patient. C, Immunohistochemistry for AR in transverse sections of human bladder neck. Immunopositive staining for AR was observed in the epithelium (*black arrows*) and stroma (*red arrows*). Endothelial cells lining blood vessels (*arrowhead*) were also stained. Magnification: *left panel*, ×50; *right panel*, ×100. Similar results were obtained in three independent experiments.

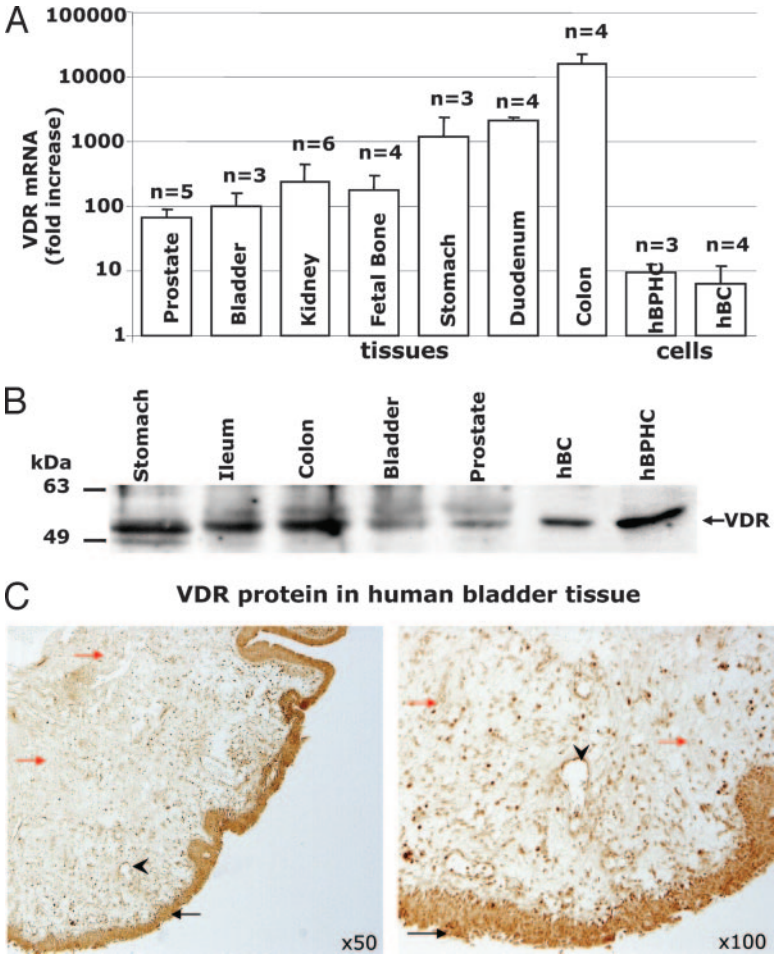

FIG. 2. Expression of VDR in human bladder neck. A, Quantitative analysis using real-time RT-PCR of VDR mRNA expression in human bladder neck homogenates and cells (hBC, n = 4, from three independent preparations) compared with androgen target tissues and prostate cells (hBPHC, n = 3, from three independent preparations). Data were first normalized to GAPDH mRNA expression. Results are expressed as fold mRNA variation compared with bladder neck (taken as 100). B, Western blot detection of AR in human bladder neck tissue and cells. Thirty micrograms of proteins from each tissue or cell type were separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and probed for VDR expression with anti-VDR antibody (1:1000). A band of about 52 kDa (*arrowhead*) was present in bladder tissue and cells (hBC) as well as in several VDR target tissues and prostate cells (hBPHC), which were used as a positive control. The blot is representative of three different experiments, each performed with samples derived from a different patient. C, Immunohistochemistry for VDR in transverse sections of human bladder neck. Immunopositive staining for VDR was observed in epithelium (*black arrows*) and stroma (*red arrows*). Endothelial cells lining blood vessels (*arrowhead*) were also stained. Magnification: *left panel*, ×50; *right panel*, ×100. Similar results were obtained in three independent experiments.

in EC_{50} (shared $-\log EC_{50}$, 11.2 ± 0.34) and E_{max} (shared E_{max}, 125 ± 2%). Figure 3A shows a shared sigmoid relationship for both androgens.

Androgen-induced hBC growth was slightly, but significantly ($P < 0.05$), inferior to that induced by a potent peptide growth factor, such as KGF (E_{max}, 135 ± 4%; $-\log EC_{50}$, 10.2 ± 0.17). Although epithelium is generally considered the main target for KGF, we previously also demonstrated biologically active KGFR in prostate stromal cells (13, 22). The *lower* and *upper insets* in Fig. 3A demonstrate the gene (RT-PCR) and protein (immunofluorescence) expression of KGFR in bladder-derived stromal cells. Different VDR ligands reduced basal hBC proliferation to the same extent (I_{max}, 22.7 ± 0.4%), although with different IC_{50} values (Fig. 3B). BXL-628 was almost 2 log units more potent than calcitriol ($-\log IC_{50}$, 13.9 ± 0.37 and 16.2 ± 0.38 respectively; $P < 0.005$). The antiproliferative effect of BXL-628 on hBC was even more evident when growth was stimulated by T or KGF (Fig. 3C). In this case, BXL-628 reduced T- and KGF-induced hBC growth with similar IC_{50} values (shared $-\log IC_{50}$, 14.8 ± 0.21), but different I_{max} values (I_{max} for T, 63.5 ± 3%; for KGF, 36.5 ± 1.1%; $P < 0.01$). Hence, the growth inhibitory effect of BXL-628 in hBC was definitely more evident in androgen-stimulated than in KGF-stimulated cells.

BXL-628 inhibits basal and androgen-stimulated human bladder cell growth and enhances their apoptosis

To investigate the BXL-628-induced inhibition of androgen-promoted hBC growth, we compared the antiproliferative effects of BXL-628 (1 nM) with those of other antiandrogens, such as the AR antagonist Cyp (100 nM) and the type 2 5α-reductase inhibitor F (1 nM; Fig. 4A). As expected, F abrogated only T-induced proliferation, whereas both Cyp and BXL-628 completely counteracted the effect of both T and DHT. However, at variance with the other antiandrogens, BXL-628 also reduced cell proliferation in unstimulated cells. Interestingly, we found that also in hBC, BXL-628 reduced the expression of the survival oncoprotein Bcl-2 (Fig. 4B), as previously observed in hBPHC (12, 13). This reduction was maintained even in the presence of steroid or peptide growth factors. Consistent with our previous observations in prostate cells (8, 12, 13, 16), BXL-628 markedly enhanced nuclear fragmentation in hBC, which was still present, although attenuated, in T- and KGF-stimulated hBC (Fig. 4C).

Effect of BXL-628 on starvation-induced hBC phenotype changes

To address the possible mechanisms involved in mediating the effects of BXL-628 on hBC, we studied the time-dependent variations in the expression of intermediate fila-

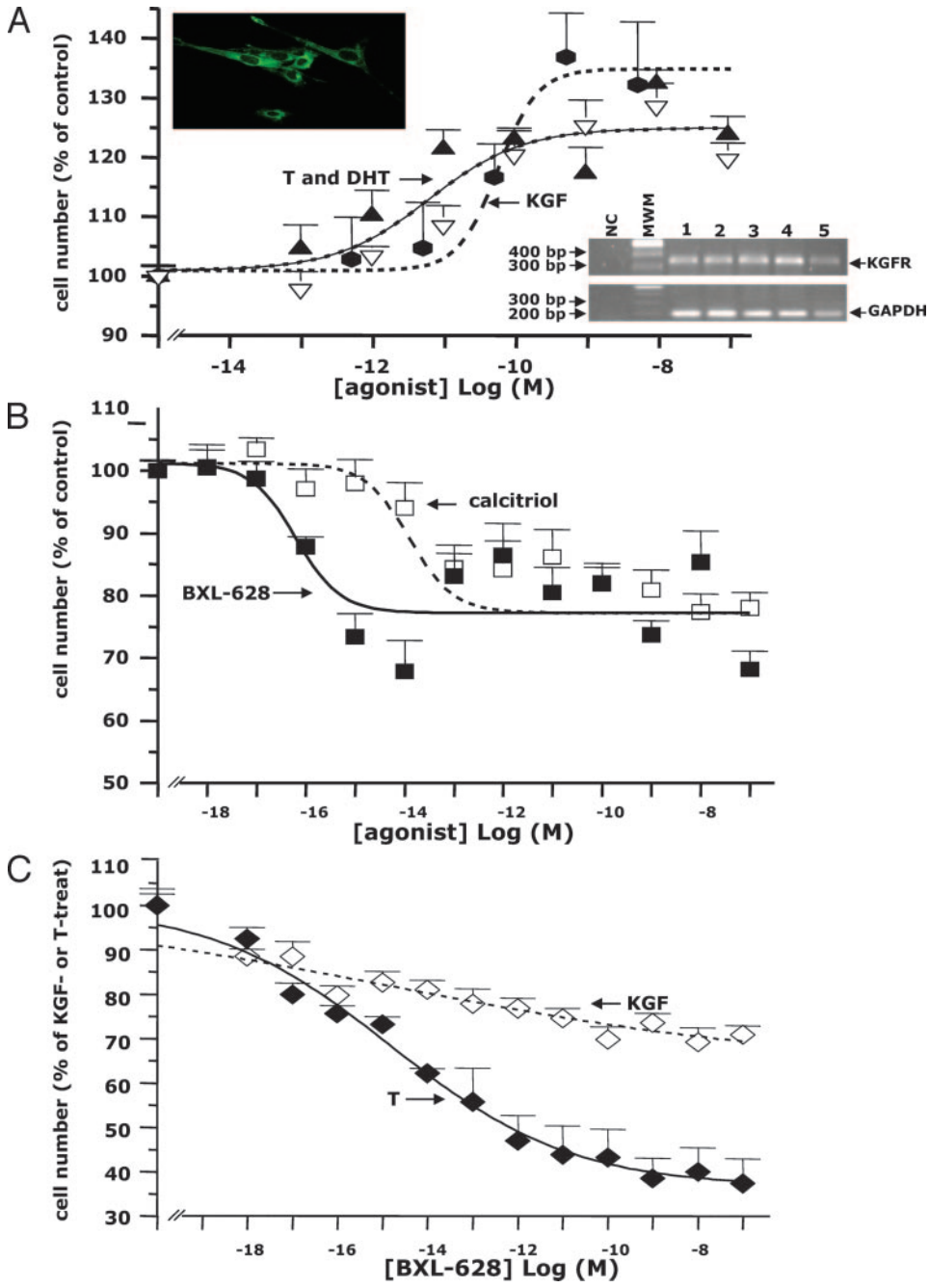

FIG. 3. Effects of AR and VDR ligands on bladder cell growth. A, Effect of a 48-h incubation with increasing concentrations of androgens (10^{-13}–10^{-7} M; ▲, T; △, DHT) or KGF (♦; 5×10^{-13} to 5×10^{-9} M) on hBC proliferation. Note that T and DHT proliferation curves are described by the same equation. *Ordinate*, Cell number expressed as percent variation (mean ± SEM) over the relative control. Data are derived from two (KGF) or three (androgens) separate experiments, derived from three distinct hBC preparations. *Lower right inset*, KGFR expression in two different bladder neck tissues (lanes 1 and 2) and in their corresponding cell cultures (lanes 3 and 4). KGFR expression in prostate tissue (lane 5) is also reported as a positive control. A single band of the expected molecular weight is present in all the specimens for KGFR (340 bp) and GAPDH (194 bp). NC, Negative control (without template); MWM, molecular weight markers (100 bp). *Upper left inset*, Immunofluorescence for KGFR in hBC. Similar results were obtained in two separate cell preparations. B, Effect of a 48-h incubation with increasing concentrations (10^{-18}–10^{-7} M) of calcitriol (□) or BXL-628 (■) on hBC. *Ordinate*, Cell number expressed as percent variation (mean ± SEM) over the relative control value. Data are from two (calcitriol) or three (BXL-628) separate experiments, derived from three distinct hBC preparations. C, Effects of increasing concentrations (10^{-18}–10^{-7} M) of BXL-628 on the growth of T-stimulated (10 nM; ♦) or KGF-stimulated (10 ng/ml; ◇) hBC. *Ordinate*, Cell number expressed as percent variation (mean ± SEM) over the relative T- or KGF-stimulated controls. Data are derived from three separate experiments using three distinct hBC preparations.

ment proteins, vimentin and desmin, which are considered the main fibroblast- and smooth muscle-associated cytoskeletal markers. Gene expression of other smooth muscle-related protein, such as smoothelin, α-SMA, MLCK, and MHC, were also studied. Experiments were conducted in serum-deprived cells, and cells were observed up to 12 d of culture. Analysis of specific desmin transcripts (quantified by real-time RT-PCR) showed a significant and progressive mRNA expression increase as a function of time in stressed bladder cells, and this increase was inhibited, at any time point tested, by BXL-628 (10 nM; Fig. 5A). Western blot and immunocytochemical studies, performed using the same antidesmin antibody, confirmed this inhibition at the protein level (Fig. 5, B and C). In particular, BXL-628 markedly reduced, at any time point investigated, the number of desmin-immunopositive hBCs (Fig. 5D).

Also, smoothelin gene expression showed a starvation-induced, time-dependent increase, which, again, was significantly abrogated by chronic exposure to BXL-628 (Table 1). Conversely, other contractile-related proteins, such as α-SMA, MLCK, and MHC, neither changed as a function of time nor were significantly affected by chronic treatment with BXL-628 (Table 1). Figure 6 reports results on gene (A) and protein (B–D) expression of the fibroblast-related intermediate filament vimentin, which was unaffected by starvation or VDR ligation (Fig. 6).

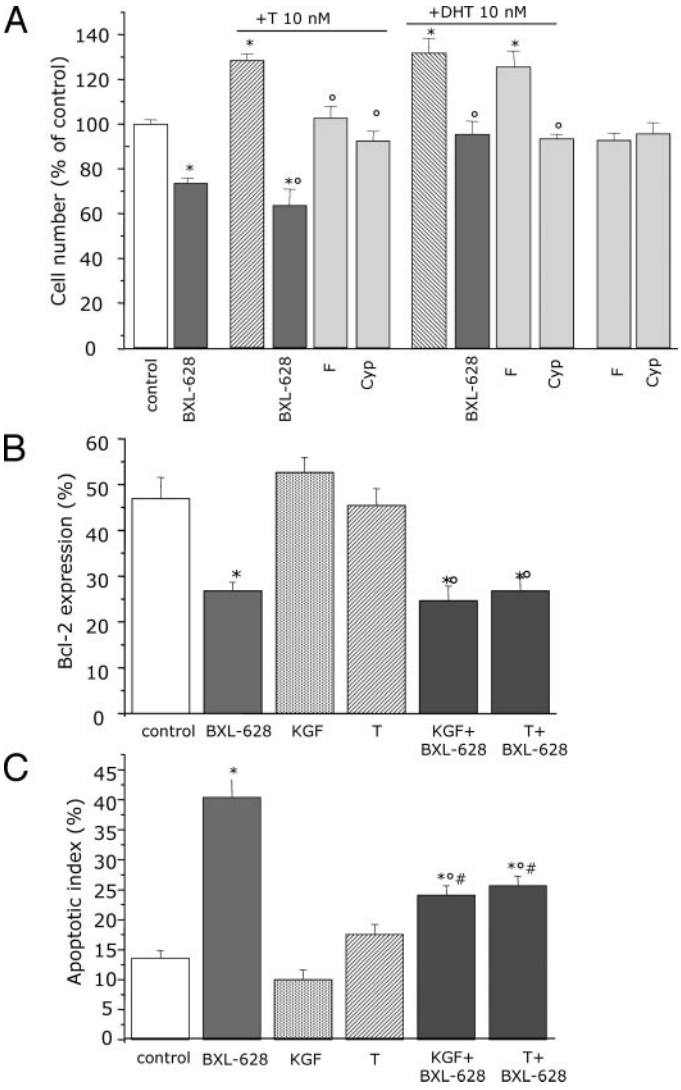

FIG. 4. Effects of BXL-628 on basal and stimulated hBC proliferation and apoptosis. A, Effects of antiandrogens or BXL-628 on androgen-stimulated hBC growth. Cells were incubated for 48 h with BXL-628 (1 nM) or antiandrogens (F, 1 nM; Cyp, 100 nM) in the presence or absence of T (10 nM) or DHT (10 nM). Results are expressed as the percent variation (mean ± SEM) over the relative control value and are derived from at least three different experiments obtained from three distinct hBC cell preparations. *, $P < 0.05$ (*vs.* control); ○, $P < 0.01$ (*vs.* androgen-treated cells). B, Effects of BXL-628 (10 nM), KGF (10 ng/ml), and T (10 nM) on Bcl-2 expression in hBC. The percentage of Bcl-2 stained cells was calculated by counting the number of immunopositive cells divided by the total cell number in each of at least five separate fields per slide. Data are derived from three different experiments obtained from three separate hBC preparations. *, $P < 0.05$ (*vs.* control); ○, $P < 0.05$ (*vs.* KGF or T-treated cells). C, Effects of BXL-628 (10 nM), KGF (10 ng/ml), and T (10 nM) on DNA fragmentation in hBC. The apoptotic index was obtained from ISEL experiments and represents the number of stained nuclei divided by the total cell number in each of at least five separate fields per slide. Results are expressed as the mean ± SEM and were obtained from three different experiments derived from three distinct hBC preparations. *, $P < 0.05$ (*vs.* control); ○, $P < 0.05$ (*vs.* BXL-628-treated cells); #, $P < 0.05$ (*vs.* KGF- or T-treated cells).

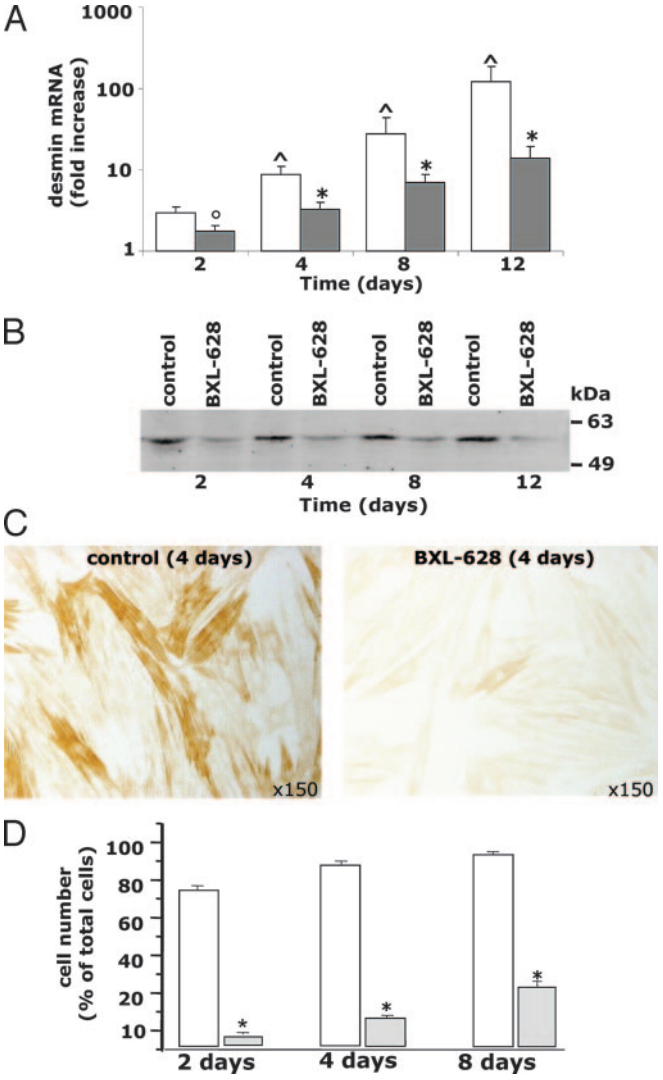

FIG. 5. Effect of BXL-628 on desmin gene and protein expression in hBC. A, Quantitative analysis using real-time RT-PCR of desmin mRNA expression in serum-starved hBC treated with BXL-628 (10 nM) examined at different time points (2–12 d). Results are derived from five different experiments from three distinct hBC preparations. Data were first normalized to GAPDH mRNA expression and are expressed as the fold mRNA increase compared with the time zero value. *, $P \leq 0.01$; ○, $P = 0.04$ (*vs.* the respective control). ^, $P < 0.01$ (*vs.* 2 d control). □, Control; ▩, BXL-628. B, Western blot detection of desmin in hBC. Thirty micrograms of proteins were separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and probed with antidesmin antibody (1:1000). A band of about 58 kDa was detected in each sample of hBC. BXL-628 (10 nM) decreased desmin protein expression at any time point tested. Molecular mass markers (kilodaltons) are indicated at the *right* of the blot. Results are representative of three independent experiments performed using separate hBC preparations. C and D, Immunocytochemical detection of desmin in hBC. Cells were seeded onto sterile glasses, treated with BXL-628 (10 nM), and processed at the indicated time points with an antidesmin antibody (1:1000). The microphotographs reported in C show results obtained after a 4-d incubation with BXL-628 (10 nM; *right* microphotograph; magnification, ×150) or vehicle (*left* microphotograph; magnification, ×150). Quantification of three separate experiments from three distinct preparations of hBC is shown in D (□, control; ▫, BXL-628). The percentage of desmin-positive cells was calculated by counting the number of stained cells divided by the total cell number in each of at least five separate fields per slide. *, $P < 0.01$ *vs.* the relative control.

TABLE 1. Expression of smooth muscle cell-specific genes in hBC

Days	Gene							
	Smoothelin (n = 7)		α-SMA (n = 5)		MLCK (n = 5)		MHC (n = 5)	
	Control	BXL-628	Control	BXL-628	Control	BXL-628	Control	BXL-628
2	0.21 ± 0.07	0.16 ± 0.06	0.40 ± 0.20	0.35 ± 0.21	0.20 ± 0.10	0.09 ± 0.07	0.23 ± 0.10	0.16 ± 0.10
4	0.50 ± 0.07[a]	0.35 ± 0.06[b]	0.72 ± 0.10	0.79 ± 0.11	0.39 ± 0.07	0.34 ± 0.05	0.40 ± 0.09	0.25 ± 0.07
8	0.55 ± 0.08[a]	0.46 ± 0.07[b]	0.82 ± 0.16	0.88 ± 0.15	0.43 ± 0.07	0.44 ± 0.07	0.48 ± 0.17	0.42 ± 0.15
12	0.61 ± 0.10[a]	0.41 ± 0.06[b]	0.84 ± 0.17	0.86 ± 0.14	0.46 ± 0.07	0.55 ± 0.10	0.44 ± 0.15	0.34 ± 0.10

Quantitative analysis using real-time RT-PCR of smooth muscle-related marker mRNAs in serum-deprived hBC treated with BXL-628 (10 nM) for 2, 4, 8, and 12 d. Serum-deprived hBC are used as control at each time point. Results were derived from three different hBC preparations (n, number of time-course experiments) and were calculated by the comparative Ct method. Data were first normalized to GAPDH mRNA expression, then *vs.* time zero (after 24-h starvation), and expressed as [log] ± SEM.

[a] $P \leq 0.01$ *vs.* 2-d control.

[b] $P < 0.05$ *vs.* time point respective control.

Discussion

This study demonstrates for the first time that the human bladder is a new potential target for VDR ligands, at least in its inferior portion, the bladder neck. Bladder neck shows VDR expression virtually identical to that in prostate, a well characterized, VDR ligand-sensitive tissue. We previously demonstrated that in prostate, calcitriol and two distinctly less hypercalcemic analogs, BXL-353 and BXL-628, markedly modulated cell proliferation and apoptosis both *in vitro* and *in vivo*, counteracting the effects of steroid and peptide growth factors (8, 12, 13, 16). We now provide evidence for similar effects in bladder. BXL-628 and, to a lesser extent, calcitriol reduced the *in vitro* growth of stromal cells derived from the bladder neck (hBC) of patients who underwent suprapubic prostatectomy for BPH. Such an effect was even more evident when hBC proliferation was stimulated by KGF, a mitogen for normal (23) and transformed (24) bladder cells that has been involved in bladder wound healing and differentiation processes (25). Although KGFR has been localized mainly in epithelial cells, we now report that bladder-derived stromal cells express biologically active KGFR, as previously demonstrated for prostate stromal cells (13, 22). Interestingly, not only KGF, but also androgens (T and DHT), stimulated hBC proliferation, an action specifically antagonized by the AR antagonist Cyp and by F-induced blockade of DHT formation.

The bladder neck is located in close proximity to the prostate gland, and it shares with the prostate the same embryological origin, the urogenital sinus (26). However, it is not generally considered a main target for androgens, although AR expression has been demonstrated in epithelial and stromal cells of rat and human bladder (27, 28), with the highest concentration in the bladder neck (29). In addition to confirming these findings using immunohistochemical and Western blot analyses, we showed by quantitation of AR mRNA abundance that bladder neck from BPH patients expresses similar levels of the AR gene as other androgen-sensitive tissues, such as vas deferens and corpus cavernosum, although 1 log unit lower than that of the corresponding prostate gland.

As recently reported for BPH cells (8), BXL-628 strongly antagonized T-stimulated hBC proliferation (~70% inhibition), with a maximal effect in the subnanomolar range. This effect was accompanied by a 2-fold reduction of the survival oncoprotein Bcl-2 and a parallel induction of programmed cell death in over one third of the entire cell population. Similar results were previously reported for calcitriol in the VDR-positive bladder cancer cell lines T24 and 253j (30).

Interestingly, BXL-628 was able to induce a significant increase in apoptotic nuclei even in the presence of growth factors, such as T or KGF. In response to chronic partial bladder outlet obstruction, enhanced expression and signaling of growth factors are believed to underlie the still reversible initial stage of bladder hypertrophy and hyperplasia, which includes increased proliferation of urothelium, fibroblast, and smooth muscle cells (2). Later, irreversible bladder decompensation and detrusor instability develop, which are often not ameliorated by relieving outlet obstruction. Hence, based on its antiproliferative and proapoptotic activities, BXL-628 might represent an interesting therapeutic opportunity to counteract bladder hypertrophy. In addition, our results, as discussed below, indicate possible beneficial effects of BXL-628 on bladder overactivity due to a modulation of smooth muscle gene expression that could lead to an increased contractile efficiency of bladder smooth muscle cells.

The initial stages of bladder hypertrophy are characterized by a tension-induced up-regulation of contractile and cytoskeleton proteins, with a net increase in the desmin/actin ratio (31). A simultaneous switch in the phenotype of the resident mesenchymal bladder cells has also been hypothesized (32–35), based on the appearance of both fibroblast and smooth muscle features, characteristic of protomyofibroblasts and differentiated myofibroblasts, respectively (36, 37). Protomyofibroblasts are vimentin-positive mesenchymal cells showing profound stress-induced cytoskeleton reorganization, characterized by increased expression of a wide range of contractile-related proteins, including desmin (36) and smoothelin (38, 39), a recently described smooth muscle-specific filament associated with α-SMA, but with unknown function and regulation (40, 41). Myofibroblasts are activated stromal cells playing an essential role in organogenesis and tissue repair, and they have also been involved in oncogenesis, inflammation, and tissue fibrosis (36, 37, 42).

In hBCs, prolonged serum starvation induced a progressive increase in the expression of some smooth muscle-specific intermediate filaments (desmin and smoothelin), as expected (43). Such an increase was almost completely counteracted by BXL-628. Conversely, gene expression for other

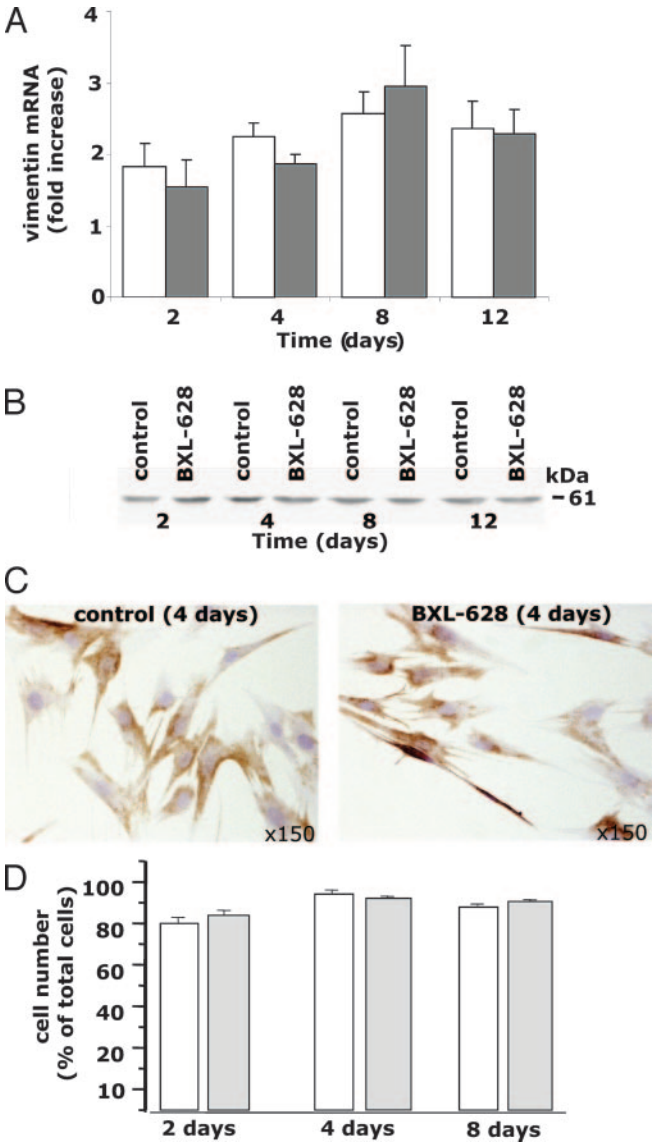

FIG. 6. Effect of BXL-628 on vimentin gene and protein expression in hBC. A, Quantitative analysis using real-time RT-PCR of vimentin mRNA expression in serum-starved hBC treated with BXL-628 (10 nM) and examined at different time points (2–12 d). Results are derived from five different experiments from three distinct hBC preparations. Data were first normalized to GAPDH mRNA expression and are expressed as the fold mRNA increase compared with the time zero value. □, Control; ▩, BXL-628. B, Western blot detection of vimentin in hBC. Thirty micrograms of proteins were separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and probed with antivimentin antibody (1:1000). A band of about 61 kDa was detected in each sample of hBC. BXL-628 (10 nM) failed to affect vimentin protein expression at any time point tested. Results are representative of three independent experiments performed using separate hBC preparations. C and D, Immunocytochemical detection of vimentin in hBC. Cells were seeded onto sterile glasses, treated with BXL-628 (10 nM), and processed at the indicated time points with an antivimentin antibody (1:1000). The microphotographs in C show results obtained after a 4-d incubation with BXL-628 (10 nM; *right* microphotograph; magnification, ×150) or vehicle (*left* microphotograph; magnification, ×150). Quantification of three separate experiments from three distinct preparations of hBC is shown in D (□, control; ▫, BXL-628). The percentage of vimentin-positive cells was calculated by counting the number of stained cells divided by the total cell number in each of at least five separate fields per slide.

contractile-related proteins, such as α-SMA, MLCK, and MHC, did not significantly change in starved cells and was not affected by BXL-628 exposure. Also, the specific fibroblastic marker vimentin was not affected by BXL-628 treatment. The most dramatic change was found in desmin mRNA abundance, which, on the average, increased with increasing time of serum starvation up to 100-fold, but remained sensitive to BXL-628-induced inhibition at any time point investigated. Although the significance of desmin overexpression in stressed bladder cells is still unclear, in the kidney, desmin was markedly up-regulated (up to 10-fold) after injury such as unilateral ureteral obstruction (44) or subtotal nephrectomy (44) In subtotal nephrectomized rats, administration of calcitriol prevented podocyte degeneration and desmin overexpression (45). Studies of desmin-deficient mice revealed the importance of this protein not only for the contractile efficiency of cardiac, skeletal, and smooth muscle cells, but also for its permissive role during myogenic development (46). Interestingly, VDR-deficient mice are characterized by an aberrant up-regulation of early markers of myogenic differentiation and myoregulatory transcription (47). The lack of VDR-mediated repression of the expression of these factors may underlie the progressive weakness and wasting of skeletal muscle reported in these mice, which resemble rachitic or osteomalacic myopathy (48). The finding also supports this observation that calcitriol administration can reduce the number of falls in elderly subjects, possibly due to increased muscle strength (49). Although no data are available about the effect of VDR gene deletion on smooth muscle tissue development, our results suggest that desmin and other smooth muscle intermediate proteins, such as smoothelin, or even their upstream regulators might be targets of VDR ligands, which could contribute to regulate smooth muscle cell overactivity.

Desmin is also important in mediating interactions between the cytoskeleton and mitochondria. Desmin-null myocytes are more prone to apoptotic cell death and show consistent abnormalities in mitochondrial integrity, including increased outer and inner membrane permeability (50). Interestingly, in mice null for desmin, cardiomyopathy and associated mitochondrial abnormalities were greatly ameliorated by cardiac overexpression of Bcl-2 (51). Bcl-2 is one of the main proteins regulating mitochondrial permeabilization, and several reports (12, 13), including the present study, suggest that its expression is negatively modulated by VDR signaling. It is still unclear whether the Bcl-2 decrease induced by BXL-628 in hBC is mediated by the observed desmin down-regulation. However, it is now evident that Bcl-2 plays an essential role during the VDR-stimulated apoptotic process, because its overexpression prevents calcitriol-induced cell death in insulinoma (52), breast cancer (53), and prostate (54) cancer cells.

In conclusion, the human bladder, which expresses VDR transcript levels virtually identical to those of the prostate gland, represents a new potential target for VDR ligands. Thus, based on our recent demonstration that BXL-628 can reduce prostate growth without affecting calcemia and without any apparent toxic effect (8), we might hypothesize its therapeutic application in the treatment of selected bladder diseases. The hyperplastic overactive bladder could repre-

sent an ideal candidate for treatment with BXL-628 in view of its antiproliferative and prodifferentiative effects on bladder cells in culture, which probably contribute to the control of smooth muscle cell overactivity, as well as considering the strong association of a higher dietary intake of vitamin D with a decreased risk of overactive bladder (55). Thus, because bladder cell overgrowth and smooth muscle overactivity have been implicated in the initial steps of bladder decompensation and LUTS, it is possible that BXL-628 might be useful in BPH patients for its effect not only on the prostate, but also that on the bladder.

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