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ORIGINAL PAPER

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Induction of anoikis by doxazosin in prostate cancer cells is associated with activation of caspase-3 and a reduction of focal adhesion kinase

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Abstract The quinazoline family of α_1-blockers (prazosin, doxazosin, and terazosin) induce apoptosis of prostate cells through an α_1-adrenoceptor–independent mechanism. The objective of this study was to gain insight into the non-adrenergic, apoptotic mechanism of action of doxazosin in the prostate and the induction of anoikis by doxazosin. Primary cultures of benign prostate stromal and epithelial cells and the LNCaP (androgen sensitive) and PC-3 (androgen insensitive) prostate carcinoma cell lines were treated with doxazosin (0–50 μM). The effects of doxazosin on cell morphology, caspase-3 activity, and the expression levels of focal adhesion kinase (FAK) and integrin-linked kinase (ILK) were examined. Doxazosin induced changes in morphology consistent with anoikis in both benign and cancerous prostatic cells and increased caspase-3 activity. The effects were similar comparing benign cells (which express α_1-adrenoceptors) and cancer cells (which do not express α_1-adrenoceptors), but were more robust in benign cells. Norepinephrine had no effect on doxazosin-induced cell morphology or caspase-3 activity. Treatment of PC-3 cells with doxazosin significantly reduced the protein levels of FAK but did not significantly affect the levels of ILK. These findings suggest that doxazosin induces apoptosis and anoikis of prostate cancer cells by a mechanism of action that is α_1-adrenoceptor independent. The apoptosis of cancer cells induced by doxazosin counteracts cell proliferation and may have the potential of retarding or reversing prostate cancer cell growth.

Keywords Apoptosis · Anoikis · Caspases · Doxazosin · Prostate

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Introduction

Along with the worldwide growth of the older population, the incidence of prostate cancer is expected to increase substantially in the next two decades. Prostate cancer, which primarily affects men aged 50 and older, is the third leading cause of death in men worldwide [1]. By the year 2020, the World Health Organization predicts there will be 393,000 prostate cancer-related deaths compared with 193,000 deaths in 1990 [1]. The American Cancer Society estimates that in 2004 there will be 29,900 deaths from prostate cancer and 230,110 newly diagnosed cases in the United States. Driven by the urgency of the projected increase, investigators are closely examining novel means of reversing the proliferation of prostate cancer cells to provide more effective therapy for patients with advanced prostate cancer.

Prostatic smooth muscle tone is regulated by the action of the sympathetic neurotransmitter norepinephrine on prostatic α_1-adrenoceptors. Relief of prostatic smooth muscle tone, the so-called dynamic component of benign prostatic hyperplasia (BPH), is obtained by treatment with α_1-adrenoceptor antagonists. Doxazosin, a quinazoline α_1-adrenoceptor antagonist related to prazosin, is a safe and well-tolerated drug for the treatment of lower urinary tract symptoms attributed to BPH [2].

In addition to improvement in BPH symptoms, doxazosin also induces apoptosis in both stromal and epithelial compartments of the prostate and is uninhibited by norepinephrine [3, 4, 5]. Furthermore, the apoptotic effects of doxazosin are not accompanied by alterations in the rate of cell proliferation [3, 4]. It appears that doxazosin induces apoptosis through utilization of a mechanism of action that is α_1-adrenoceptor independent. Doxazosin may therefore possess therapeutic efficacy for preventing or delaying tumor growth.

The objective of this research is to gain insight into the nonadrenergic, apoptotic mechanism of action of doxazosin in prostatic cells. This study focuses on the

aggressive, androgen-independent PC-3 prostate tumor cell line and reports on the effects of doxazosin treatment on anoikis and cell morphology, caspase-3 activity, and the expression levels of focal adhesion kinase (FAK) and integrin linked kinase (ILK).

Materials and methods

Materials

Human prostate tissue was obtained from male patients undergoing prostatectomy for BPH or prostate cancer in accordance with the Institutional Board of Research Associates' guidelines in effect at New York University Medical Center. The α_1-adrenergic antagonist doxazosin mesylate used in this study was obtained from Pfizer (Sandwich, Kent, UK). The ILK antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif), and the FAK antibody was from BD Biosciences (Bedford, Mass.).

Cell culture

Primary cultures of benign prostate stromal and epithelial cells were established from human prostate tissue as described by Walden et al. [6]. The LNCaP cell line, which is androgen sensitive, and the PC-3 cell line, which is androgen insensitive were maintained according to conditions recommended by the supplier (American Type Culture Collection, Manassas, Va.). For experimentation, all cell lines were plated in 9 cm culture dishes. At 70% confluence, either vehicle (0) or doxazosin mesylate was added to the culture medium to final concentrations of 0, 10, 20 or 50 μM, and cells were incubated for either 24 or 72 h. The PC-3 cells were photographed using phase contrast optics. The effects of doxazosin treatment on anoikis and cell morphology were examined.

Caspase-3 assay

Lysates from floating and adherent PC-3 cells treated with vehicle or doxazosin mesylate were assayed for caspase-3 activity using the Fluorace Apopain Fluorescent Assay kit and Versafluor Fluorometer (both from Biorad, Hercules, Calif.). Assay conditions were those described by the manufacturer. Briefly, lysates were prepared by freeze-thawing four times in buffer (10 mM HEPES, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 μg/ml pepstatin, 20 μg/ml leupeptin, pH 7.4). Caspase-3 activity in the lysates was determined by assessing the change in fluorescence of the substrate Ac-DEVD-AFC over time using the Versafluor Fluorometer (Biorad). The protein concentration in the lysates was determined using Biorad Protein Assay reagent. Positive and negative controls in the kit were also included in the assay. Caspase-3 activity was expressed as units caspase-3 activity (arbitrary) per mg of protein.

Immunoblotting for FAK and ILK

Protein extracts were prepared from control and doxazosin-treated PC-3 cells as described [7]. The protein concentration in the extracts was determined using Biorad Protein Assay reagent. The protein extracts (20 μg total protein) were electrophoresed in 10% SDS-polyacrylamide gels and the gel contents were transferred to PVDF membranes using a Multiphor II semi-dry blotting apparatus (Amersham Pharmacia Biotech, N.J.). The membranes were immunoblotted with antibodies to FAK (Upstate Biotechnology, N.Y.) or ILK (Santa-Cruz Biotechnology). Finally the membranes were re-probed with an antibody to mitogen-activated protein kinase, Erk 1/2 (MAPK) (Santa-Cruz Biotechnology). The change in FAK or ILK relative to MAPK was determined densitometrically using a Biorad GS710 calibrated densitometer and Biorad Quantity 1 software.

Results

This study focuses on the effects of doxazosin on the highly metastatic, androgen insensitive α_1-adrenoceptor negative PC-3 prostate cancer cell line. The PC-3 cell line allows the effects of doxazosin to be studied independently of adrenergic or androgen control. The other cells used in the study (the androgen sensitive LNCaP prostate cancer cell line, and primary cultures of prostate stromal and epithelial cells) were for comparison only. The PC-3 cell line was overall more refractory to the effects of doxazosin than the other cells.

Effects of doxazosin on cell morphology

Doxazosin caused a dose dependent increase in the morphological features of apoptosis and anoikis in both benign and cancerous prostatic cells. These features included the rounding up of cells, DNA-degradation in the nucleus, cell shrinkage, the appearance of vacuoles, and cell detachment from the tissue culture plate.

The effect of doxazosin on the morphology of the PC-3 cells is evident in the phase-contrast photographs (Fig. 1). Cultures that received vehicle alone (0 μM doxazosin) revealed no morphological changes indicative of apoptotic activity. Cultures that received 10 μM doxazosin for 72 h were highly granulated and vacuolated. In cultures that received 20 μM doxazosin for 72 h, anoikis was evident in the cells that appear round and separated. In the cultures treated with 20 and 50 μM doxazosin, many cells were seen floating in the culture medium, and in the culture treated with 50 μM

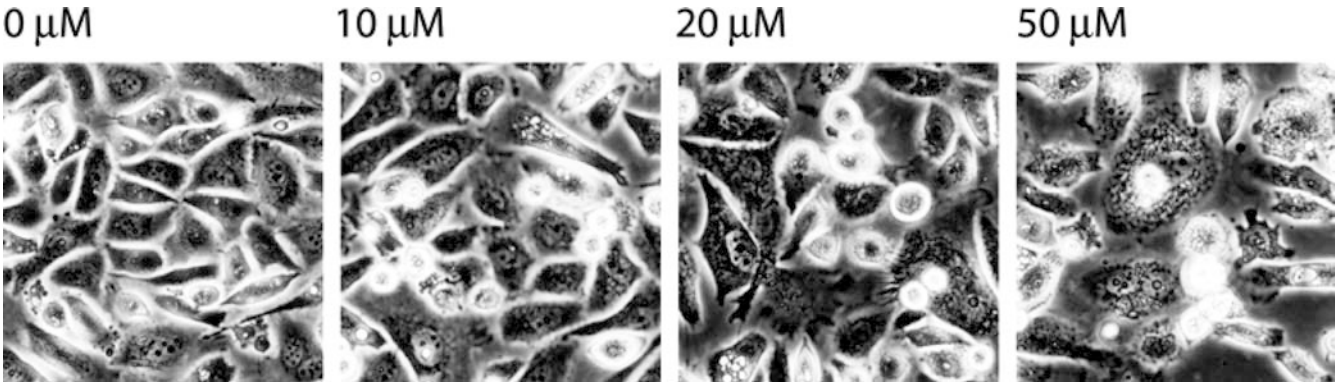

Fig. 1 Effects of doxazosin on the morphology of PC-3 cells. Cells were incubated for 72 h with 0, 10, 20 or 50 μM doxazosin and photographed using phase contrast optics

doxazosin for 72 h, few cells remained attached. Benign stromal and epithelial cells were more sensitive to the apoptotic effect of doxazosin; results were produced with lower doses of doxazosin in less time. Norepinephrine did not prevent or have any obvious effect on the cell morphological changes induced by doxazosin (data not shown), indicating that the effects of doxazosin on cell morphology were independent of adrenoceptors.

Effects of doxazosin on caspase-3 activity

Doxazosin stimulated an increase in caspase-3 activity in PC-3 cells that was both concentration- and time-dependent (Fig. 2). Caspase-3 activity was significantly elevated in the 50 μM treatment group at 24 h and in the 20 and 50 μM treatment groups at 72 h. Once again, the effect of doxazosin was more robust in the benign cells, where lower doses of doxazosin caused much greater activation of caspase-3. Norepinephrine did not significantly effect doxazosin-induced caspase-3 activation in either benign or cancer cells. Based on these initial findings, the concentration of doxazosin used in subsequent tests was 20 μM. Compared to PC-3 cells at doxazosin concentrations of 20 μM, caspase-3 activity was 4.1-fold, 5.8-fold and 8.2-fold greater in LNCaP, primary stromal and primary epithelial cells, respectively.

Expression levels of FAK and ILK

The morphology of the PC-3 cells treated with doxazosin suggested that the therapeutic agent might be stimulating anoikis by reducing cell adhesion to the extracellular matrix (ECM). The integrins are central to a number of biological processes including adhesion to extracellular matrix, intercellular adhesion, the maintenance of cell morphology, cell migration and the regulation of cell growth and differentiation [8]. Integrins are heterodimeric, transmembrane proteins that can simultaneously bind to proteins of the ECM, such as fibronectin, and to components of the actin cytoskeleton, such as talin and α-actinin. In addition, the integrins are involved in signal transduction; signals are transduced from integrins by interaction with FAK, and signals are transduced to integrins by interaction with ILK. To determine if doxazosin had an effect on the expression levels of FAK and ILK, immunoblotting of protein extracts from control and doxazosin-treated PC-3 cells was done. The expression levels of FAK were reduced in every extract treated with 20 μM doxazosin (Fig. 3). By densitometry, the mean ± SEM levels of FAK relative to levels of MAPK, were reduced by 47.8 ± 5.4%, 86.6 ± 8.7% and 38.8 ± 5.2% at 24, 48 and 72 h, respectively. All of these were statistically significant. In contrast, doxazosin had no significant effect on the levels of ILK (Fig. 3), which were not changed by more than 10–15% relative to MAPK following treatment with doxazosin.

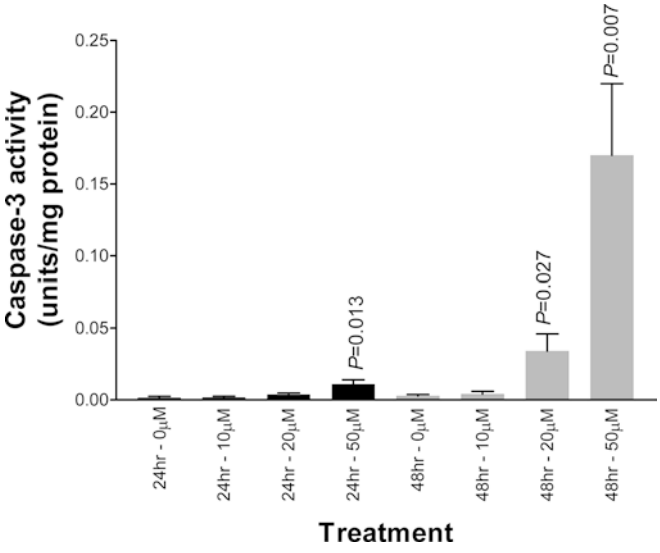

Fig. 2 Time-dependent influence of doxazosin activity on caspase-3 activity in PC-3 cell lysates. Cells were incubated with 0, 10, 20 or 50 μM doxazosin for 24 or 72 h. Lysates from these cells were assayed for caspase-3 activity using a fluorescent substrate (Biorad). Results are expressed as the mean ± SEM for six independent determinations. *P* values are given for those significant differences compared to 0 μM doxasozin at 24 or 72 h

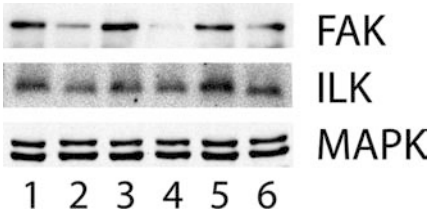

Fig. 3 Immunoblot analysis of the effects of doxazosin on focal adhesion kinase and integrin linked kinase protein levels. Protein extracts from control and doxazosin-treated PC-3 cells were immunoblotted with antibodies to FAK, ILK, and mitogen-activated protein kinase (MAPK; control for loading). Cells were treated with 0 μM doxazosin (*lanes 1*, *3*, *5*) or with 20 μM doxazosin (*lanes 2*, *4*, *6*). Times of treatment were 24 h (*lanes 1*, *2*), 48 h (*lanes 3*, *4*), and 72 h (*lanes 5*, *6*)

Discussion

The importance of developing more effective therapies for prostate cancer is intensified by the projected increase in its incidence. While radical prostatectomy is considered curative for cancer localized to the prostate, advanced prostate cancer is often treated with androgen ablation therapy to curtail the growth of cancer cells [5]. However, this treatment is effective only against androgen-dependent prostatic cancer cells and fails to block the proliferation of androgen-independent cells [5].

Doxazosin, which provides effective treatment for the relief of BPH symptoms, also induces apoptosis in both stromal and epithelial compartments of the prostate [3, 4]. The apoptotic effects of doxazosin are not accompanied by alterations in the rate of cell proliferation as determined by immunohistochemistry for Ki-67, the antigen associated with proliferation [3, 4]. Norepinephrine, the neurotransmitter that mediates smooth muscle tone, was unable to inhibit the apoptotic effects of doxazosin on the prostate cell lines [5].

Similar effects were also observed for the α_1-blocker terazosin, another quinazoline derivative structurally related to doxazosin [4]. In contrast, the structurally unrelated α_1-adrenoceptor antagonist tamsulosin had no effect on cell kinetics [5]. In other studies, doxazosin and prazosin reduced DNA synthesis and cell number in growth factor-stimulated vascular smooth muscle cells, also by an adrenoceptor independent mechanism [9]. Taken together, it is apparent that the quinazoline family of α_1-blockers (prazosin, doxazosin, and terazosin) induces apoptosis through an α_1-adrenoceptor independent mechanism.

The goal of the studies presented here was to investigate the α_1-adrenoceptor independent, apoptotic mechanism of action of doxazosin in prostate cells. Anoikis induced by doxazosin in PC-3 cells was evident in the rounding of cells, appearance of other apoptotic features, and detachment from the tissue culture plate. Anoikis is a specific form of apoptosis that occurs in normal epithelial cells when integrin-mediated matrix contacts are disrupted or lost [10]. Anoikis prevents epithelial cells that are shed from their base from colonizing elsewhere [10]. Resistance to anoikis may be caused by the aberrant expression of oncogenes or tumor suppressor genes, contributing substantially to malignancy [10]. However, even in PC-3 cells the stimulation of anoikis reduces the number of cancer cells and counteracts the growth of the tumor.

Doxazosin also induced concentration- and time-dependent increases in caspase-3 activity on PC-3 cells. Early in the apoptotic process, the initiator caspases (e.g., caspase-8, caspase-9) are activated [11]. They activate other caspases (e.g., caspase-3, caspase-7) downstream, which are primarily responsible for the morphological characteristics of apoptosis [11]. Caspase-3, identified as a key mediator of apoptosis in mammalian cells, cleaves major structural elements of the cytoplasm and nucleus, DNA components, and protein kinases, causing cells to become round in shape, detach from the plate, and undergo nuclear fragmentation [12, 13]. Furthermore, caspase-3 also amplifies apoptotic signals by the cleavage of BCL-2 [11]. Though initiation of the caspase cascade in anoikis remains to be explained [14], the increase of caspase-3 activity by doxazosin promotes anoikis and, subsequently, apoptosis of cancer cells.

Doxazosin may be stimulating anoikis by influencing cell adhesion. Immunoblot analysis revealed that the expression levels of FAK in PC-3 cells treated with doxazosin were less than in control cells. FAK is a nonreceptor tyrosine kinase that modulates crucial cell functions such as proliferation, migration, and survival [15]. When FAK is activated, it can suppress anoikis in normal epithelial and endothelial cells [16]. However, when FAK is cleaved by caspases, its disruption contributes to the morphological changes of apoptosis [17]. This study suggests that by increasing caspase-3 activity, doxazosin may reduce the expression levels of FAK and promote apoptotic activity. It is interesting in this regard that the increased metastatic potential of the PC-3 prostate cancer cell line correlates with elevated FAK expression [18]. If, as our study suggests, doxazosin-mediated anoikis acts at least in part by reducing FAK levels, then the elevated FAK expression seen in PC-3 cells compared to benign cells could potentially explain the increased resistance of PC-3 cells to the effects of doxazosin.

Results of the immunoblot analysis indicated that doxazosin did not reduce ILK protein levels; however, ILK is involved in the regulation of integrin binding properties as well as cell survival and proliferative pathways [19]. When ILK is overexpressed in epithelial cells, it disrupts cell-extracellular matrix and cell-cell interactions and suppresses anoikis [20].

Taken together, our findings indicate that doxazosin acts at least in part to reduce FAK levels. Additional research is now under way to determine whether the expression of FAK is reduced at the transcriptional level or at the post-transcriptional level (by increased protein

degradation due to elevated caspase-3 activity). Because the quinazoline ring is structurally similar to the purine ring, doxazosin may also interfere with the activity of certain kinases. Additional experiments are under way to address this possibility and to globally examine transcriptional events influenced by doxazosin using cDNA microarrays.

Conclusions

This study indicates that doxazosin induces anoikis and increases caspase-3 activity in PC-3 cancer cells, suggesting that it may provide a means of retarding or reversing prostate cancer cell proliferation. Doxazosin also reduces FAK protein expression, which obstructs anoikis, thus advancing the process of apoptosis. This line of investigation offers new potential for utilizing the α_1-adrenoceptor independent mechanism of action of doxazosin in a novel approach for more effective therapy for advanced prostate cancer.

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