

Testosterone promotes apoptotic damage in human renal tubular cells

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Testosterone promotes apoptotic damage in human renal tubular cells.

Background. Apoptosis is a mode of cell death that participates in the kidney physiologic remodeling processes and is thought to contribute to cell loss and kidney structural damage in chronic renal diseases. Gender is one factor which contributes to accelerated nephron loss, with progression more rapid in men than in women in diabetic and nondiabetic chronic renal diseases. Mechanisms by which androgens may cause higher rate of progression of chronic renal diseases in men are poorly explored.

Methods. In this study, to investigate the role of androgens on apoptotic damage and its associated mechanisms, we examined the effects of testosterone (T) (0.1 nmol/L to 1 μ mol/L) on apoptosis, and apoptosis-related proteins in a proximal human tubule cell line (HK-2 cells). Additional experiments were performed in primary cultures of proximal tubular epithelial cells (PTECs). Cells were grown to subconfluence in normal growth medium, and apoptotic damage was induced by serum deprivation for 24 to 48 hours. Cycloheximide, flutamide (a T-receptor antagonist), 17- β estradiol, or caspase inhibitors were added to cultures that were successively processed for terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end-labeling (TUNEL) analysis, annexin V/propidium iodide staining, immunofluorescence, or immunoblots to identify effects and apoptotic pathways that could be modulating cell survival.

Results. Both morphologic analysis by annexin V/propidium iodide staining and TUNEL showed that physiologic T levels (1 to 10 nmol/L) induced a significant increase in apoptosis both in HK-2 cells and PTECs. In both types of cell lines pretreatment with the androgen receptor antagonist flutamide prevented the T-induced apoptosis. T-induced apoptosis was enhanced by treatment with cycloheximide and prevented by 17 β -estradiol. Fas, Fas ligand (FasL), and Fas-associating death domain containing protein (FADD) were clearly up-regulated within 48 hours of T treatment in HK-2 cells. Also, T significantly increased the expression of Bax protein ($P < 0.01$ vs. control) (an effect which was blocked by flutamide), and decreased the ex-

pression of Bcl-2. Western blot analysis showed that caspase-3 was activated. Moreover, cleavage into an 85-kD poly(ADP-ribose) polymerase-1 (PARP-1) terminal breakdown product was detectable. The changes in cellular morphology induced by T at 48 hours were no longer observed after the addition of caspase-8, caspase-9, and caspase-3 inhibitors to the culture medium.

Conclusion. These results indicate that T increases the permissiveness of proximal tubule kidney cells to apoptotic effects by triggering an apoptotic pathway involving caspase activation, Fas up-regulation, and FasL expression, thus potentially interacting with mechanisms of cell loss which have been already shown to be activated in chronic renal diseases. This is consistent with a role for T in promoting renal injury in men.

Apoptosis is a mode of cell death that participates in kidney physiologic remodeling processes [1, 2] and is thought to contribute to cell loss and kidney structural damage in chronic renal diseases [1, 3–6]. Gender is one factor that likely contributes to accelerated nephron loss, with progression more rapid in men than in women in diabetic and nondiabetic chronic renal diseases [7]. The specific mechanisms underlying this clinical finding may be related to genetically determined differences between the sexes in renal structure and function and/or to the direct effects of sex hormones on renal tissues [6]. Estrogen has been shown to stimulate renal nitric oxide generation and to modulate the synthesis and release of various growth factors, hormones, and cytokines [7]. Female hormones may also induce protective effects in mesangial cells, including suppression of collagen gene transcription and protection from transforming growth factor β 1 (TGF- β 1)-induced apoptosis [8]. By contrast, the effects of androgens on processes contributing to the progression of chronic renal diseases have been less extensively studied. Androgens can interact with the renin-angiotensin system and potentiate the damage induced in the kidney and extra-renal tissues by hypertension [9]. Androgens can also increase collagen synthesis by vascular smooth muscle cells and promote the accumulation of collagen and elastin in vessels [10]. In addition, androgens can induce apoptosis in human vascular endothelial cells, and

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in this way may contribute to the increased risk of vascular events in men [11]. However, the interaction of androgens with cell survival and death is complex and appears to be variably regulated in various tissues of the body [12, 13]. Although the clinical significance of cell death by apoptosis in chronic kidney diseases is yet to be fully established, several observations indicate that some kidney structures may be particularly involved by apoptotic processes. In normal rat kidney, proximal tubular epithelium is the main site of Fas and FasL expression, which can promote apoptosis by caspase activation [14, 15]. Moreover, the Fas/FasL system is up-regulated during renal injury [1, 15]. In experimental chronic kidney disease, cellular apoptosis outweighs proliferation, thus favoring cell deletion and progressive tubular atrophy [16]. Finally, in patients with chronic glomerulonephritis, an increase in apoptotic tubular cells has been detected [17].

Here, we examine if T induces apoptosis in human kidney proximal tubular cells. We also examine alterations in the expression and/or activity of a wide variety of apoptosis-associated proteins after T exposure and investigate the role of Fas/FasL and caspases in the execution of cell death. Our results demonstrate that T causes apoptosis of tubular cells through Fas sensitization and activation of caspase-dependent apoptotic pathway, thus potentially interacting with mechanisms of cell loss which have been already shown to be activated in chronic renal diseases.

METHODS

Reagents

Media and additives for culture of HK-2 cells, as well as annexin fluoroscein isothiocyanate (FITC), propidium iodide, 3,3-diaminobenzidine (DAB), testosterone, flutamide, 17 β -estradiol, phenazine methosulfate, p-iodonitrotetrazolium violet, β -NAD, L-(+)-lactic acid, cycloheximide, acrylamide, and other reagents for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Caspase inhibitors were purchased from R&D Systems Europe, Ltd. (Oxon, UK). Coomassie protein assay reagent was obtained from Pierce (Rockford, IL, USA). Tissue culture plates and dishes were from Corning (New York, NY, USA). Antihuman Bcl-2 monoclonal antibody, antihuman Bax polyclonal antibody, antihuman Fas and FasL antibodies were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Antihuman-cleaved poly (ADP-ribose) polymerase-1 (PARP-1) polyclonal antibody was purchased from Alexis Biochemicals (Lausen, Switzerland). Antihuman FADD (monoclonal antibody) and antiactive caspase-3 (p17) monoclonal antibody were purchased from BioVision Research Products (Mountain View, CA, USA).

The biotin-streptavidin-amplified detection system was obtained from Biogenex (Biogenex Laboratories, San Ramon, CA, USA) and Eukitt from O Kindler GmbH and Co. (Freiburg, Germany). Terminal deoxynucleotidyl transferase (TdT), TdT buffer, and biotinylated dUTP were obtained from Boehringer Mannheim (Mannheim, Germany). Hybond-c-nitrocellulose membrane, enhanced chemiluminescent reaction ECL, and Hyperfilm-ECL were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL, USA).

Cell culture

HK-2 cells, an immortalized proximal tubular epithelial cell line from normal adult human male kidney, were obtained from American Tissue Culture Collection (ATCC, Manassas, VA, USA). Primary human proximal tubular epithelial cells (PTECs) were cultured according to the method of Detrisac et al [18]. Portions of renal cortex not involved by disease were obtained from male kidneys removed by surgery for renal cell carcinoma. Small fragments of renal tissue were placed on a matrix of type I bovine collagen and fetal bovine serum (FBS). Outgrowth of PTECs was confirmed by their morphologic appearance, enzyme cytochemistry (reactivity for alkaline phosphatase, gamma-glutamyltranspeptidase, acid phosphatase, and leucine aminopeptidase), and immunofluorescence. PTECs were used between the third and fifth passage. Cells were grown in phenol red-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 5% (v/v) FBS, 100 U/mL penicillin-streptomycin, 2 mmol L-glutamine, 5 μ g/mL insulin, 5 μ g/mL transferrin, 5 ng/mL sodium selenite, 5 pg/mL T3, 5 ng/mL hydrocortisone, 5 pg/mL PGE1, and 10 ng/mL epidermal growth factor. Cells were grown at 37°C in a humidified 5% CO₂ condition.

Cell treatments

Cells were grown to subconfluence in normal growth medium, and apoptotic damage was induced by additives and serum deprivation for 24 to 48 hours in the presence of T (0.1 nmol/L to 1 μ mol/L). Control cells (C) were not treated with hormone. In order to determine if T is directly involved in apoptosis, the antiandrogen flutamide (100 nmol/L) was added 4 hours earlier than 10 nmol/L testosterone. In separate settings of experiments, cycloheximide (10 μ g/mL), a combination of T and cycloheximide, or buffer control, respectively, was used. In other experiments, 17 β -estradiol (10 nmol/L) was used in combination with T. To determine a role of caspases in 10 nmol/L T-induced cell death, HK-2 cells were preincubated for 30 minutes with 50 μ mol/L caspase inhibitors. Caspase-8 inhibitor II (Z-IEDT-FMK), caspase-9 inhibitor I (Z-LEHD-FMK), and caspase-3 inhibitor I (DEVD-CHO) were used.

Cell viability

Experiments were carried out by adding T at scalar doses to 10^5 cells cultured in 12-well plates. Nontreated cells were used as control. After 24 and 48 hours of incubation, cells were counted using a NeuBauer chamber (Corning Costar Co., Cambridge, MA, USA). Cell viability was assessed by Trypan blue. For each treatment group the number of adherent cells at $t = 0$ served as baseline value (100%), and was used to express the percentage of adherent cells.

Lactate dehydrogenase release

The amount of lactate dehydrogenase (LDH) activity released into the medium was measured to assess the leakage of normal components of the cytoplasm into the medium. For LDH assay, supernatant aliquots were transferred to the corresponding wells of a 96-well plate. Then 100 μ L of substrate containing L-(+)-lactic acid, β -NAD, p-iodonitrotetrazolium violet, phenazine methosulfate, and Tris buffer were added at each well and incubated at 37°C until reaching a plateau. The absorbance was calculated at 540 nm using a scanning multiwell spectrophotometer.

Annexin V-FITC/propidium iodide (PI) staining

In multiple experiments, HK-2 cells were stained with annexin V/propidium iodide and observed under a fluorescence microscope. The assay is based on the ability of annexin V (green fluorescence) to bind to the phosphatidylserine exposed on the surface of cells undergoing apoptosis and the capacity of propidium iodide (red fluorescence) to enter cells that have lost their membrane integrity. HK-2 cells were grown on chamber slides, washed with Dulbecco's phosphate buffer solution (DPBS), and incubated in the dark for 5 minutes at room temperature with 200 μ L of 1 \times binding buffer, 1 μ L of FITC-labeled recombinant annexin V, and 1 μ L of propidium iodide. Cells were observed and counted (300 cells for each condition and experiment) under a fluorescence microscope using dual filter set for FITC and rhodamine. Cells that lost membrane integrity showed red staining throughout the nucleus and a halo of green staining on the cell surface. To evaluate apoptotic phenomena, we considered the percentage of cells annexin V-positive/propidium iodide-negative.

Terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end-labeling (TUNEL)

HK-2 cells grown on chamber slides to subconfluence were incubated for 48 hours with or without T (10 nmol/L). After a five-minute incubation in 2% paraformaldehyde, cells were fixed in 2:1 vol/vol ethanol:acetic acid at room temperature for 10 minutes. After three washes with phosphate-buffered saline

(PBS), cells were incubated with 100 U/mL TdT, 0.5 μ g/mL biotinylated uridine triphosphate in 140 mmol/L potassium cacodylate, 125 mmol/L Tris-HCl, 2.5 mmol/L cobalt chloride, pH 6.6, for 1 hour at 37°C in a humidified chamber. After stopping the reaction by transferring the slides to tris-borate (TB) buffer (300 mmol/L sodium chloride, 30 mmol/L sodium citrate) and washing in PBS, cells were incubated for 30 minutes at room temperature with extra-avidin-peroxidase diluted 1:20 in PBS, and then incubated with the peroxidase-substrate solution (0.04% 3,3-diaminobenzidine in 50 mmol/L Tris-HCl buffer containing 0.03% hydrogen peroxide) for 5 minutes. After rinsing with PBS, sections were counterstained with hematoxylin and examined by light microscopy.

Cell immunofluorescence and immunostaining

The expression of Fas, FasL, FADD, and p17 fragment of the active caspase-3 protein were initially documented by immunofluorescence. HK-2 cells were grown on chamber slides to subconfluence and incubated for 48 hours in the presence or absence of 10 nmol/L T. Cells were then washed with cold PBS and fixed in cold methanol for 5 minutes. After a 30-minute incubation with anti-FAS (polyclonal Ab 1:40 in PBS), anti-FASL (polyclonal Ab 1:40 in PBS), anti-FADD (monoclonal Ab, 20 mg/mL), or antiactive caspase (polyclonal Ab, 20 mg/mL) antibodies at room temperature, cells were washed extensively with PBS and exposed to biotinylated conjugated secondary antibody for 30 minutes. After being washed, cells were incubated with FITC-streptavidin for 30 minutes. Slides were observed under a fluorescence microscope. The expression of cleaved PARP-1, Bax, and Bcl-2 proteins was documented by immunostaining. Both attached and floating cells were harvested by trypsin and incubated on poly-L-lysine coated glass slides for 40 minutes at 4°C. Then, the spots were air-dried and fixed in cold acetone for 30 seconds and stored at -20°C until the immunodetermination of cleaved PARP-1, Bax, and Bcl-2. After rehydration in PBS, spots were incubated with the antihuman cleaved PARP-1 polyclonal antibody (dilution 1 μ g/mL in PBS), anti-Bax polyclonal antibody (1:200 in PBS), and anti-Bcl-2 monoclonal antibody (1:200 in PBS) at room temperature. The second and third steps were performed using the improved biotin-streptavidin-amplified detection system. Briefly, cells were incubated with the secondary antibody (biotinylated IgG; 1:100 in PBS) for 20 minutes. After several washes with PBS, cells were incubated with the concentrated enzyme label (biotin-streptavidin-peroxidase) for 20 minutes at room temperature. Peroxidase was developed with 0.04% 3,3-diaminobenzidine in 50 mmol/L Tris-HCl buffer containing 0.03% hydrogen peroxide for 15 minutes. After rinsing with PBS, slides were counterstained with

hematoxylin, coverslipped with Eukitt, and examined by light microscopy.

Image analysis

Image analysis was performed by the Leica Q500 MC Image Analysis System (Leica, Cambridge, UK). For each sample 300 cells were randomly analyzed and the optical density of the signals was quantitated by a PC computer. The video image was generated by a CCD Camera (Leica, Cambridge, UK) connected through a frame grabber to a PC computer. Single images were digitized for image analysis at 256 gray levels. Imported data were analyzed quantitatively by Q500MC Software-Qwin (Leica). The single cells were randomly selected by the operators using the cursor, and then positive areas automatically estimated. Constant optical threshold and filter combination were used [19, 20].

Western blot analysis

The cell layers (including the floating cells) were washed twice with cold DPBS and cold lysis buffer [20 mmol/L HEPES, 150 mmol/L NaCl, 10% (v/v) glycerol, 0.5% (v/v) NP-40, 1 mmol/L EDTA, 2.5 mmol/L DTT, 10 μ g/L aprotinin, leupeptin, pepstatin A, 1 mmol/L PMSF, and Na_3VO_4]. Cells lysates were incubated for 1 hour at 4°C, and the insoluble material was removed by centrifugation at 13,000 rpm for 20 minutes at 4°C. Protein concentration was determined by using the Coomassie protein reagent. Protein (30 or 50 μ g) was loaded on 10% to 15% SDS-PAGE under reducing or nonreducing conditions after being heated for 3 minutes at 100°C. The proteins were electrotransferred to a nitrocellulose membrane. Blots were blocked for 1 hour at room temperature in PBS 5% milk. The membrane was incubated overnight at 4°C with antihuman BAX, antihuman BCL-2, antihuman Fas, antihuman FasL, anti-FADD, antiactive caspase-3, (p17 fragment), anti-cleaved PARP-1 (p85 fragment), followed by three washes (total 30 minutes) with PBS 0.05% (v/v) Tween 20. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Immunoblots were developed with the ECL Western blotting detection system.

Statistical analysis

Statistical analysis was performed with the InStat software package version 2.01 (Graph Pad, San Diego, CA, USA). The one-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test were used to test the significance of differences. Results are expressed as mean \pm SD and are the expression of at least three experiments, with two wells for each experiment. Differences were considered statistically significant if $P < 0.05$.

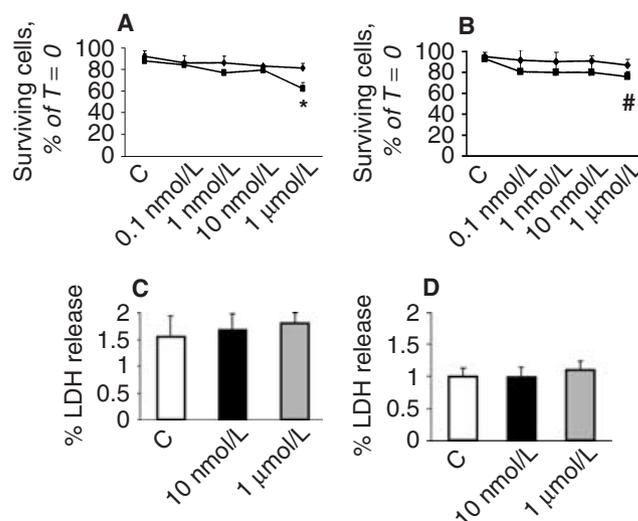


Fig. 1. Effects of increasing doses of testosterone (T) on proximal human tubule cell line (HK-2) (A) and proximal tubular epithelial cells (PTEC) (B) viability at 24 (\blacklozenge) and 48 (\blacksquare) hours. Cell viability was assessed by counting the number of adherent and floating cells. For each treatment group the number of cells at $t = 0$ served as baseline value 100% and was used to express the percentage of living cells. * $P < 0.001$ vs. control (C); # $P < 0.05$ vs. C. Effects of 10 nmol/L and 1 μ mol/L of T on HK-2 (C) and PTECs (D). LDH release at 48 hours. Results are expressed as mean \pm SD and are representative of 3 separate experiments. Each one was carried out in triplicate. The differences were not statistically significant.

RESULTS

Cell viability

During the course of the experiments, the majority of cells remained viable and attached to culture dishes in control cultures over a 48-hour period ($88 \pm 6.5\%$ of HK-2 cells and $93 \pm 6.4\%$ of primary PTECs were viable, respectively, at 48 hours) (Fig. 1). To examine the apoptotic effects of androgens, cells were treated with different concentrations of T (0.1 nmol to 1 μ mol/L). When T was used at the lowest physiologic doses (0.1 to 10 nmol/L), $83 \pm 8\%$ and $90.5 \pm 9\%$ of HK-2 cells and PTECs, respectively, were viable at 24 hours ($P > 0.05$ vs. untreated cells). These percentages did not significantly change at 48 hours ($80 \pm 2\%$ and $80 \pm 11\%$, respectively, for HK-2 cells and PTECs) (Fig. 1A and B). With 1 μ mol/L T (a concentration which is greater than that commonly found circulating in vivo in man) [21], cell viability was significantly decreased at 48 hours ($62 \pm 5\%$ of HK-2 cells; $76 \pm 5\%$ of PTECs viable).

There were no significant changes in LDH release (Fig. 1C and D) in untreated cells at 48 hours. Similarly, the addition of T did not cause cell injury, as represented by LDH release.

Effects of T on apoptosis in renal tubular cells

Cultured HK-2 cells and primary PTECs were incubated with different concentrations of T for 24 and

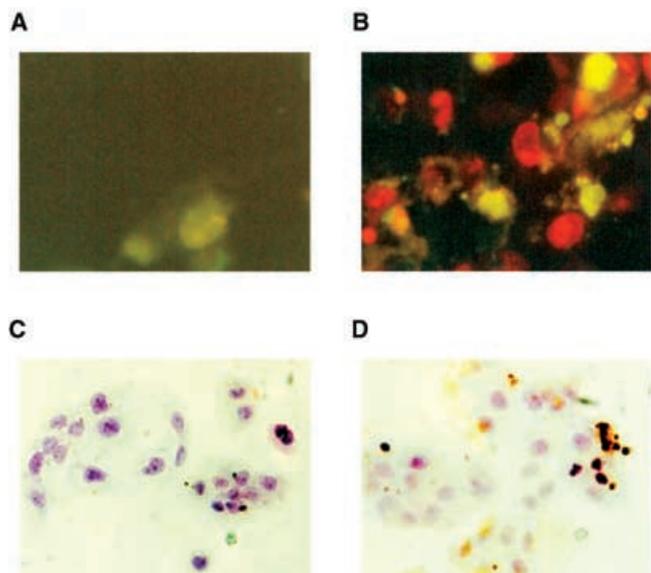


Fig. 2. Apoptosis in proximal human tubule cell line (HK-2) cultures stained with annexin V (green fluorescence) and propidium iodide (red fluorescence). Control HK-2 (A), HK-2 treated with 10 nmol/L testosterone (T) for 48 hours ($\times 400$) (B). DNA strand breaks as assessed by terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end-labeling (TUNEL) staining of HK-2. Cells were exposed or not exposed to 10 nmol/L T for 48 hours and subjected to TUNEL staining. Control HK-2 (C), HK-2 exposed to 10 nmol/L T ($\times 400$) (D).

48 hours in multiple experiments and subjected to apoptosis assay(s). Treatment with T induced several apoptotic features both in HK-2 cells (Fig. 2A-D) and PTECs. Features of the T-treated cells stained by TUNEL observed by light microscopy included rounding and detachment of the cells with fragmentation and shrinking of nuclei. Apoptotic cells showed chromatin condensation and fragmentation of nuclear material. The treatment with T also increased the percentage of annexin V/propidium iodide-positive cells, as shown by halo of green staining on cell surface (Fig. 2B). During the course of these experiments, only $3.60 \pm 1.93\%$ of the untreated HK-2 cells was annexin V-positive at 24 hours ($P = \text{NS}$ vs. $t = 0$), and this percentage increased slightly ($9.7 \pm 3.01\%$) at 48 hours. T induced a progressive rise in apoptotic HK-2 cells (Fig. 3A), with a 2- to 3-fold increase being observed at 24 hours ($12.3 \pm 2.5\%$ and $14.6 \pm 2.46\%$ for T 0.1 to 10 nmol/L, $P < 0.05$ to 0.01 vs. control). Analogous findings were observed at 48 hours (Fig. 3A). A similar trend was observed when cells were grown for 48 hours at different concentrations of T in 0.5% charcoal-stripped FBS (data not shown). For both T 10 nmol/L and 1 $\mu\text{mol/L}$ the apoptotic index was significantly higher than in experiments performed at 24 hours ($P < 0.05$ to 0.01).

Similar proapoptotic effects of T were obtained in primary PTECs (Fig. 3B). At 24 hours, $4.3 \pm 2\%$ of the untreated cells was annexin V-positive, and this percent-

age rose to $10.7 \pm 2.4\%$ at 48 hours ($P = \text{NS}$ vs. $t = 24$ hours). When primary PTECs were incubated with scalar doses of T, we observed a 3-fold increase in the apoptotic index at 24 hours ($13.3 \pm 2.3\%$ and $12 \pm 1\%$ for 1 to 10 nmol/L T; $P < 0.05$ vs. C), which rose further at 48 hours ($18 \pm 1.8\%$ and $28.5 \pm 6.6\%$ for T 1 nmol/L and 1 $\mu\text{mol/L}$, respectively; $P < 0.05$ to 0.001). Similar to what was observed with the immortalized line, the apoptotic index at 48 hours was significantly higher than in experiments performed at 24 hours ($P < 0.05$ to 0.01).

Cycloheximide strongly potentiated the apoptotic effect induced by T. Apoptotic cell death involved approximately 15% of HK-2 cells after 48 hours, and this figure raised to 40% when cycloheximide was added to T (Fig. 4).

Proapoptotic effects of T were abolished by the administration of the androgen-receptor antagonist flutamide (100 nmol/L). Only $10.8 \pm 4.2\%$ (in HK-2 cells) or $7 \pm 1\%$ (in PTECs) of flutamide + T treated cells showed apoptotic features at 48 hours ($P < 0.01$ to 0.001 vs. T, and $P = \text{NS}$ vs. C) (Fig. 5A and B).

To examine whether these effects were specific to androgens, in a separate setting of experiments, cells were treated with 17β -estradiol (10 nmol). Estradiol, per se, had no effects on apoptosis, but the coincubation of T + estradiol prevented the increase in the apoptosis induced by T both in HK-2 cells (Fig. 6A) and PTECs (Fig. 6B-E).

Effect of T on Fas, FasL, and FADD proteins

As a following step, we studied the expression of possible apoptosis-inducing proteins in HK-2 cells in order to individuate the underlying apoptotic pathway(s). Because of the evidence of the Fas-FasL system in proximal tubule cells [1], we first looked at the expression of Fas, FasL, and FADD induced by T treatment. Fas, a 45-kD member of the tumor necrosis factor (TNF) receptor superfamily, binds to its cognate ligand, FasL. Ligation of Fas causes rapid death-inducing FADD signaling complex formation. The protein is recruited to the receptor's DD and primes the apoptotic pathway [1]. HK-2 cells were analyzed by immunofluorescence, SDS-PAGE, and Western blotting with antibodies to Fas, FasL (a 37-kD protein), and FADD protein (24-kD). The basal low amount of FasL in the cell surface is consistent with data from murine tubular cells [15], as well as other cell types where most FasL is bound to intracellular membranes [22]. Our study revealed a clear up-regulation of Fas, FasL, and FADD in proximal tubule cells within 48 hours of T treatment (Figs. 7 and 8).

Effect of caspase inhibition

Starting from the above data, we sought to evaluate the downstream effectors of the Fas-FasL pathway, including the caspase family [23]. Two main pathways leading

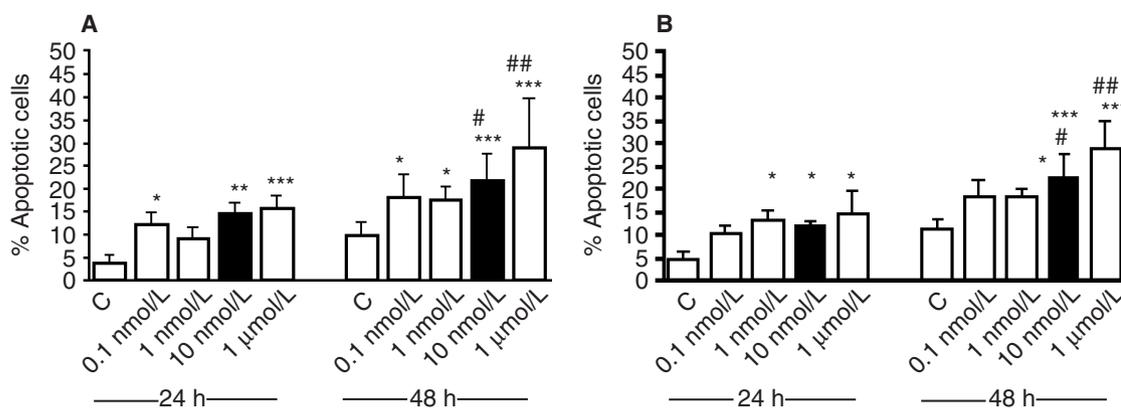


Fig. 3. Apoptosis detection by annexin V/propidium iodide. Subconfluent proximal human tubule cell line (HK-2) (A) and proximal tubular epithelial cells (PTECs) (B) were growth factor- and serum-deprived for 24 and 48 hours in presence of testosterone (T) (0.1 nmol/L to 1 μ mol/L). Cells were stained with annexin V/propidium iodide and examined under fluorescence microscope. Apoptotic cells were expressed as percent of total cells counted (350 cells for each condition). Data shown as mean \pm SD. Each experiment was performed in triplicate. * P < 0.05; ** P < 0.01; *** P < 0.001 vs. untreated cells (control); # P < 0.05 vs. 10 nmol/L 24 hours; ## P < 0.01 vs. 1 μ mol/L 24 hours.

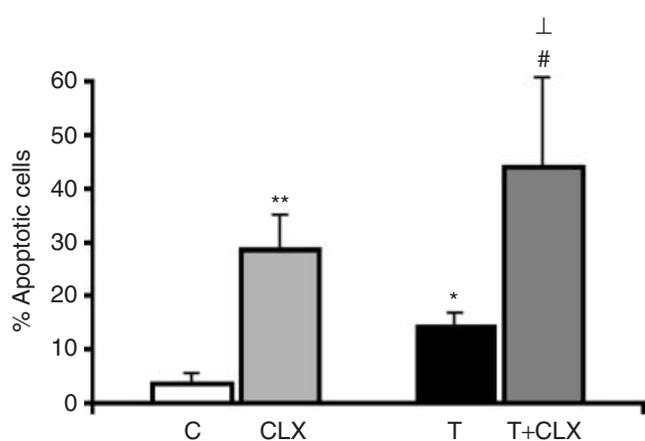


Fig. 4. Apoptosis detection by annexin V/propidium iodide. Subconfluent proximal human tubule cell line (HK-2) were growth factor- and serum-deprived for 24 hours in the presence or absence of testosterone (T) (10 nmol/L) and cycloheximide (10 μ g/mL) (CLX). * P < 0.05 vs. control (C); ** P < 0.001 vs. T; # P < 0.001 vs. T; † P < 0.001 vs. CLX.

to apoptosis through caspase activation have been described. On one way, the “extrinsic pathway” is activated by receptors of the TNFR superfamily through mechanisms that involve the adapter protein FADD, which activates the initiator caspase-8. On the other way, the “intrinsic pathway” is activated by mitochondrial proteins through the action of the initiator caspase-9. Since the inhibition of caspase functions has been shown to block the development of programmed cell death [24], we evaluated whether T-induced HK-2 cell apoptosis could be prevented by treatment with different inhibitors. The results revealed that the change in cellular morphology induced by T at 48 hours were no longer observed after the addition of Z-IEDT-FMK (caspase-8 inhibitor II), Z-LEHD-FMK (caspase-9 inhibitor I) and DEVD-CHO

(caspase-3 inhibitor I) to the culture medium. The anti-apoptotic effects were of similar entity for all inhibitors (Fig. 9).

T-induced apoptosis is associated with the activation of caspase-3

Caspase-3 is one of the key executioners of apoptosis and is responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear enzyme PARP-1. HK-2 cells incubated for 48 hours with 10 nmol/L T were analyzed by immunofluorescence, SDS-PAGE, and Western blot with a polyclonal antibody to caspase-3, which detects only the p17 fragment of active caspase-3. Results showed that caspase-3 is activated during T treatment (Figs. 10A and B, 11). We next checked for the cleavage of PARP-1, an endogenous substrate for caspase-3. During apoptosis, caspase 3 cleaves PARP-1 to yield an 85-kD and a 25-kD fragment. We examined PARP-1 expression by immunostaining and Western blot (Fig. 11). Cleavage into an 85-kD terminal breakdown product was detectable on Western blots in T-treated cells, confirming that caspase-3 was acting on cellular substrates.

Effects of T on Bax and Bcl-2

Because T-induced apoptosis is inhibited by caspase-9 inhibitor I (Z-IEDT-FMK), we examined the expression of Bax and Bcl-2. T treatment significantly increased the expression of Bax protein (P < 0.01 vs. C) (Fig. 12B and D). The pretreatment with flutamide did not induce this increase, and the expression of Bax was similar to control. The expression of Bcl-2 was opposite respect to Bax (Fig. 12A and C).

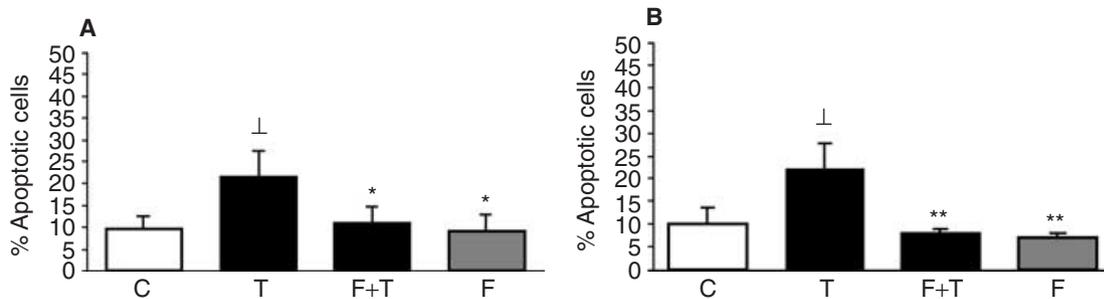


Fig. 5. Apoptosis detection by annexin V/propidium iodide. Subconfluent proximal human tubule cell line HK-2 (A) and proximal tubular epithelial cells (PTECs) (B) were growth factor- and serum-deprived for 48 hours in the presence of testosterone (T) (10 nmol/L), flutamide (F) (100 nmol/L) alone, or T plus F (added 4 hours earlier than T). Data are shown as the mean \pm SD from three representative experiments, with two wells of the cells for each treatment. * $P < 0.01$ vs. 10 nmol/L T; † $P < 0.001$ vs. C; ** $P < 0.001$ vs. 10 nmol/L T.

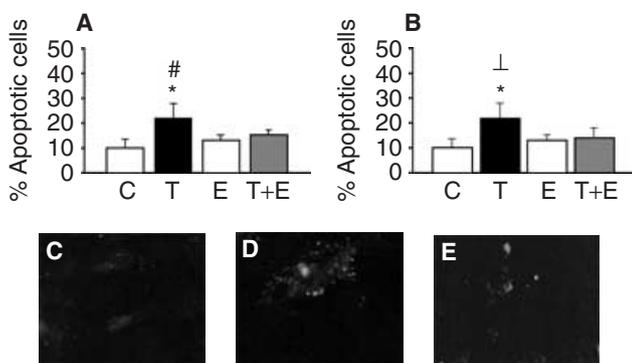


Fig. 6. Apoptosis detection by annexin V/propidium iodide. Subconfluent proximal human tubule cell line (HK-2) (A) and proximal tubular epithelial cells (PTECs) (B) were growth factor- and serum-deprived for 48 hours in the presence of T (10 nmol/L), 17 β -estradiol (E) (10 nmol/L) alone, or T plus E. Data are shown as the mean \pm SD from three representative experiments, with two wells of the cells for each treatment. * $P < 0.001$ vs. control (C); # $P < 0.001$ vs. E; † $P < 0.01$ vs. E. Apoptosis in primary PTEC cultures stained with annexin V (green fluorescence) and propidium iodide (red fluorescence): Control PTECs (C); PTECs treated with 10 nmol/L for 48 hours (D); PTECs exposed to T and E (10 nmol/L) for 48 hours ($\times 400$) (E).

DISCUSSION

The present study shows that androgens promote apoptotic damage in human proximal tubule cells, as evaluated by multiple apoptosis-associated determinations, including morphologic observation of TUNEL-stained cells, annexin V binding, and detection of apoptosis-related proteins. Moreover, these effects are observed at T levels that are in the physiologic range and are blocked by androgen receptor inhibition and estrogens. These findings are observed both in immortalized and primary proximal tubular cells. According to our data, androgens increase the permissiveness of tubular cells to apoptotic effects by triggering apoptotic pathways involving Fas up-regulation, FasL expression, and caspase(s) activation.

When we blocked survival signals with cycloheximide, apoptosis occurred rapidly. We observed that combined

treatment with androgens and cycloheximide generated additive apoptotic effects compared to T alone, suggesting a correlation between the inhibition of protein synthesis and T-mediated apoptosis. Both conditions induced the typical morphologic findings and the cell surface program characteristic for apoptosis.

Fas has been recognized in several nonlymphoid organs, including the kidney [1, 14, 24]. In contrast, FasL, a 40-kD type II membrane protein, is expressed in a more restricted number of tissues, where it can promote apoptosis through the activation of Fas receptors [1]. The regulation of FasL-induced apoptosis is complex, and some cell types express FasL but are unable to promote apoptosis [25]. Fas is expressed in renal tubular epithelial cells, but the role of the Fas-FasL system on apoptosis has been demonstrated only recently [1]. It has been previously observed that Fas/FasL-induced apoptosis promotes parenchymal cell damage in models of acute renal failure and glomerular injury [1]. According to our data, human proximal tubular cells are activated by T and show an up-regulated expression of Fas/FasL, which can contribute to their increased susceptibility to FasL-mediated apoptosis. In keeping with these findings, we found that the effector caspase-3 was activated by T, and that a hallmark caspase substrate, PARP-1, was cleaved. As an additional finding, our data suggest that T works primarily through a FADD- and caspase-8-dependent pathway. However, the involvement of caspase-9 in the degeneration process was somewhat unexpected. On one hand, it is possible that caspase inhibitors studied here are not equipotent, but that their effect may change according to individual substrate and inhibition kinetics. On the other hand, apoptotic changes were associated with an increased expression of Bax together with a decrease in the antiapoptotic protein Bcl-2 in T-treated cells, therefore suggesting an interaction between the extrinsic and intrinsic pathway. Caspase-8 may either directly activate procaspase-3 or cleave the proapoptotic protein Bid, which then subsequently induces cytochrome c release [27]. Nevertheless, the final result of either

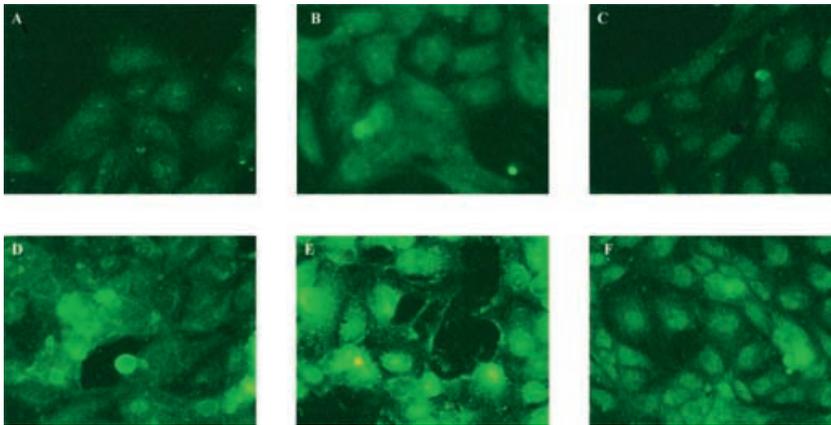


Fig. 7. Testosterone (T) treatment induces Fas (D), FasL (E), and Fas-associated death domain containing protein (FADD) (F), with respect to untreated cells (A, B, and C for Fas, FasL, and FADD, respectively) ($\times 400$).

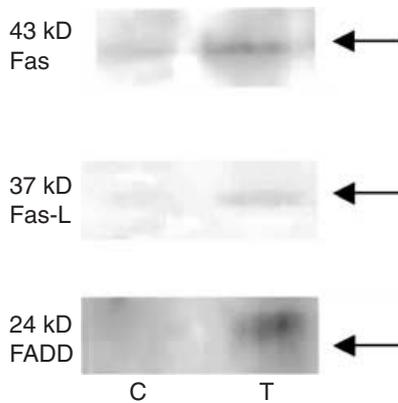


Fig. 8. Testosterone (T)-induced apoptosis is associated with up-regulation of Fas, Fas-L, and Fas-associated death domain containing protein (FADD). Cells incubated for 48 hours with 10 nmol/L of T were subjected to Western analysis with the various antibodies as shown. The results are representative of 3 different experiments.

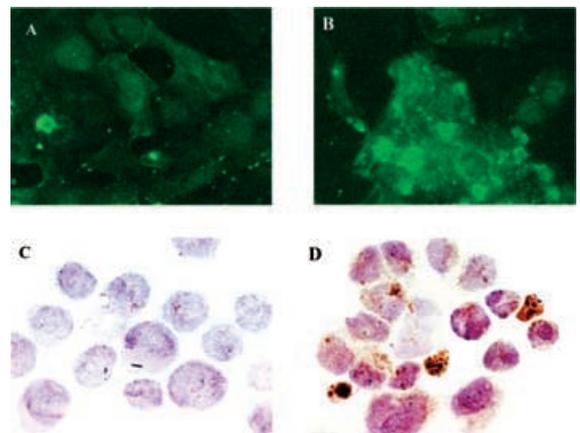


Fig. 10. Expression of p17 fragment active caspase-3 by immunofluorescence (upper panel) and cleaved poly(ADP-ribose) polymerase (PARP-1) by immunocytochemistry (lower panel) in control (A, C) and testosterone (T)-treated cells (B, D). T activates caspase-3 and its activation is documented by PARP-1 cleavage ($\times 400$).

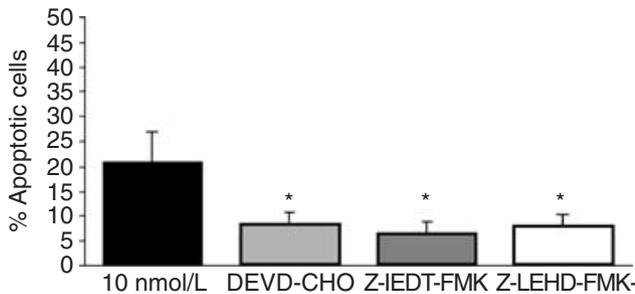


Fig. 9. Effects of the caspase inhibitor 3 (DEVD-CHO), caspase inhibitor 8 (Z-IEDT-FMK), and caspase inhibitor 9 (Z-LEHD-FMK) (50 μ mol/L) on 10 nmol/L testosterone (T)-induced apoptosis at 48 hours. Apoptotic cells were assessed by annexin V/propidium iodide and the values are the mean \pm SD of 3 different experiments. * $P < 0.01$ vs. 10 nmol/L T.

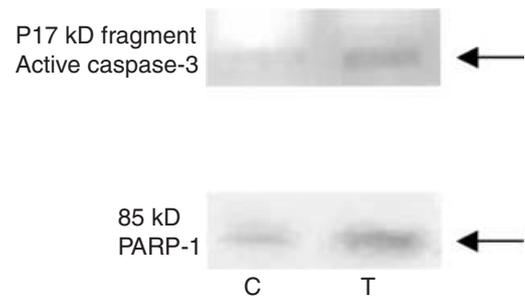


Fig. 11. Testosterone (T)-induced apoptosis is associated with activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase (PARP-1). Cells incubated for 48 hours with 10 nmol/L of T were subjected to Western analysis with the various antibodies as shown. The caspase-3 antibody detects only the p17 fragment active; the PARP antibody detects only the cleaved 85-kD fragment. The results are representative of 3 different experiments.

pathway is caspase activation, resulting in the biochemical and morphologic changes associated with the apoptotic phenotype.

The mechanisms responsible for gender differences in progression rates of chronic diseases are not yet clear and continue to be the object of investigation. Changes ob-

served in tubule cells under serum-free culture in vitro appear very similar to the apoptotic damage occurring in vivo in the tubuli subjected to ischemia or chronic renal disease [28–30]. The androgen-promoted apoptotic

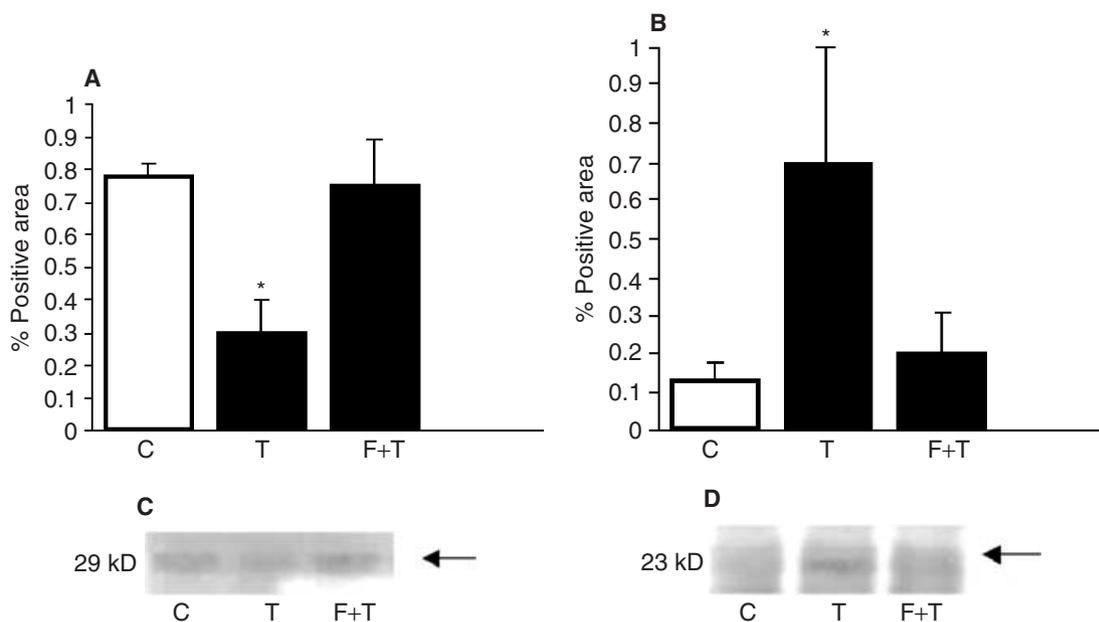


Fig. 12. Expression of antiapoptotic Bcl-2 (A) and proapoptotic Bax (B) proteins were evaluated by immunocytochemistry and image analysis. Cells subjected to 10 nmol/L testosterone (T) for 48 hours showed alteration in Bcl-2 and Bax proteins with respect to control (C) cells and cells pretreated with flutamide (F). Data are expressed as mean \pm SD of 4 separate experiments. * $P < 0.001$ vs. C. Expression of antiapoptotic Bcl-2 (C) and proapoptotic Bax (D) proteins evaluated by Western blot. Results are representative of 3 different experiments.

damage might interact with the apoptotic pathway already activated in several chronic kidney diseases. It has been previously shown that renal FasL may potentially regulate the immune response and promote parenchymal cell death in several experimental models of chronic kidney disease [22]. The Bax/Bcl-2 ratio (through caspase-3 activation) has been shown to modulate renal cell apoptosis associated with the progressive renal damage in immune-mediated experimental glomerulonephritis [26], in the subtotal nephrectomy model of chronic renal scarring [29], in adult polycystic kidney disease (APKD) [30], and in experimental obstructive nephropathy [31]. Therefore, the pathways to cell death, which are primed by androgens, as shown by the present study, are shared by several conditions leading to the loss of renal cells in chronic disease.

CONCLUSION

Physiologic T levels cause intracellular events, allowing Fas sensitization and leading to activation of caspase-dependent apoptotic pathway in human renal tubule cells. This is consistent with a role for T in promoting renal injury in men.

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