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ORIGINAL ARTICLE

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Dihydrotestosterone (DHT) modulates the ability of NSAIDs to induce apoptosis of prostate cancer cells

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Abstract *Purpose:* Recent evidence indicates that nonsteroidal antiinflammatory drugs (NSAIDs) are effective in the treatment and prevention of prostate cancer. In the study reported here, we investigated the ability of the steroid hormone dihydrotestosterone (DHT) to modulate NSAID-induced apoptosis of prostate cancer cells. *Materials and methods:* Using in vitro models of androgen-sensitive and androgen-insensitive human prostate cancer cells, we evaluated the ability of a specific cyclooxygenase-2 inhibitor (NS-398) and a nonspecific cyclooxygenase inhibitor (indomethacin) to induce apoptosis in the presence of various concentrations of DHT. Apoptosis was quantified using the TUNEL method and verified by electron microscopy. *Results:* We found that increasing concentrations of DHT significantly enhanced the ability of NS-398 and indomethacin to induce apoptosis of androgen-sensitive LNCaP cells. The ability of NSAIDs to induce apoptosis of androgen-insensitive PC-3 cells, however, was not affected by the presence of DHT. Higher levels of DHT in the incubation medium both before as well as following exposure to NSAIDs enhanced apoptosis of LNCaP cells. Another steroid hormone that interacts with the androgen receptor in LNCaP cells (progesterone) also promoted apoptosis of these cells. Increasing concentrations of DHT caused LNCaP cells to shift from the S and G_2/M to the G_0/G_1 stages of the cell cycle. *Conclusions:* These observations support the use of DHT in combination with NSAIDs in the treatment of prostate cancer, and indicate that DHT is an important issue to address in clinical trials of NSAIDs since androgen ablation is a common treatment for prostate cancer.

Keywords Androgen receptor · Apoptosis · DHT · NSAIDs · Prostate cancer

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Introduction

An increasing number of studies have shown that nonsteroidal antiinflammatory drugs (NSAIDs) may be effective in the prevention and treatment of many common cancers including prostate cancer. Early in vivo studies using rodents indicated that NSAIDs can decrease the size of prostate tumors [7] and suppress the metastasis of these tumors [7, 26]. More recently, in vitro studies have shown that NSAIDs can induce apoptosis in human prostate tumor cell lines [14, 18, 19], as well as reduce the invasiveness of these cells [1]. Finally, recent retrospective studies have indicated a reduced risk of prostate cancer associated with regular use of NSAIDs [23, 24]. Because these and other studies [10, 30] have called for clinical trials to investigate the use NSAIDs in the treatment of prostate cancer, it is important to determine what factors can modulate the anticarcinogenic effectiveness of NSAIDs.

The naturally occurring androgen, dihydrotestosterone (DHT) is biphasic in its effect on prostate cancer cells. At low concentrations, DHT stimulates prostate cancer cell proliferation and secretion, while at higher concentrations, DHT inhibits the proliferation of prostate cancer cells [6, 13]. Clinical studies investigating the treatment of prostate cancer with exogenous androgen have, in many cases, shown a significant regression of the cancer in response to such treatment [2, 21, 28, 36]. Also, DHT has been shown to induce apoptotic cell death of other cancer cell lines (i.e. breast cancer cells) that express the androgen receptor [17]. These findings, together with the fact that androgen ablation ultimately fails in patients with advanced prostate cancer, has led to a call for a

reevaluation of supplementary androgen in the treatment of the disease [27].

We report here that the ability of NSAIDs to induce apoptosis of human prostate cancer (LNCaP) cells is significantly enhanced by DHT. This observation represents the first demonstration that a naturally occurring steroid hormone can significantly affect the ability of NSAIDs to induce apoptosis of prostate cancer cells. Although there have been reports that chemotherapeutic drugs such as the anthracyclines (e.g. doxorubicin) and vincristine can work synergistically with NSAIDs to enhance the apoptosis of cancer cells [8, 29], the generalized toxic side effects of these chemotherapeutic agents limit their usefulness. DHT, on the other hand, appears to target its apoptotic-enhancing effects to proliferating cells with androgen receptors (i.e. prostate cancer cells). In view of the facts that both the onset of prostate cancer and current treatment regimens are usually associated with reduced levels of DHT, these observations also have important implications regarding the possible treatment/prevention of prostate cancer with NSAIDs.

Materials and methods

Cell cultures

The LNCaP and PC-3 cell lines were purchased from the American Type Culture Collection (Rockville, Md.). The LNCaP cell line is an androgen-responsive human prostate adenocarcinoma, which was isolated from a biopsy of a lymph node aspirated from a patient with a confirmed diagnosis of metastatic prostate carcinoma [13]. The PC-3 cell line is an androgen-insensitive human prostate cancer cell line. The cells were grown in flasks containing Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, Va.) 5% supplemented with fetal calf serum, and were seeded onto six-well cluster dishes. LNCaP cells were grown in the presence of 10^{-9} *M* DHT (maintenance level) except where otherwise indicated. Culture dishes were kept in a humidified atmosphere of 10% CO_2 in air at a temperature of 37ºC. Once the cells had grown to approximately 50% confluency, they were washed with phosphate-buffered saline (PBS) and the medium changed to phenol red-free medium containing 5% dextran-coated charcoal-treated (i.e. steroid-depleted) fetal calf serum.

TUNEL analysis of apoptotic cells

The cells were preserved for TUNEL assay by the addition of 10% neutral formalin (2 ml per well). The fixed cells were harvested by scraping and analyzed using an in situ apoptosis detection kit (ApopTag; Intergen Company, Purchase, N.Y.) according to the manufacturer's instructions. Labeled cells were examined and counted using an OM-2 Olympus microscope equipped for fluorescence. Two independent observers undertook cell counts blindly and statistical analysis was performed using ANOVA. The significance of differences between groups was determined using Student's *t*-test with *P*-values < 0.05 being considered statistically significant.

Electron microscopic analysis

For transmission electron microscopy, the cells were fixed overnight in 2% phosphate-buffered glutaraldehyde, postfixed for 45 min in 1% phosphate-buffered osmium tetroxide, dehydrated and embedded in Spurr medium. Ultrathin sections (20–50 nm) were mounted on grids, stained with uranyl acetate and lead citrate, and examined with a JEOL transmission electron microscope operating at 60 kV. For scanning electron microscopy, the cells were fixed overnight in 2% phosphate-buffered glutaraldehyde, postfixed in 1% phosphate-buffered osmium tetroxide, dehydrated in acetone, dried using the critical point method (Samdri-780, Rockville, Md.), mounted on stubs, coated with a thin layer of palladium/gold in a sputter coater (Hummer X; Anatech, Alexandria, Va.), and viewed and photographed using a Hitachi Model 570 scanning electron microscope operating at 15 kV.

Cell cycle analysis

The cells were washed twice in PBS and trypsinized. The cells ($1–2\times10^6$) were then pelleted in triplicate by centrifugation and resuspended in 100 µl citrate buffer (40 m*M* trisodium citrate·$2H_2O$, 250 m*M* sucrose, and 5% DMSO, pH 7.6). Nuclei were prepared for flow cytometric cell cycle analysis by members of the Vincent T. Lombardi Cancer Research Center Flow Cytometry Core Facility (Georgetown University Medical Center, Washington, D.C.) using the method of Vindelov et al. [40], with propidium iodide as the stain for nucleic acid. Cell cycle analysis was performed using a FACStar Plus fluorescence-activated cell sorter (Becton Dickinson Immunocytochemistry Systems, Mountain View, Calif.) equipped with the ModFit cell cycle analysis program (Verity Software House, Topsham, Me.).

Results

DHT promotes the ability of NSAIDs to induce apoptosis of LNCaP cells

When the concentration of DHT was increased in the incubation medium of LNCaP cells, exposure to the specific COX-2 inhibitor NS-398 (100 µ*M*) induced an increasing percentage of apoptosis (Fig. 1). Incubation

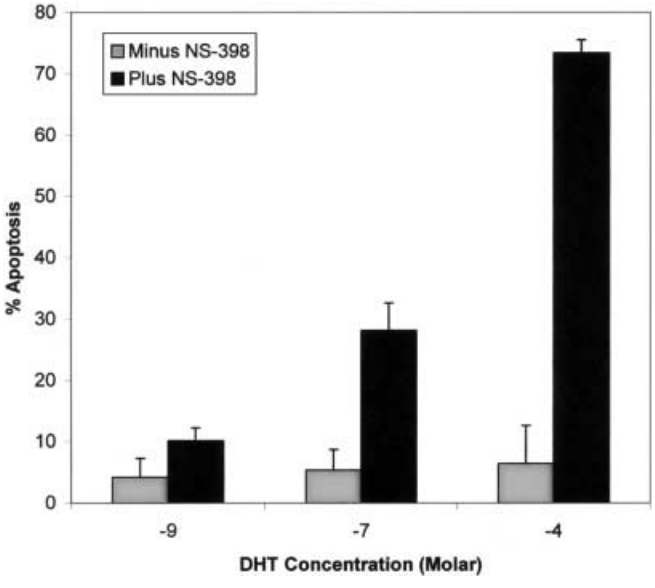

Fig. 1 Percentage apoptosis exhibited by LNCaP cells following incubation with different concentrations of DHT plus (*black*) and minus (*gray*) 100 µ*M* NS-398. Incubation with 10^{-9} *M* DHT resulted in very little increase in apoptosis, while incubation with 10^{-7} and 10^{-4} *M* DHT resulted in significant increases in apoptosis ($P < 0.05$). The *error bars* represent one standard deviation

in NS-398 plus 10^{-9} *M* DHT (i.e. maintenance level) for 48 h induced a significantly higher percentage of apoptosis (10%) than seen in LNCaP cells not exposed to NS-398 ($P < 0.05$). However, when the concentration of DHT was increased to 10^{-7} *M* and 10^{-4} *M*, NS-398 induced near 30% and 75% apoptosis, respectively, both significantly higher than seen in the presence of NS-398 plus 10^{-9} *M* DHT ($P < 0.05$). When incubated in the presence of 10^{-9} *M* DHT plus the nonspecific cyclooxygenase inhibitor indomethacin (100 μ*M*), a similar synergistic effect was found, but only in the presence of higher concentrations of DHT (Fig. 2). In the foregoing studies, apoptosis was quantified using the TUNEL assay and verified by electron microscopy (see Electron microscopic analysis).

Prior exposure of LNCaP cells to high concentrations of DHT promotes apoptosis

In the experiments described above, the LNCaP cells were preincubated in the presence of 10^{-9} *M* DHT (i.e. maintenance level) prior to being exposed to NSAIDs plus various concentrations of DHT. In another series of experiments, we evaluated the effects of incubation for 48 h in either no DHT or a higher concentration of DHT (i.e. 10^{-4} *M*) prior to the addition of indomethacin plus high concentrations of DHT to the incubation medium (Fig. 3). When LNCaP cells were cultured in the absence of DHT for 48 h (following prior maintenance in 10^{-9} *M* DHT) and then cultured in the presence of indomethacin (100 μ*M*) plus high concentrations of DHT (i.e. 10^{-5} or 10^{-4} *M* DHT) for an additional 48 h, the mean percentage of apoptosis was 0.5% and 2% in 10^{-5} and 10^{-4} *M* DHT, respectively. However, when LNCaP cells were incubated with 10^{-4} *M* DHT for 48 h (again, following prior maintenance in 10^{-9} *M* DHT) and subsequently incubated with indomethacin (100 μ*M*) plus high concentrations of DHT (i.e. 10^{-5} or 10^{-4} M DHT) for an additional 48 h, the mean percentage of apoptosis increased to 9% and 91% with 10^{-5} and 10^{-4} *M*, respectively.

These results indicate that prior exposure of LNCaP cells to DHT predisposed these cells to subsequent indomethacin-induced apoptosis. Also, pre- and postincubation with high concentrations of DHT alone resulted in an increase in the percentage of apoptosis from exposure to DHT ($P < 0.005$). It therefore appears that long-term exposure of LNCaP cells to very high concentrations of DHT can itself induce apoptosis independently of the effects of NSAIDs.

DHT does not promote NSAID-induced apoptosis in an androgen-insensitive prostate cancer cell line

In this series of experiments the PC-3 cell line, which is insensitive to androgen, was subjected to the same

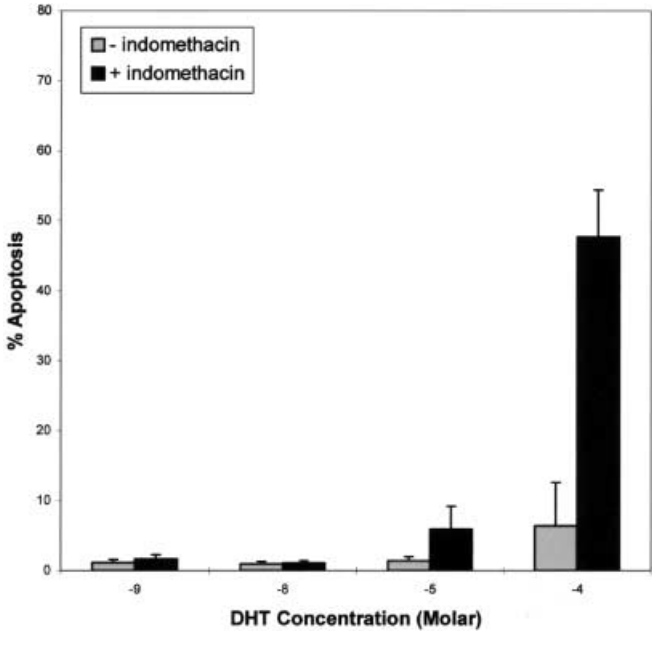

Fig. 2 Percentage apoptosis exhibited by LNCaP cells following incubation with different concentrations of DHT plus (*black*) and minus (*gray*) indomethacin (100 μ*M*). Incubation with 10^{-9} and 10^{-8} *M* DHT resulted in very little increase in apoptosis, while incubation with 10^{-5} and 10^{-4} *M* DHT resulted in significant increases in apoptosis ($P < 0.05$). The *error bars* represent one standard deviation

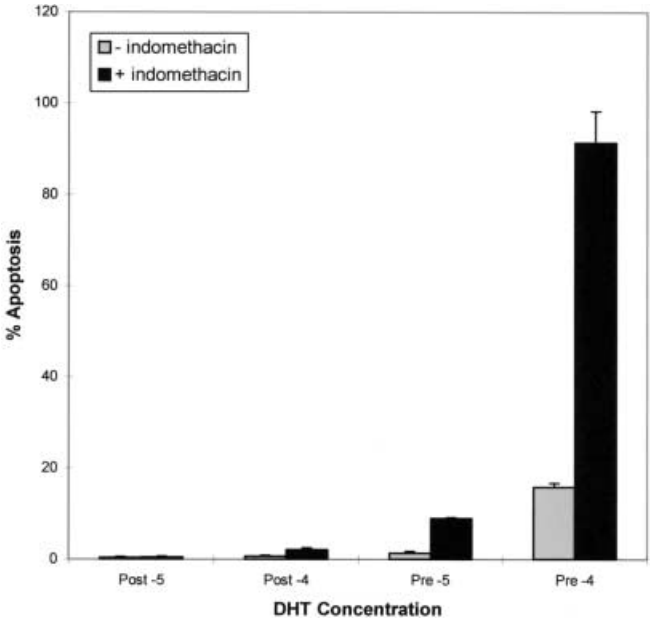

Fig. 3 Percentage apoptosis seen in LNCaP cells which were either shifted from medium containing 10^{-9} *M* DHT to medium containing 10^{-4} *M* DHT for 48 h prior to exposure to indomethacin (100 μ*M*) plus 10^{-4} *M* or 10^{-5} *M* DHT (*Pre-5* and *Pre-4*, respectively), or shifted from medium containing 10^{-9} *M* DHT to medium containing no DHT for 48 h prior to exposure to indomethacin (100 μ*M*) plus 10^{-4} *M* or 10^{-5} *M* DHT (*Post-5* and *Post-4*, respectively). Preincubation with DHT significantly increased apoptosis ($P < 0.05$) when subsequently exposed to indomethacin plus high concentrations of DHT (i.e. 10^{-4} *M* and 10^{-5} *M*). The *error bars* represent one standard deviation

experimental conditions which were found to maximize the ability of DHT to promote indomethacin-induced apoptosis of LNCaP cells. Specifically, PC-3 cells were incubated with high concentrations of DHT (10^{-4} *M*) for 48 h prior to being exposed to indomethacin (100 μ*M*) with or without 10^{-4} *M* DHT. Under these conditions, DHT did not promote indomethacin-induced apoptosis in the PC-3 cell line ($P > 0.05$; Fig. 4).

Other steroid hormones which interact with the androgen receptor of LNCaP cells can also promote NSAID-induced apoptosis

LNCaP cells contain an abnormal androgen receptor system with broad steroid-binding affinity. In this series of experiments, we evaluated whether or not the addition of the steroid hormones hydroxycortisone or progesterone to the incubation medium would promote indomethacin-induced apoptosis in a manner similar to the effect of DHT. The addition of a high concentration (10^{-5} *M*) of hydroxycortisone did not significantly increase the percentage of indomethacin-induced apoptosis of LNCaP cells when compared with cells cultured with indomethacin plus 10^{-9} *M* DHT alone ($P > 0.05$; Fig. 5). The addition of 10^{-5} *M* progesterone, however, did result in a statistically significant increase in the percentage of apoptosis of LNCaP cells when compared with samples incubated with progesterone alone and samples incubated with indomethacin plus 10^{-9} *M* DHT ($P < 0.05$; Fig. 5). The increased apoptosis seen in the presence of progesterone plus indomethacin was not significantly different from that seen in the presence of an equivalent amount of DHT plus indomethacin (10^{-5} *M*; $P > 0.05$).

High concentrations of DHT cause a shift in the cell cycle of LNCaP cells

Cell cycle analysis indicated that following 48 h of incubation with high concentrations of DHT (10^{-4} *M*), LNCaP cells exited from the S and G_2/M phases of the cell cycle and accumulated in the G_0/G_1 phases of the cell cycle (Table 1). Incubation in the presence of indomethacin or absence of DHT for 48 h induced similar but not as prominent shifts of the LNCaP cells into the G_0/G_1 phases of the cell cycle ($P < 0.05$; Table 1).

Electron microscopic analysis confirms apoptosis of LNCaP cells

As illustrated in Figs. 6, 7, scanning and transmission electron microscopy revealed that the LNCaP cells exposed to high concentrations of DHT plus indomethacin undergo ultrastructural alterations characteristic of apoptosis. When incubated with indomethacin (100 μ*M*) plus low concentrations of DHT (10^{-9} *M*), LNCaP cells were irregularly shaped, flattened, elongated cells, with elongated cell processes, irregularly shaped nuclei, dispersed chromatin, and a normal complement of cytoplasmic organelles (Fig. 6). When exposed to

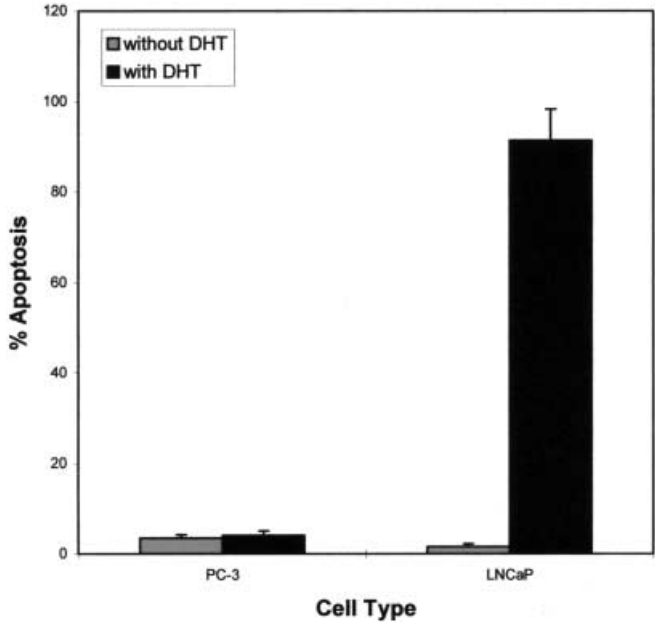

Fig. 4 Percentage apoptosis seen in PC-3 and LNCaP cells following incubation for 48 h with DHT (10^{-4} *M*) without (*gray*) and with (*black*) indomethacin (100 μ*M*). PC-3 cells did not exhibit any increase in apoptosis, while LNCaP cells exhibited a dramatic increase in apoptosis ($P < 0.05$). The *error bars* represent one standard deviation

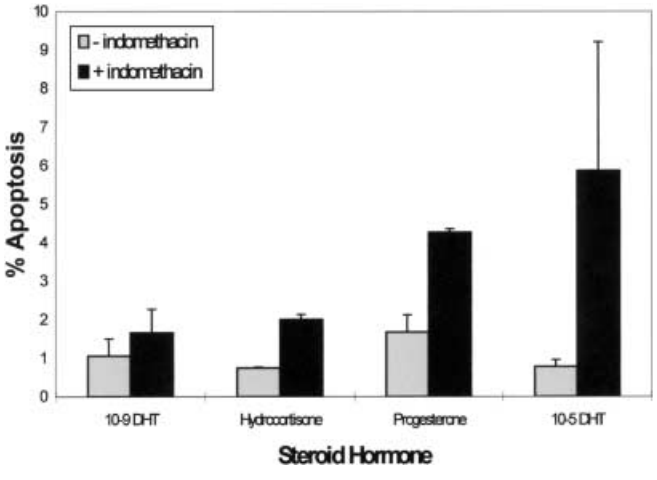

Fig. 5 Percentage apoptosis seen in LNCaP cells following 48 h incubation in medium containing steroid hormones without (*gray*) or with (*black*) indomethacin (100 μ*M*). Compared with LNCaP cells incubated with low concentrations of DHT (10^{-9} *M*), cells incubated with high concentrations of hydrocortisone (10^{-5} *M*) plus indomethacin did not exhibit a significant increase in apoptosis ($P > 0.05$). LNCaP cells incubated with 10^{-5} *M* progesterone plus indomethacin, however, did exhibit a small but significant increase in apoptosis ($P < 0.05$), but this was not significantly different from the increase in apoptosis in LNCaP cells incubated with 10^{-5} *M* DHT plus indomethacin ($P > 0.05$)

Table 1 Effects of dihydrotestosterone and indomethacin on the cell cycle in LNCaP cells analyzed following 48 h of incubation in medium containing different concentrations of DHT or indomethacin (100 μM). The results indicate that high concentrations of DHT or indomethacin (100 μM) caused LNCaP cells to shift from the S/G_2M phases into the G_0/G_1 phases of the cell cycle. The differences in the percentages in each phase of the cell cycle between cells incubated with 10^{-9} *M* DHT and those incubated with 10^{-4} *M* DHT or indomethacin are all statistically significant ($P < 0.05$)

Medium	Cell cycle phase					
	G_0/G_1		G_2/M		S	
	%	SD	%	SD	%	SD
No DHT	82.61	0.33	10.73	0.09	6.67	0.28
10^{-9} *M* DHT	78.46	0.40	12.89	0.21	8.65	0.19
10^{-4} *M* DHT	89.38	0.73	8.26	0.68	2.39	0.17
Indomethacin	87.25	0.71)	8.59	0.75	4.16	0.45

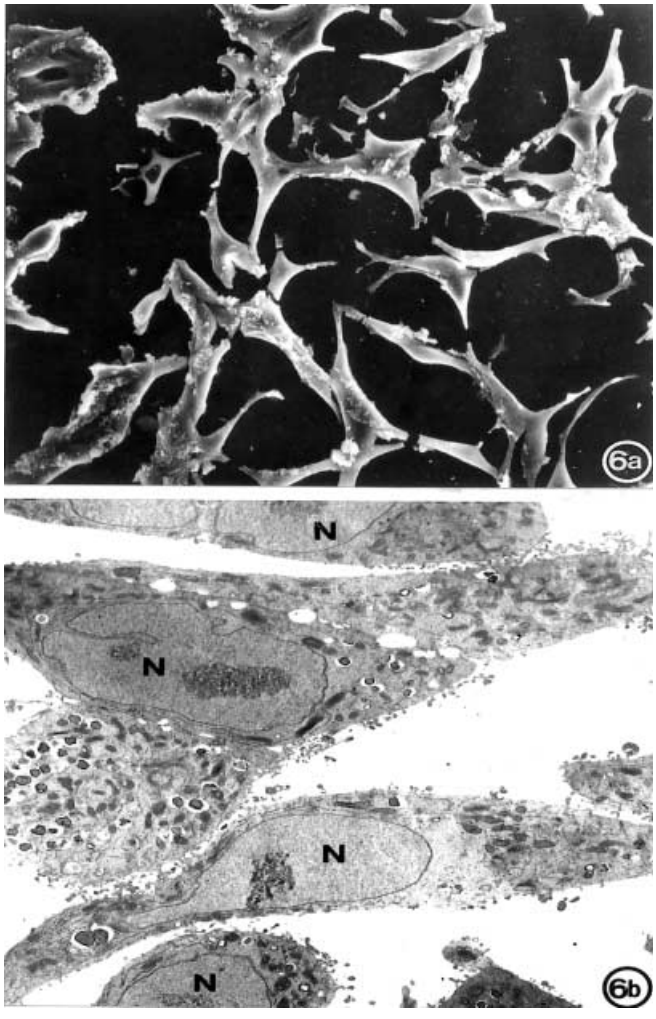

Fig. 6a, b Scanning (**a**) and transmission (**b**) electron micrographs of LNCaP cells treated for 24 h with indomethacin (100 μ*M*) in the presence of 10^{-9} *M* DHT. The LNCaP cells appear elongated, flattened, and spindle-shaped with extended cell processes, euchromatic nuclei (*N*), and a normal complement of cytoplasmic organelles (**a** ×425, **b** ×4300)

indomethacin (100 μ*M*) plus high concentrations of DHT (10^{-4} *M*), however, LNCaP cells were transformed into small, rounded cells with rounded nuclei containing highly condensed clumps of chromatin (Fig. 7).

Discussion

NSAIDs show promise for the prevention and treatment of prostate cancer. In the present study, the steroid hormone DHT potentiated the ability of the NSAIDs NS-398 and indomethacin to induce programmed cell death of a human prostatic cancer cell line (LNCaP). This synergistic effect of DHT in promoting NSAID-induced apoptosis could be dramatic, with the percentage of apoptosis being affected by the concentration of DHT in the incubation medium both before and after the addition of NSAID. The specific COX-2 inhibitor NS-398 induced significant increases in apoptosis when the concentration of DHT was increased from 10^{-9} *M* to 10^{-7} *M*, which approaches the levels of DHT reported to be present in the human prostate (5 ng/g of tissue) [22, 31]. In addition, it has been shown that DHT is very rapidly metabolized by LNCaP cells in vitro with less than one-third of the initial DHT reported to be present following 3 h of incubation [32]. As a result, the amount of DHT in the incubation medium after 48 h of incubation is significantly reduced relative to the amount that was originally added. Therefore one might expect a greater effect in vivo, wherein the metabolized DHT is being continuously replenished by recirculating DHT.

Previous studies have shown that NSAIDs vary in their ability to inhibit the growth of prostate cancer cells [14, 18, 43]. At the concentrations tested in the present study, NS-398 was not only more effective than indomethacin in inducing apoptosis of LNCaP cells, but worked synergistically with lower concentrations of DHT to induce apoptosis. There are indications that other NSAIDs (e.g. celecoxib) may also be able to induce apoptosis of prostate cancer cells at relatively low concentrations [14]. Therefore, the ability of DHT to work synergistically with other NSAIDs needs to be explored. In addition, investigations have shown that the ability of DHT to activate the androgen receptor can be significantly enhanced through the synergistic action of other agents [5]. Therefore, further in vitro studies together with in vivo studies are warranted in order to develop effective treatment regimens for treating prostate cancer with NSAIDs.

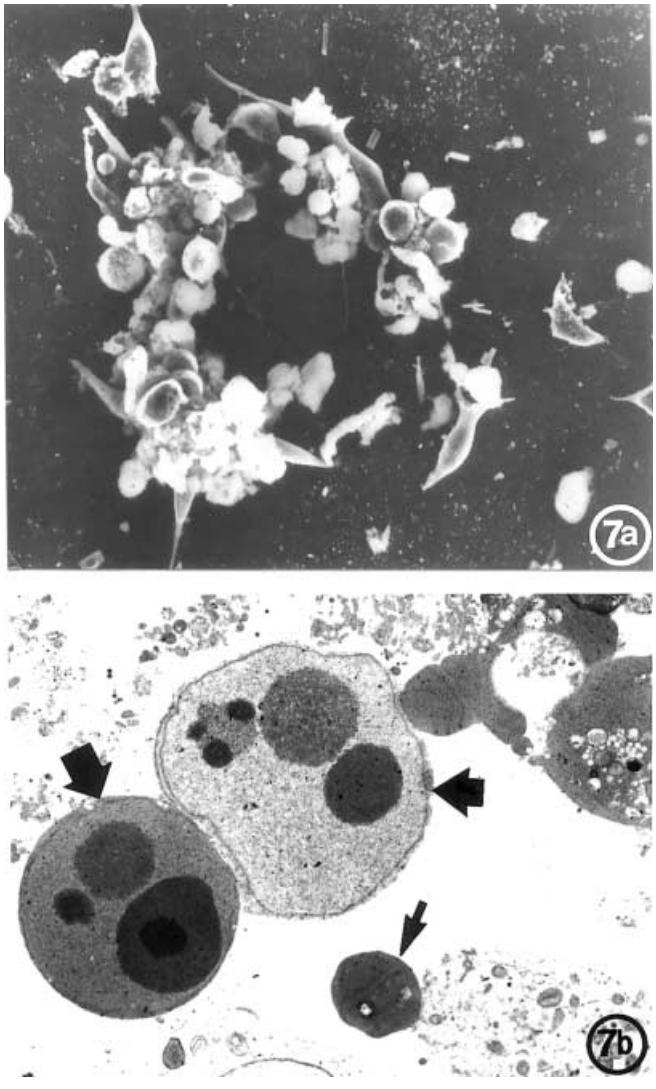

Fig. 7a, b Scanning (**a**) and transmission (**b**) electron micrographs of LNCaP cells treated for 24 h with indomethacin (100 μ*M*) in the presence of 10^{-4} *M* DHT. The LNCaP cells have become transformed into smaller rounded cells, with nuclear condensation characteristic of cells in late stages of apoptosis (*large arrows*). An apoptotic body (*small arrow*) is also evident (**a** ×425, **b** ×5400)

The mechanism for the anticarcinogenic effects of NSAIDs is the subject of ongoing investigation. One of the proposed mechanisms is inhibition in the production of cyclooxygenase-2 (COX-2), one of two isoforms of cyclooxygenase. COX-2 is induced by growth factors, cytokines, oncogenes, and tumor promoters, and is upregulated in transformed cells and malignant tumors [12, 20, 35, 38]. Recently, it has been reported that there is an overexpression of COX-2 and lipoxygenase-5 in human prostate adenocarcinomas [9, 10]. Liu et al. [19] have reported that NS-398 causes downregulation of the expression of the antiapoptotic protein, bcl-2, in LNCaP cells. Hsu et al. [14] have reported that while a specific COX-1 inhibitor, piroxicam, has no effect on LNCaP cells, the specific COX-2 inhibitor celecoxib induces apoptosis of LNCaP cells. However, Hsu et al. [14] provided evidence to indicate that celecoxib induces apoptosis by blocking the antiapoptotic kinase Akt, and that this apoptotic effect is independent of bcl-2. Other recent studies have indicated that NSAIDs may inhibit tumorigenesis by inhibiting the peroxisome proliferator-activated receptor beta (PPAR-beta), a downstream transcription mediator for prostaglandins and fatty acids [11]. This suppression of PPAR-beta appears to be mediated in part by the ability of NSAIDs to directly inhibit the DNA-binding activity of PPAR-beta. A recent study, however, has indicated that PPAR-alpha and -beta are not expressed in LNCaP cells and activators for these receptors do not induce cell death [3].

Just how DHT works synergistically with NSAIDs to promote apoptosis is not clear. A recent study has indicated that DHT downregulates the expression of bcl-2 in breast cancer cells with androgen receptors [17]. In the present study, we found that high concentrations of DHT caused LNCaP cells to shift into the G_0/G_1 stages of the cell cycle, thereby perhaps rendering them more susceptible to apoptosis. This latter observation is in line with previous observations indicating a reduction in the growth rate of LNCaP cells associated with high concentrations of DHT [6, 13].

We found that in addition to DHT, progesterone, but not hydrocortisone, promoted indomethacin-induced apoptosis. The finding that progesterone behaves in a manner similar to DHT is perhaps not unexpected in that LNCaP cells possess a mutated androgen receptor with a high affinity for progesterone [39]. Furthermore, the presence of progesterone, like DHT, both stimulates and inhibits the growth of LNCaP cells [32, 33]. Our observation that androgen-insensitive cells (i.e. PC-3 cells) did not respond to indomethacin-induced apoptosis in the presence of high concentrations of DHT, further indicates that the androgen receptor is needed to elicit the synergistic effect between DHT and NSAIDs.

Prostate cancer is one of the leading causes of cancer-related deaths in men in the United States [16]. Although androgen ablation produces significant palliation of symptoms in patients with advanced prostate cancer, the proportion of tumors that regress is actually relatively small [4]. More important, however, is the fact that this treatment regimen is doomed to failure in the long-term because it promotes hormone-refractory disease, which is ultimately lethal to the patient. In a recent article, Prehn [27] has argued that declining rather than high levels of androgens contribute to human prostate carcinogenesis and that androgen supplementation may lower the incidence of the disease. In fact, high levels of androgen appear to be inhibitory to prostatic cells (due to a mechanism mediated by the androgen receptor), and excess androgen can inhibit prostate cancers under experimental conditions [15, 25, 34, 37, 41, 42]. Significant regression of prostate cancer in response to exogenous androgen has also been seen in a substantial proportion of the few patients in whom this treatment has been tried [2, 21, 28, 36]. With respect to future treatment regimens, our observations indicate that

prolonged exposure to high concentrations of DHT may predispose prostate cancer cells to effective NSAID treatment. However, since all our observations were undertaken using an in vitro model of prostate cancer, additional in vivo studies are needed to determine the usefulness of this treatment regimen.

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