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Gonadotropin Releasing Hormone Analogs Induce Apoptosis by Extrinsic Pathway Involving p53 Phosphorylation in Primary Cell Cultures of Human Prostatic Adenocarcinomas

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BACKGROUND. Gonadotropin-releasing-hormone (GnRH) analogs are widely used to block hypothalamic–pituitary–gonadal axis and inhibit blood androgen levels in patients with prostate cancer (PCa). In addition, GnRH analogs induce proliferation arrest and apoptosis through GnRH receptors expressed on the membrane of PCa cells. Possible molecular mechanisms involved in GnRH-mediated apoptosis on prostate cancer cells were studied.

METHODS. Primary cultures from PCa and benign prostatic hyperplasia (BPH) (non-malignant control) were derived from samples provided by our Institutional Hospital. Cell cultures were incubated for 24 hr with 20 ng/ml of GnRH agonist Leuprolide (Lp) or antagonist Cetrorelix (Cx). Apoptosis was evaluated by studying the expression of Bax and Bcl-2 and the activation of caspase-9 (intrinsic pathway), caspase-8 (extrinsic pathway), and caspase-3. Also, mRNA level, protein expression and phosphorylation of p53 were studied.

RESULTS. Cleaved caspase-8 and -3, but not -9, increased in presence of Lp and Cx in PCa cell cultures. Bax and Bcl-2 mRNA levels showed no changes after GnRH-analog treatments. Only Bax protein showed an increase after Cx treatment in PCa cell cultures. p53 mRNA level was higher in PCa than in BPH cell cultures. Lp and Cx increased p53 expression and phosphorylation in PCa cell cultures.

CONCLUSIONS. Apoptosis induced by GnRH analogs seems to be mediated by extrinsic pathway involving p53 phosphorylation. Phosphorylated-p53 might be associated with the increase in apoptotic NGF receptor, p75, previously reported by our laboratory. These findings reinforce the concept of clinical use of GnRH analogs for PCa suggesting that intraprostatic treatment may be more effective. *Prostate* © 2009 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; GnRH analogs; primary cell culture; apoptosis; p53**

INTRODUCTION

Prostate cancer (PCa) occurs mostly in males over 50-year-old and morbidity and mortality of the disease show a progressive increase with age. The increase in longevity of male population in the last decades reinforces the importance of PCa diagnosis and treatment from the epidemiological perspective [1]. Today, PCa is considered the second leading cause of cancer-related deaths in men over the world [2]. In the last

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20 years, the most widely used therapy for advanced PCa is based on the treatment with gonadotropin releasing hormone (GnRH) analogs. This hormone-therapy blocks the hypothalamic–pituitary–gonadal axis, inhibitis the secretion of gonadotropins and lowers plasma levels of testosterone [3,4]. In addition, direct effects of GnRH analogs on tumors may also occur. Moreover, evidence for presence of GnRH receptor (GnRHR) in PCa tumor samples and established cell lines has led to proposals of some local action for these peptides [3,4].

It has been observed that apoptosis in prostate cells is preceded by an increase in the expression of proto-oncogene c-fos, which can alter the synthesis of p53 and the activation of other genes, as apoptosis regulators of the Bcl-2 family [5]. Also, it has been reported in different tissues, that some cytotoxic drugs induce apoptosis by p53 activation [6,7]. The activation and accumulation of p53 is regulated by phosphorylation and acetylation of the protein. At least 20 sites located within the N-terminal or C-terminal transactivation domains of human p53 can be modified in response to the activation of different stress signaling pathways [8,9]. Moreover, the phosphorylation of p53 in the N-terminal serine increases the interaction with the transcriptional co-activators p300/CBP and PCAF [8,9]. However, it has been reported that p53 is mutated in most advanced cancers [10], losing its anti-oncogenic properties and thus cannot prevent genomic instability. On the other hand, p53 also regulates the expression of p21, GADD45 and Bax, which are involved in controlling the cell cycle and apoptosis [11]. Furthermore, the increase in Bax can trigger the activation of caspase-9 pathway [12].

There is evidence that p53-induced cell arrest could be irreversible, inhibiting further cell proliferation [13]. Alternatively, p53 may act as a temporary barrier (G1 arrest) providing the cell with enough time to repair damaged DNA [14]. Most of p53-mediated cell cycle arrest is produced by the induction of p21 transcription, which prevents cell cycle progression to phase S by the inhibition of cyclin-cdk complex activity [15]. Using a primary cell culture system from human PCa, we have recently reported gene and protein expression of GnRHR [16]. In addition, treatments of these primary cultures with GnRH analogs induce proliferation arrest and an increase in apoptosis rate [16]. It has also been observed that GnRH agonists induce the expression of p53 as well as the Fas/Fas-ligand system in uterine leiomyoma and in endometrial and ovarian cancer cell lines [17–20]. In normal prostate, the nerve growth factor (NGF) receptors, TrkA and p75, have been found [21]. It is known that NGF is produced mainly by prostatic stromal cells and regulates epithelial cell survival in a paracrine manner [22,23]. Therefore, the activation of TrkA tyrosine-kinase receptor promotes prostate epithelial cell proliferation while the Fas-like receptor p75 induces apoptosis [24–28]. In PCa cells, TrkA receptor can be normal or overexpressed [29–31]. In contrast, p75 receptor is poorly expressed or absent in malignant prostate cells [32,33]. In this fashion, TrkA/p75 system is unbalanced in PCa favoring cell proliferation. We have recently reported that treatment of primary PCa cell cultures with the GnRH analog Leuprolide (Lp) or the antagonist Cetrorelix (Cx) increases the expression of both mRNA and protein of p75 receptor [21]. The induction of this receptor might activate the caspase-8-dependent apoptotic pathway.

Analyzed together, these data prompted us to investigate the possible participation of the caspase-8 and -9 pathways and p53 in the apoptotic effect of GnRH analogs on PCa cells in primary culture.

MATERIALS AND METHODS

Reagents

All chemicals and reagents, unless otherwise indicated, were purchased from Sigma Chemical Co. (St. Louis, MO).

Prostatic Tissue

The prostatic samples were obtained from patients undergoing radical prostatectomy due to adenocarcinoma, in our Institutional Hospital. Prostate fragments were collected in sterile culture medium containing RNAse inhibitors. The tissue was then brought to the laboratory and, if present, soft hyperplasic tissue was separated from harder malignant nodes. For control purpose, sections of each sample were processed by routine histological technique for Gleason classification [34]. In this study, prostate samples with a Gleason score range of 5–7 were included. This protocol was approved by Bioethics Committee of our Institution.

Cell Isolation and Culture

Prostatic cells were isolated, cultured and characterized essentially as described previously [35]. Briefly, small tissue fragments (1 mm^3) were digested with collagenase (2.5 mg/ml), hyaluronidase (1 mg/ml) and deoxyribonuclease (0.01 mg/ml), for 2–3 hr at 37°C in a shaking water bath. The resulting large epithelial cell aggregates were washed and further digested with collagenase for another 8–12 hr in the same conditions. Resulting small aggregates of prostatic cancer cells

were mechanically dispersed, washed and plated on cell culture plates (Falcon, Becton Dickinson Co., NJ). During the first days of culture media were supplemented with 5% of fetal bovine serum (FBS) (Gibco, Invitrogen Co., CA).

GnRH Analogs Treatments

After first medium change, cells were washed and fresh medium without FBS, supplemented with human transferrin 5 mg/L, insulin 2 mg/L, epidermal growth factor 10 μg/L, vitamin A and E 200 μg/L, hydrocortisone 10 nM, sodium selenite 2 μg/L, dihydrotestosterone (DHT) 10 nM, was added. Media were changed every 48 hours and cultures maintained for 2 weeks. GnRH-analog treatments were carried out during days 4 and 5 of culture. At the end of the treatment, cells were processed for RT-PCR or western blot. According to our previous studies, both Leuprolide (Sigma) and Cetrorelix (Serono, Novartis, Chile) were used at a concentration of 20 ng/ml.

Immunocytochemistry

For these studies, cells were cultured on coverslips. After reaching 50% confluence, cultures were washed with PBS and fixed for 30 min with a solution containing phosphate buffer 0.2 M, sucrose 1 M and paraformaldehyde 16%. Later on, cells were permeabilized with a solution containing Triton X-100 0.1% in PBS-glycine and, then, treated with a solution containing BSA 1% in PBS-glycine to avoid unspecific binding. Then, cells were incubated with 1/500 polyclonal anti-active caspase-3 (BD PharMingen, USA) for 1 hr at 37°C. Epitopes of primary antibody were localized by immunoperoxidase technique, using the secondary antibody–avidin–biotin complex and peroxidase substrate LSAB kit (Dakar, USA), according to the manufacturer. Negative controls were obtained by omitting the first antibody. The cells were then treated with chromogen 3′-3′-diaminobenzidine tetrahydrochloride (Zymed Laboratories) to detect bound antibody complex.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from prostate cells using Trizol Reagent (Gibco BRL, Rocksville, MD) according to the manufacturer's instructions. Aliquots of RNA (2 μg) from each culture were used in a RT reaction, in a final volume of 20 μl. Resulting cDNA was subjected to polymerase chain reaction (PCR). The specific primers used were as follows:

Primer	Sense	Antisense
bax	5′-GGC CCA CCA GCT CTG AGC AGA-3′	5′-GCC ACG TGG GCG TCC CAA AGT-3′
bcl-2	5′-ACA ACA TCG CCC TGT GGA TGA C-3′	5′-ATA GCT GAT TCG ACG TTT TGC C-3′
p53	5′-GTC TGG GCT TCT TGC ATT CT-3′	5′-GCC CAT GCA GGA ACT GTT AC-3′
β-Actin	5′-TCA CCA ACT GGG ACG ACA T-3′	5′-GAA GTC CAG GGC GAC GTA G-3′

The amplification was carried out using a Thermal Cycler (PT-100, MJ Research, Inc.) in a reaction mix that included: 2 μl of cDNA, 2.5 μl of PCR Buffer 10×, 0.75 μl of $MgCl_2$ 25 mM, 0.625 μl of dNTPs 10mM, 0.125 μl of Taq polimerase 500 UI (Biotools), 0.5 μl of each primer and 19 μl of DEPC water. β-Actin was used as an internal standard. Amplification was performed for 38 cycles for bax, 36 cycles for bcl-2, 33 cycles for p-53 and 28 cycles for β-actin. The PCR products were electrophoretically resolved on agarose gel 1.2% and stained with ethidium bromide. The bands were evaluated using an image analyzer UN-SCAN-IT gel version for Windows 4.1 and Kodak Digital Science 1D Software (Rochester, NY), and normalized relative to β-actin product. Data were expressed as the ratios between mRNAs of p-53, bax, bcl-2 and β-actin.

Western Blot Analysis

Cells were lysed in buffer containing Tris–HCl 500 mM, pH 7.4, NaCl 150 mM, EDTA 2 mM, Triton X-100 1%, PMSF 1 μg/ml and aprotinin 3 μg/ml. Homogenates were centrifuged at 13,000 rpm for 15 min at 4°C and protein concentration of the supernatants were determined by Bradford method. Subsequently, 150 μg of protein was separated by SDS–PAGE 7.5% and transferred into nitrocellulose membranes (Bio-Rad, CA). The membranes were blocked in fatless milk 5% during 1 hr at room temperature, and then incubated with the following first antibodies for 1 hr at room temperature:

Antibody	Dilution
Actin (MP Biomedicals, USA) monoclonal	1/15,000
Bax (Santa Cruz Biotechnology, USA) polyclonal	1/200
Bcl-2 (Calbiochem, USA) monoclonal	1/60
Active Caspase-9 (BD PharMingen, USA) polyclonal	1/1,500
Active Caspase-8 (BD PharMingen, USA) polyclonal	1/2,000
p53-p (Santa Cruz Biotechnology, USA) polyclonal	1/150

Later on, membranes were incubated for 45 min at room temperature with secondary antibodies coupled to HRP. The detection of antibodies complex was carried out by a chemoluminescence kit (Amersham Biosciences). The films were evaluated using an image analyzer UN-SCAN-IT gel version for Windows 4.1 and Kodak Digital Science 1D Software (Rochester, NY), and normalized relative to β-actin product. Data were expressed as the protein ratios between the Bax, Bcl-2, cleaved caspase-8 and -9, phosphorylated-p53 and β-actin.

Statistical Analysis

Statistical evaluation of data was performed using ANOVA analysis and non-parametric test of Kruskal–Wallis followed by Dunn's post-test. All experiments were repeated al least 3–4 times and results are expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Bax Determination

No significant differences were found comparing Bax expression between primary cultures of BPH and PCa, for both mRNA and protein (Fig. 1A,C). Also, no differences were observed in Bax gene expression between PCa cultures treated with GnRH analogs (Fig. 1B) and untreated control. However, unlike mRNA, Bax protein expression increased significantly ($P < 0.05$) after treatment with Cx (Fig. 1D).

Bcl-2 Determination

Both BPH and PCa are diseases with increased proliferation activity. Bcl-2 is a well-documented marker in PCa and it has been associated with resistance to anti-cancer therapies. Our results show that there were no significant differences in the gene expression of Bcl-2 between cultures from BPH and PCa (Fig. 2A), although, a slight increase in Bcl-2 protein level was found in BPH cultures (Fig. 2C). Treatment of PCa cultures with GnRH analogs showed no changes in gene or protein expression of Bcl-2 (Fig. 2B,D).

Caspases Evaluation

In order to study the apoptotic mechanism involved in GnRH effect, caspases from the extrinsic (caspase-8) and intrinsic (caspase-9) pathways were evaluated. We analyzed pro-caspase and active (cleaved) forms of the initiator caspases (8 and 9) as well as the effector caspase-3. The protein expression of the pro-caspase-8 and -9 was similar in both BPH and PCa cultures.

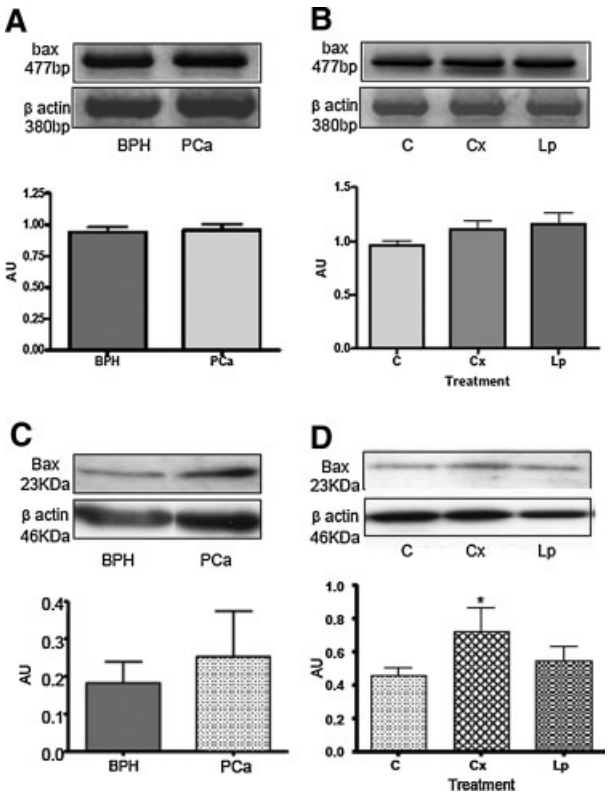

Fig. 1. Effect of leuprolide and cetrorelix on Bax mRNA and protein expression. **A**: RT-PCR for Bax in primary cultures of epithelial cells from BPH and PCa. **B**: RT-PCR for Bax in PCa primary cultures in the absence or presence of 20 ng/ml of GnRH analogs. **C**: Western blot analysis for Bax in BPH and PCa cultures. **D**: Western blot analysis for Bax in PCa cultures in absence or presence of 20 ng/ml of GnRH analogs. The results are expressed in arbitrary units (AU) as the mean ± SD from at least three independent experiments (*$P < 0.05$).

However, the cleaved forms of these caspases were only evident in PCa cultures (Figs. 3A and 4A). Treatment of PCa cultures with Cx and Lp induced a significant increase in the active form of caspase-8 (Fig. 4B). However, no differences were observed in the active caspase-9 (Fig. 3B). Caspase-3 was analyzed by immunocytochemistry in PCA cultures (Fig. 5), showing an increase in the presence of both GnRH analogs used compared with untreated control cultures (C).

p53 Study

P53 is a key regulator and marker in several malignancies. At the level of mRNA, p53 showed a significantly higher expression in PCa than in BPH cultures (Fig. 6A). This p53 level was increased after 24 hr of GnRH analogs treatment in PCa cultures (Fig. 6B). On the other hand, it is well established that p53 protein is highly regulated by phosphorylation at

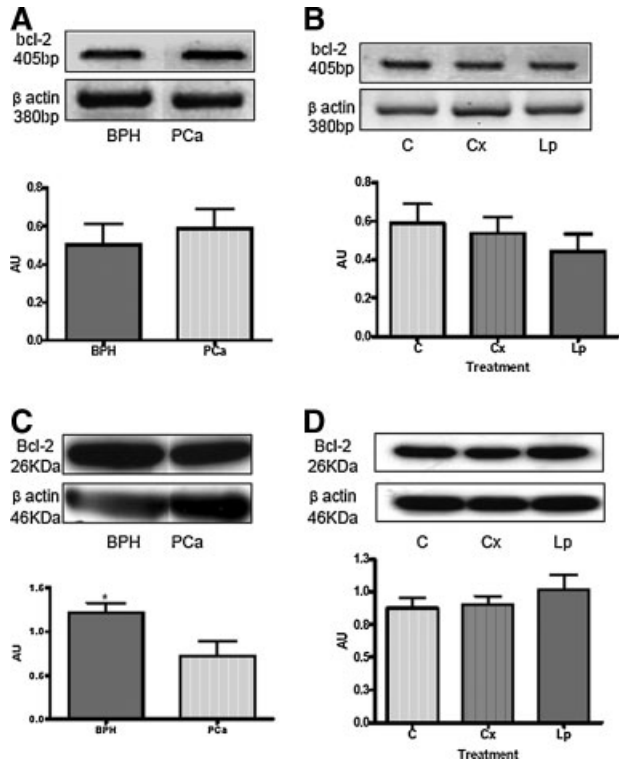

Fig. 2. Effect of leuprolide and cetrorelix on Bcl-2 mRNA and protein expression. **A**: RT-PCR for Bcl-2 in primary cultures of epithelial cells from BPH and PCa. **B**: RT-PCR for Bcl-2 in PCa primary cultures in the absence or presence of 20 ng/ml of GnRH analogs. **C**: Western blot analysis for Bcl-2 in BPH and PCa cultures. **D**: Western blot analysis for Bcl-2 in PCa cultures in absence or presence of 20 ng/ml of GnRH analogs. The results are expressed in arbitrary units (AU) as the mean ± SD from at least three independent experiments ($^*P < 0.05$).

different amino acid residues. We investigated p53 phosphorylation at serine 20 (Ser20p-p53) in our culture conditions and treatments. The expression of Ser20p-p53 was significantly higher in PCa than in BPH cultures (Fig. 7A). In addition, treatment with GnRH analogs increased the expression of Ser20p-p53 in PCa cultures (Fig. 7B).

DISCUSSION

Therapy with GnRH analogs has been widely used to inhibit androgens level in patients with PCa. In addition, GnRHR have been reported in malignant prostate tissue as well as in cell lines derived from prostate carcinoma, suggesting the possibility of a local action of GnRH analogs [3]. GnRHR have also been observed in several other malignancies [36]. However, various GnRH analogs have shown differing effects in cell lines, inducing apoptotic or proliferative effects depending on the cell line and the experimental model used [37–40]. There is extensive information about the

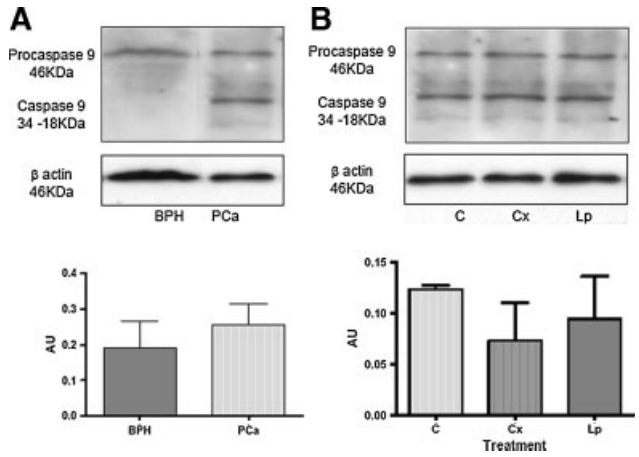

Fig. 3. Expression of pro-caspase-9 and cleaved caspase-9. **A**: Western blot analysis for caspase-9 in primary culture of epithelial cells from BPH and PCa. **B**: Effect of 20 ng/ml of GnRH analogs on the protein expression of caspase-9 in PCa cultures. The results are expressed in arbitrary units (AU) as the mean ± SD from at least three independent experiments ($^*P < 0.05$).

presence of GnRHR and the effect of GnRH analogs, using cell lines derived from prostate cancer metastasis, as DU-145, PC-3, and LNCaP [37,41,42]. However, these cell lines represent advanced metastatic cancers, which may not be suitable for studying the multiple steps during the carcinogenesis process and tumor progression in the prostate. In our laboratory, we have characterized a primary culture system of epithelial cells from human prostate carcinoma [35]. The main advantage of this model is that it represents more closely the characteristics of the prostate tumor cells. Previous works has shown that Lp and Cx have

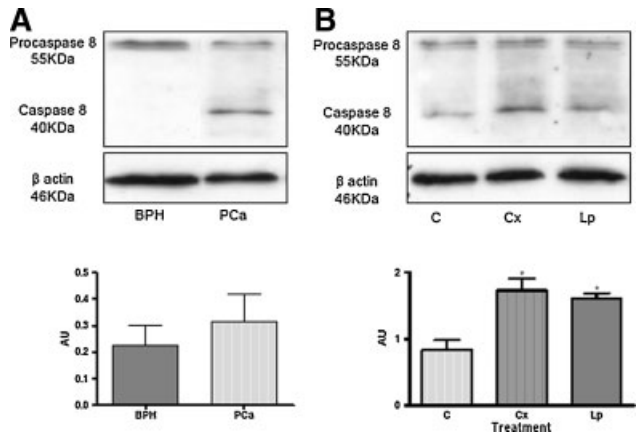

Fig. 4. Expression of pro-caspase-8 and cleaved caspase-8. **A**: Western blot analysis for caspase-8 in primary culture of epithelial cells from BPH and PCa. **B**: Effect of 20 ng/ml of GnRH analogs on the protein expression of caspase-8 in PCa cultures. The results are expressed in arbitrary units (AU) as the mean ± SD from at least three independent experiments ($^*P < 0.05$).

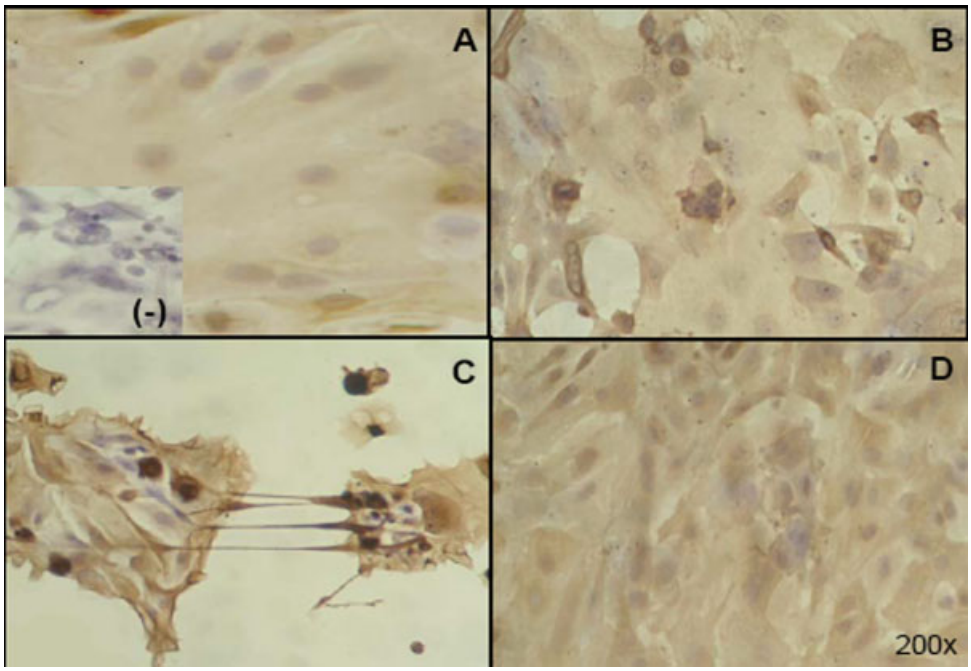

Fig. 5. Expression of caspase-3. Immunocytochemistry for caspase-3 in primary cultures of prostatic epithelial cells from PCa untreated cultures (**A**), BPH cultures (**B**), PCa cultures treated with 20 ng/ml of cetrorelix (**C**) and PCa cultures treated with 20 ng/ml of leuprolide (**D**). Insert shows control cultures without first antibody (200×).

antiproliferative and apoptotic effects on PCa cell cultures [16]. In the present work, it was explored the possible molecular mechanism of the apoptotic effect of these GnRH analogs in our primary culture system. The tumor suppressor protein p53 plays a key role in the apoptosis induction [43]. The increase in the expression of p53 in neoplasic cells may indicate a physiological response to damaged DNA. It has been reported that p53 is one of the most frequently mutated gene in human cancer. However, several studies have shown that mutations in the p53 gene are rare in the PCa, and when present, these mutations are associated with advanced stages of the disease [44]. In agreement with other authors [45], we have found a high gene and protein expression of p53 in PCa cultures compared with BPH, suggesting that PCa genomic damage is

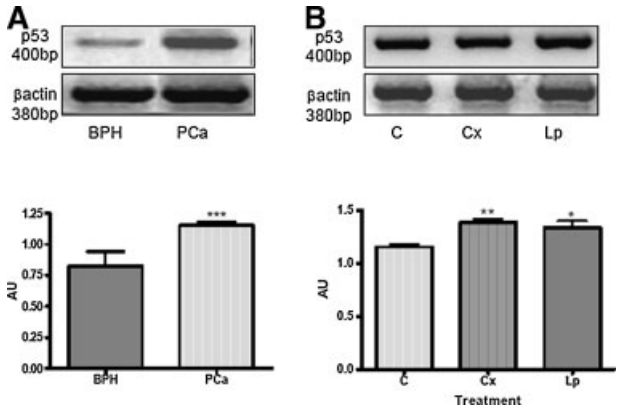

Fig. 6. Effect of leuprolide and cetrorelix on p53 mRNA expression. **A**: RT-PCR for p53 in primary culture of epithelial cells from BPH and PCa. **B**: RT-PCR for p53 in PCa primary cultures in the absence or presence of 20 ng/ml of GnRH analogs. The results are expressed in arbitrary units (AU) as the mean ± SD from at least three independent experiments (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

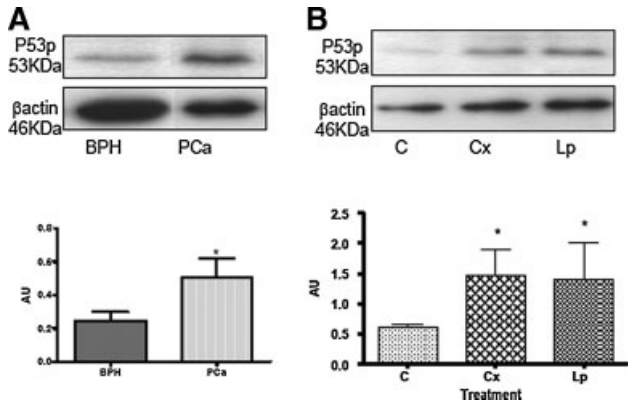

Fig. 7. Effect of leuprolide and cetrorelix on phosphorylation of p53. **A**: Western blot analysis for serine 20 phosphorylated p53 in primary cultures of epithelial cells from BPH and PCa. **B**: Western blot analysis for serine 20 phosphorylated p53 in PCa cultures in absence or presence of 20 ng/ml of GnRH analogs. The results are expressed in arbitrary units (AU) as the mean ± SD from at least three independent experiments (*$P < 0.05$).

higher than in BPH. Some authors have reported, in patients with leiomyoma [17], that treatment with GnRH analogs induces an increase in the expression of p53. Consistent with these results we have shown an increase in mRNA expression of p53 in PCa cultures treated with both Cx and Lp. Moreover, we observed an increase in p53 phosphorylated at serine 20 (Ser20p-p53) in PCa cultures treated with GnRH analogs. This amino acid residue is one of the phosphorylation sites involved in the integration of various signaling pathways that stabilize and activate p53 [46]. It is interesting to note that the phosphorylation of p53 at serine 20 is also induced by some chemotherapeutic drugs [47]. For these reasons, we evaluated Ser20p-p53 as a possible pathway for effects of GnRH analogs in our system. In response to a severe genome damage, p53 can direct the cell into apoptosis, promoting the expression of genes coding for death receptors and pro-apoptotic members of the Bcl-2 family (Bax, Bad, etc.) [48,49]. The expression of Bax is controlled by p53 [50] or by independent mechanisms such as the proteasome inhibition. However, this later mechanism may be important in advanced stages of PCa [51]. Then, it would be reasonable to expect an increase of Bax consistent with the increase in p53. We observed an increase in Bax protein expression in PCa cultures treated with Cx. This increase may be independent of proteasome inhibition as our cultures were not obtained from advanced stages of PCa. However, this Bax increase in PCa cultures treated with Cx was not associated with changes in the expression of caspase-9. A possible explanation for these results is that some of the molecules involved in the mitochondrial apoptotic pathway, downstream Bax, might be altered or mutated in PCa cells. The proper balance between Bcl-2 and Bax is important to determine the survival or apoptosis of cells exposed to DNA damage [52]. Probably, the absence of a high expression of Bcl-2 in our PCa cultures might be due to the fact that positive regulation of this molecule is associated with PCa that are androgen independent and resistant to chemotherapy [52–55]. As mentioned above, our cultures came from androgen sensitive and non-metastatic PCa samples. On the other hand, it has been observed that p53 is involved in apoptosis mediated by the TNF-receptor family [55–57]. The p75 NGF receptor is present in normal prostate cells and, in association with the tyrosine kinase receptor TrkA, regulates the prostate growth in response to locally produced NGF [22,25]. Interestingly, the p75 NGF receptor is expressed to a very low extent in PCa [32,33]. There is strong evidence that this TNF-family receptor induces apoptosis in a variety of cell types [58–60], including prostate cell lines [25]. It is reasonable to postulate that the decrease or loss of p75 NGF receptor during the carcinogenic process could partly explain the apoptotic resistance of PCa cells. In previous work, we have demonstrated that GnRH analogs increase gene and protein expression of p75 NGF receptor [21]. This receptor contains death domains that bind the corresponding protein adapters to trigger the activation of caspase-8 (extrinsic apoptotic pathway) [61]. We have found a significant increase in cleaved caspase-8 in PCa cultured treated with both Cx and Lp, compared with untreated cultures, suggesting that this mechanism might account, at least in part, for the apoptotic effect of GnRH analogs. There is consistent evidence that p53 is involved in caspase-8 activation in cancer cells [62,63]. This p53-mediated caspase-8 activation has been associated with death receptors [11,64,65]. Interestingly, p75 NGF receptor-mediated apoptosis has been functionally associated with p53 activity in neurons [66].

Although most of clinical benefits observed in patients with PCa treated with GnRH analogs are due to androgen deprivation, the plasmatic concentration of GnRH analog after a standard treatment seems to be lower than the medium concentration at which we observed the highest apoptotic effect in vitro. We suggest that increasing prostatic concentration of GnRH analog through intraprostatic injections, may improve the direct apoptotic drug effect on PCa cells.

CONCLUSIONS

Taken together, our results suggest that the increase in p53, Ser20p-p53 and caspase-8 activation induced by GnRH analogs in PCa cultures might be associated with the increase in p75 NFG receptor level and apoptosis described previously [16,21]. Finally, the findings presented in this study reinforce the concept of direct effect of agonists and antagonists of GnRH on the survival of PCa cells suggesting that intraprostatic administration of these analogs might be a more efficient approach for the treatment of this disease because a higher tissue concentration of the GnRH analog could improve its direct apoptotic effect.

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