The DDAH/NOS pathway in human prostatic cancer cell lines: Antiangiogenic effect of L-NAME

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Abstract. Benign prostate hypertrophy (BPH) and prostate cancer (PC) are chronic diseases that require a long period for development from a small lesion to clinical manifestation. PC is the most common cancer in men in Europe and the Americas. Tumor growth and metastasis depend upon the development of neovascularization around the tumor. This process, called angiogenesis, may be regulated by NO, and thus modulation of NO production could play an important role in tumor progression. Recent studies report the involvement of DDAH, an enzyme which metabolizes the endogenous NOS inhibitor ADMA, in the development of tumor vasculature. The aim of the present study was to verify the involvement of the DDAH/NOS pathway in the progression of prostate cancer. The effect of the NOS inhibitor L-NAME was evaluated in the human prostate cancer cell line LnCap and in BPH-1 cells which represent benign prostatic hypertrophy. Higher DDAH-2, eNOS, iNOS and VEGF expression was found in LnCap cells compared to BPH-1 cells. L-NAME treatment of LnCap cells resulted in a reduction in VEGF, iNOS and eNOS expression. VEGF, iNOS and eNOS inhibition is a promising approach for targeting tumor vasculature and certain NOS inhibitors could potentially serve as experimental agents for treatment of certain chemoresistant tumors, including prostate tumors. Moreover, since in our experimental conditions L-NAME was unable to reduce DDAH activity and expression, it is plausible to hypothesize the development of a targeted polypharmacological approach by developing dual and specific inhibitors of DDAH and NOS to better control NO biosynthesis.

Introduction

Benign prostate hypertrophy (BPH) and prostate cancer (PC) are chronic diseases that require a long period for development from a small lesion to clinical manifestation. PC is the second leading cause of death in men of Western world (1) (American Cancer Society 2007 Prostate Cancer Statistics- http://www.cancer.org; American Society of Clinical Oncology Prostate Cancer Statistics- http://www.cancer.net/prostate).

Despite significant improvements in local and systemic therapies, most deaths from prostate cancer are due to metastasis which resist conventional therapies (2-4). Therefore, novel therapeutic strategies targeting specific molecular markers are being pursued to allow early detection and cure.

Tumor growth and metastasis depend upon the development of a neovascularization around the tumor (5-9). This process, called angiogenesis, is critical to tumorigenicity and metastasis (10). Similarly to carcinogenesis, angiogenesis is a multistep process, regulated by a balance between stimulatory and inhibitory factors released by the tumor and its microenvironment (7,11-17).

Angiogenesis facilitates tumor growth through a series of steps including dissociation of endothelial cells (EC) from adjacent pericytes, remodelling of extracellular matrix, proliferation and migration of EC and capillary differentiation.

Nitric oxide (NO) is a signalling molecule produced by three isoforms of nitric oxide synthases (neuronal NOS, endothelial NOS and inducible NOS); it mediates a variety of actions such as vasodilatation, neurotransmission, host defence against bacteria and tumor cells (18,19).

Strong evidence suggests that NO is a regulator of angiogenesis (20,21), which enhances vascular permeability, induces extracellular matrix degradation, endothelial cell proliferation and migration (22-24) and stimulates the expression of vascular growth factor (VEGF) (25,26). Increased iNOS expression and NO production in BPH and in high grade PC occur when compared to normal tissue (27). Prostate cancer cells express lower levels of antioxidant enzymes than BPH cells and compared to normal prostatic cells, activity of antioxidant enzymes is decreased in BPH cells (28,29). Although conflicting data have been reported, an overwhelming amount of clinical and experimental evidence suggested a positive association between NO production and tumor progression (30-39). Modulation of NO production may therefore play an important role in regulation of angiogenesis and consequently in tumor progression. Overexpression of dimethylarginine dimethylaminohydrolase (DDAH), enzyme which metabolizes the endogenous NOS inhibitor asymmetric
dimethylarginine (ADMA), reduces tissue ADMA levels and enhances angiogenesis (40).

The involvement of DDAH in cerebral tumor growth and the development of tumor vasculature has been reported (41). Two isoforms of DDAH have been identified: DDAH-1 and DDAH-2. Although there is some overlapping between the sites of expressions of DDAH-1 and nNOS (neuronal NOS) and between DDAH-2 and eNOS (endothelial NOS), it is now evident that both DDAHs are widely expressed and not confined to NOS expressing cells or tissues (42). Both isoforms have been identified in the prostate tissue, but the expression of DDAH-2 isoform appears more abundant (43).

In order to verify the involvement of DDAH/NOS pathway in the progression of prostate cancer, two different cell lines were used in the present study: BPH-1 cells which represent benign prostatic hypertrophy and LnCap, which are a model of human prostatic carcinoma (44,45). In addition, the effect of the NOS inhibitor $N^\omega$-nitro-L-arginine methyl ester (L-NAME), which is not degraded by DDAH (40), was examined.

Materials and methods

Cell culture conditions. Human prostate BPH-1 cells were purchased from Deutsche Sammlung Von Mikroorganism Und Zellkulturen-GmbH (DSMZ-GmbH) and grown in 80% RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS) and 20 ng/ml testosterone, 5 µg/ml sodium selenite, 5 µg/ml insulin and a trace elements mix. Human prostate cancer LnCap cells were purchased from American Type Culture Collection (Manassas, VA) and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 0.1% streptomycin-penicillin, 1% L-glutamine, 1% sodium pyruvate and 1% glucose. Cells were incubated at 37°C in a 5% CO$_2$ humidified atmosphere and maintained at subconfluency by passaging with trypsin-EDTA (Gibco).

Cell viability. To monitor cell viability, BPH-1 and LnCap cells were seeded 2x10$^5$ cells per well in a 96-well, flat-bottomed 200 µl microplate. Cells were incubated at 37°C in a 5% CO$_2$ humidified atmosphere and cultured for either 24 or 72 h, in the presence and absence of different concentrations of L-NAME (0.1-1 mM). Four hours before the end of treatment, 20 µl of 0.5% 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate buffered saline (PBS) were added to each microwell. After incubation with the reagent, the supernatant was removed and replaced with 100 µl DMSO. The amount of formazan produced is proportional to the number of viable cells present. The optical density was measured using a microplate spectrophotometer (Thermo Labsystems Multiskan) at 370 nm during the pyruvate-lactate transformation, as previously reported (46,47). Cells were lysed with 50 mM Tris-HCl and 20 mM EDTA pH 7.4 plus 0.5% sodium dodecyl sulfate, further disrupted by sonication and centrifuged at 13,000 g for 15 min. The assay mixture (1 ml final volume) for the enzymatic activity contained 33 µl of sample (5-10 µg of protein) in 48 mM PBS pH 7.5 plus 1 mM pyruvate and 0.2 mM NADH. The LDH released was calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cell lysate and that in the culture medium. The optical density was measured using a Hitachi U-2000 dual beam spectrophotometer (Hitachi, Tokyo, Japan).

Western blotting. BPH-1 and LnCap cells cultured for 24 h, in both the presence and absence of 0.1-1 mM L-NAME, were suspended in 25 mM Tris buffer, pH 8.5, containing 100 mM NaCl (Sigma-Aldrich, St. Louis, MO, USA), 7 mM mercaptoethanol (Merck KGaA, Darmstadt, Germany) and a protease inhibitor cocktail (1:1000) (Sigma-Aldrich) and then sonicated for 3 cycles of 5 sec. The whole lysate was collected to evaluate DDAH-2, VEGF, eNOS and iNOS expressions by Western blot analysis. Briefly, 50 µg of lysate was loaded in a 10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 3% fat-free milk in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.05% TBST buffer, at 4°C for 2 h and then incubated with polyclonal anti-DDAH-2 (Calbiochem EMD Biosciences, Inc., Darmstadt, Germany), monoclonal anti-iNOS (Santa Cruz Aldrich), anti-VEGF (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA) and anti-iNOS (Santa Cruz Biotechnologies) antibodies overnight at room temperature, with constant shaking. The same membranes were used for blotting with anti-β-actin (Santa Cruz Biotechnologies) antibody as an internal loading control. The filters were then washed and probed with horseradish peroxidase-conjugated donkey secondary anti-mouse, anti-rabbit and anti-goat IgG (Amersham Biosciences, Piscataway, NJ, USA) at a dilution of 1:5000. Chemiluminescence detection was performed with the ECL plus detection kit (Amerham) according to the manufacturer's instructions. Western blot analyses were quantified by densitometric analysis performed after normalization with β-actin. Results were expressed as arbitrary units (AU).

DDAH enzyme activity assay. Cell lysates were centrifuged at 2000 g for 45 min at 4°C and supernatants were collected for evaluating DDAH enzymatic activity and protein content by the Lowry et al assay (48). DDAH activity was assayed by determining L-citrulline formation in 96-well microtiter plate (49). One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 µmol L-citrulline/min at 37°C.

$NO_3^-/NO_2^- $ quantification. Nitrite, the stable metabolite of NO, was measured colorimetrically via Griess's reaction. Based on results regarding cell viability, for $NO_3^-/NO_2^-$ determination, cells were cultured in presence or absence of 0.1-1 mM L-NAME for 24 h, after which the media were removed and used as below described.

Aliquots of cell culture medium (100 µl) were preincubated for 30 min at room temperature with 50 µM NADPH (Sigma- Aldrich) and 24 µM of nitrate reductase (Roche Diagnostics
Samples were then treated with 0.2 U of lactate dehydrogenase (Roche Diagnostics Gmbh) and 0.5 µmol of sodium pyruvate for 10 min. The coloration was developed adding Griess reagent (Merck KGaA) (1:1, v/v). Finally, after 10 min at room temperature, absorbance was recorded using a 96-well plate microtiter (Thermo Labsystems Multiskan) at λ = 540 nm. Nitrite levels were determined using a standard curve and expressed as nmoles of NO₂⁻/NO₃⁻/1x10⁶ cells.

Statistical analyses. The data are presented as means ± SD for 4 experiments in triplicate. One-way variance analysis and Student’s t-test were used where appropriate; p<0.05 was regarded as significant.

Results

Effect of L-NAME on BPH-1 and LnCap cell proliferation. Cell viability, measured as functionality of succinate dehydrogenase, is reported in Fig. 1. The exposition of cell culture to 0.1 mM L-NAME did not modify BPH-1 and LnCap viability after either 24 or 72 h. When BPH1 and LnCap cells were exposed to 1 mM L-NAME, a significant reduction in viability was observed. This effect was less in LnCap cells when compared to BPH-1 cells and was not time-dependent since both 24 and 72 h of exposure to L-NAME elicited the same 30 or 40% reduction in cell viability. The growth of BPH-1 and LnCap cell lines was examined by direct cell counting after treatment for 72 h with L-NAME (0.1 and 1 mM) (Fig. 2). Although the results obtained show a reduction in cell number after 72 h of exposure to L-NAME, LnCap cells appeared more resistant to L-NAME treatment compared to BPH-1 cells. In fact, a significant cell number reduction was evident only at 1 mM concentration (Fig. 2A). Fig. 2B shows representative images of BPH-1 and LnCap cells cultured in presence and absence of 1 mM L-NAME treatment.

Effect of L-NAME on DDAH-2, eNOS, iNOS and VEGF expression in LnCap cells and BPH-1 cells. Higher DDAH-2 expression
was found in LnCap cells compared to BPH-1 cells. L-NAME treatment resulted in an increase in DDAH-2 in both BPH-1 and LnCap cells. However the effects were different. In BPH-1 0.1 mM L-NAME increased DDAH-2 expression (p<0.005) to a greater extent than 1 mM L-NAME. In LnCap cells both 0.1 and 1 mM L-NAME increased DDAH-2 expression to the same extent (p<0.05) (Fig. 4A). Fig. 4B reports representative Western blotting of DDAH-2 protein expression in cultured BPH-1 and LnCap cells.

Effect of L-NAME on DDAH activity. L-NAME resulted in a significant (p<0.05) increase in DDAH activity at both 0.1 and 1 mM L-NAME in BPH-1 cells. Control levels of DDAH activity were higher in LnCap cells when compared to BPH-1 cells. L-NAME resulted in a dose-dependent increase in DDAH activity (p<0.005) when compared to control in LnCap cells (Fig. 8).

Effect of L-NAME on eNOS expression. Higher eNOS expression was found in LnCap cells compared to BPH-1 cells. L-NAME had no effect on eNOS expression in BPH-1 cells. VEGF expression was unaffected by 0.1 mM L-NAME in Ln Cap cells, however 1 mM L-NAME produced a significant (p<0.005) decrease in VEGF expression (Fig. 7A). Fig. 7B reports representative Western blotting of VEGF protein expression in cultured BPH-1 and LnCap cells.

Effect of L-NAME on nitrite/nitrate levels. The effect of both concentrations, 0.1 and 1 mM, of L-NAME was examined in BPH-1 and LnCap cells. The dose of 0.1 mM L-NAME had no effect on nitrite/nitrate levels in either cell line. In contrast, 1 mM L-NAME caused a significant (p<0.05) decrease in nitrite/nitrate levels in both cell lines (Table I).
Discussion

Angiogenesis is a multi-step process that may be considered a major factor affecting the metastatic spread of malignant cells (50,51). Investigations published on experimental tumor models and on several types of human tumor including gynecologic, head and neck, breast, central nervous system, colorectal and prostate cancer (32,33,35,39,52-58) have shown an increased expression of iNOS. Such overexpression has been correlated to...
The increased DDAH-2 expression and activity reported after L-NAME treatment, both of BPH-1 and LnCap cells, may represent an adaptational response to the presence of L-NAME (an inhibitor that is not degraded by DDAH) by reducing endogenous ADMA levels. iNOS and eNOS inhibition is a promising approach for targeting tumor vasculature and certain NOS inhibitors potentially serve as experimental agents for treatment of certain chemoresistant tumors, including prostate tumors (Fig. 9). Moreover, since L-NAME was unable to reduce DDAH activity and expression, as reported by Wang et al (84), it is plausible to hypothesize the development of a targeted polypharmacological approach by developing dual and specific inhibitors of DDAH and NOS to better control NO biosynthesis.

References


Figure 9. DDAH/NOS pathway: effect of NOS inhibitor L-NAME.


