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Expression of inducible nitric oxide synthase in tumoral and non-tumoral epithelia from bladder cancer patients

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Abstract

We have previously demonstrated that nitric oxide (NO) is elevated in the urine from bladder cancer patients. As the inducible nitric oxide synthase (iNOS) produces high NO output, the aim of this study was to examine iNOS expression and activity in tumoral (BT) and non-tumoral bladder tissue (NT). iNOS expression was determined by Western blot in 42 BT, 22 NT, and 4 normal bladders (normal B). iNOS activity was evaluated by conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline plus NO, in additional 15 BT, 8 NT, and 1 normal B. iNOS tissue localization was studied by immunohistochemistry. iNOS expression and activity were found in almost 50% of bladder cancer patients, in both BT and in NT. A similar positive or negative iNOS expression in each pair of NT and BT tissue compared was observed, suggesting that high urine NO levels could be generated by an active iNOS present not only in the tumor but also in the non-tumoral bladder tissue. By immunohistochemistry, heterogeneous iNOS staining was detected in tumor cells from superficial and invasive tumors, while it was not evident in the normal bladder epithelium. A follow-up of 21 patients during 2 years showed recurrences in 80% with positive iNOS. On the contrary, no recurrences were observed in 73% of iNOS negative patients. Our results suggest that iNOS expression in bladder tissue may predispose to cancer recurrences.

Keywords: Tumorigenicity; Nitric oxide; Inducible NO synthase; Bladder cancer; Tumor marker

Nitric oxide (NO) is generated by a family of nitric oxide synthase enzymes (NOS). The endothelial and neuronal NOS isoforms are expressed constitutively and require the presence of intracellular Ca^{2+} and calmodulin, generating nanomolar levels of NO. Inflammatory cytokines and/or bacterial products usually activate the inducible NOS (iNOS), generating large amounts of NO (about micromolar). It is expressed in a wide range of mammalian cells as macrophages, hepatocytes, endothelial cells, and chondrocytes [1].

iNOS is also expressed in a variety of tumor cells and it has been suggested to play a controversial role in tumor biology, either promoting or inhibiting tumor growth, depending on the tumor model levels of NO delivered, genetic background, and cell type all of which can determine NO sensitivity [2–4]. For example, it has been reported that continuous NO production may be involved in the inflammatory process associated with the appearance of many human malignancies, including bladder cancer [5–7]. Besides, it is known that *N*-nitroso compounds, of which NO is a precursor, induce a signifi-

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cant number of cancers [8]. On the other hand, mechanisms involved in bacterial or tumor cell death are mediated by cells of the immune system such as macrophages, which can produce high levels of NO [9,10]. The instillation with bacillus Calmette–Guerin, an effective immunotherapy for recurrent superficial bladder cancer and carcinoma in situ, induces long-term local formation of NO in the bladder [11].

In our knowledge, there are many reports showing expression of NOS in solid tumors [12–14], but only few show iNOS expression in human bladder neoplasia [15,16], without analyzing the expression of this enzyme in non-tumor bladder tissue from the same patients. Therefore, taking into account this information, in the present study, we examined iNOS expression and activity in human bladder tumors (BT) and in random non-tumoral epithelium (NT) samples collected from patients with bladder cancer. Our results show that approximately half of the patients analyzed express iNOS in tumor tissue as well as in NT. Inducible NOS was not detected in normal bladder mucosa. Our results also show that patients with iNOS positive expression have lower relapse free disease rate (RFD) than iNOS negative patients, suggesting that iNOS activity and expression may be a previous event to tumor appearance.

Materials and methods

Tumor samples

Tumor samples were obtained when transurethral resection or total cystectomy were performed at the Department of Urology of the Institute of Oncology "Angel H. Roffo" as conventional treatment to 57 patients (50 men and 7 women) with a median age of 64 (38–82) year. Random bladder samples without evidence of tumor presence (named non-tumoral bladder: NT) confirmed during surgery by pathological examination were also collected when indicated for diagnosis. Pathological examination of the samples was performed by a single pathologist according to the UICC [17] and World Health Organization [18].

iNOS expression was determined by Western blot in 42 tumor samples (3 adenocarcinomas and 39 transitional carcinomas). Bladder tumors were classified according to invasion status as superficial (2 in situ, 1 pTa, 15 pT1) and invasive tumors (15 pT2, 3 pT3, 4 pT4) while 2 remained without specification. According to histological grade, transitional tumors were classified as low grade (19 GII) or high grade (17 GIII and 3 GIV). NT tissues were obtained from 22 patients. Four samples of normal bladder mucosa were obtained during prostate transbladder adenomectomy of patients without bladder cancer. In 14 patients tumor tissue and non-tumor mucosa from the same bladder were paired for Western blot assays.

iNOS activity was determined in additional independent samples of 4 adenocarcinomas (1pT1, 2 pT2, 1 pT3) and 11 transitional carcinomas (histological grade: 5 GII, 3 GIII, 3 GIV; invasive status: 2 pTa, 6 pT2, 1 pT3, and 2 pT4), 8 NT samples (4 being diagnosed as inflammatory), and 1 normal bladder.

Informed consent has been obtained from all patients and procedures followed were in accordance with the ethical standards of the Ethics Committee from the Institute of Oncology "Angel H. Roffo" and the Helsinki Declaration of 1964 as revised in 1983.

Western blot analysis

Samples were processed after surgery as previously described [19]. Briefly, samples were minced in homogenization buffer (50 mM Tris–HCl, pH 8.0; 100 μg/ml NaCl; 1% Triton, 1 μg/ml aprotinine, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 10 mM EDTA/EGTA) and then centrifuged for 40 min at 13,000*g*. Protein content in the resultant supernatant was determined by Bradford method using bovine serum albumin as standard [20]. Samples were stored at −80 °C until use. Electrophoresis of the samples (120 μg protein/lane) was performed on 7.5% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) [21]. Proteins were transferred to nitrocellulose membranes and blotted with human specific polyclonal anti-iNOS antibody (H-174, sc-8310 from Santa Cruz Biotechnology, USA) diluted 1:50. A goat anti-rabbit IgG, conjugated with alkaline phosphatase (Sigma–Aldrich, St. Louis, Missouri, USA), was used as second antibody. Immunoreactive proteins were detected with NBT and BCIP (Sigma–Aldrich, St. Louis, Missouri, USA). As seeding control, actin expression revealed in the same stripped membrane with an anti-actin antibody (#Ab Mo 090M, Biogenex, 4600 Norris Canyon Road, San Ramon, CA 9453) was used. Paired BT and NT samples were run in the same membrane and the blotted proteins were scanned with a computerized densitometer (GS-700 Calibrated Densitometer Bio-Rad Laboratories, Richmond, CA). iNOS protein was expressed in units of optical density per milligram of total protein (OD/mg protein). As all samples showed similar actin blot, OD was not normalized by actin expression

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections from nine bladder tissues (two normal bladders, four superficial tumors (two in situ), and three invasive tumors) were used to analyze iNOS tissular distribution. Immunohistochemical staining was performed using an avidin–biotinyl peroxidase complex. Briefly, samples were treated with