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ORIGINAL ARTICLE

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Inducible nitric oxide synthase expression in human urinary bladder cancer

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Abstract Nitric oxide (NO) is generated by a family of enzymes, nitric oxide synthases (NOS), in a wide range of mammalian cells. NO produced by the inducible NOS isoform (iNOS) has been suggested to play an important role in tumor biology with both tumor promoter and antitumor activity. Here, the cellular localization of iNOS in tissue of 100 cases of urinary bladder cancer was assessed immunohistologically using a commercially available antiserum. Positive iNOS immunostaining was detected in all samples of tumor tissue, whereas nonmalignant tissue adjacent to malignant areas did not show any iNOS positivity. The tumor tissue revealed a highly inhomogeneous staining pattern. In addition to uniformly stained tumor specimens, we also found markedly iNOS-positive tumor islets in the midst of unstained tumor tissue and scattered individual tumor cells expressing marked staining. In some cases, the tumor tissue showed no or only weak staining intensity. In some instances, the superficial epithelial layer of papillary carcinomas was extremely immunoreactive, in other cases it was not. Thus we were unable to show a clear correlation to tumor grade or stage. Further studies with a diversity of tumor markers including molecular genetics techniques will be necessary to elucidate how and to what extent NO and bladder cancer of different grades and stages are functionally interrelated.

Keywords Nitric oxide synthase · Immunohistochemistry · Transitional cell carcinoma

Introduction

Nitric oxide (NO) is a short-lived pleiotropic biomolecule with a multitude of biologic functions. Since its discovery as a biologically active molecule in the late 1980s, NO has been thought to play a role as a signal molecule in many parts of the organism, in immunological and defense mechanisms [14, 17, 18,31] and in cancerogenesis [2, 3, 9, 18,32]. This small molecule, NO, is a product of the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS), which exists as three enzyme classes: the calcium-dependent endothelial (eNOS) and the neuronal or brain isoforms (nNOS) and a calcium-independent inducible or immunologic NOS (iNOS) [5, 6, 23]. iNOS expression in human diseases has long been a matter for investigation, and the importance of NO in tumor biology is poorly understood.

At present, the role of NO during tumor development appears to be complex. There is a growing body of evidence that high-output NO production by infiltrating macrophages is a part of their cytotoxic repertoire [12, 17,28]. The observation that levels of calcium-independent NOS (iNOS) are higher in malignant tissue and the localization of iNOS to intratumoral macrophages and endothelial cells of the tumor vasculature suggest that the intratumoral environment of these cancers is conducive to induction of this special isoform (iNOS) [9, 13, 16, 27,28]. Studies showing an increase in growth rate, vascular density, and invasiveness of a human tumor cell line transfected to constitutively expressed iNOS support this assumption [1,3]. Consistent with this, administration of a highly selective inhibitor of iNOS limited the invasion and growth rate of iNOS-transfected tumor cell lines and of other tumors expressing this isoform [20, 21, 23,24]. So far, iNOS has been detected in tumor cells of human brain, breast, lung, stomach, colon, prostate, cervix, ovary, kidney, liver, and pancreas, among others [7, 10, 13, 15, 22, 25, 27, 29, 30,35]. In most cases, assays for iNOS were performed using frozen tissue or cultures of cell lines by means of immunocytochemistry, mRNA detection (in situ hybridization), or by measuring iNOS activity by NO production (nitrite/nitrate levels) or by conversion of radiolabeled arginine to citrulline. Little is currently known about the expression of iNOS in urinary bladder cancer [11,26].

In the present study, the local evidence of iNOS in tissue of 100 cases of urinary bladder cancer was investi-

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gated immunohistochemically using a commercially available iNOS antiserum. The study aimed to compare iNOS expression in different tumor grades and stage in order to discuss its potential roles in tumor biology.

Materials and methods

Urinary bladder cancer tissue of different grades was selected from the files of the Department of Pathology of the University of Texas, M.D. Anderson Cancer Center, Houston, Texas (mean age, 46.2 years). Specimens (n=100, highest stage was T3, defined as N0 and M0 according to the TNM Classification of Malignant Tumours, fifth edition, ICD-O C67, see Table 1) were fixed in 4% paraformaldehyde and embedded in paraffin. Deparaffinized and rehydrated 4-µ tissue sections were washed in tap water, distilled water, and then in 0.05 *M* Tris buffer (pH 7.6). Nonspecific binding sites were blocked with a commercially available protein-blocking agent (Ultra-Tech., Coulter-Immunotech, Marseille, France) for 10 min. After removing the excess blocking reagent, sections were incubated with the primary polyclonal rabbit antibody (Transduction Laboratories, Biomol, Hamburg, Germany), diluted to 1:1200 in Roswell Park Memorial Institute (RPMI) medium (Life Technologies, Eckenstein, Germany). The antibody is directed against the mouse iNOS C-terminal peptide (1131–1144) plus additional N-terminal Cys conjugated to KLH (CKKGSALEEPKATRL). According to the manufacturer, the antibody recognizes the iNOS 130-kDa protein in humans, rats, and mice without cross-reaction to eNOS or nNOS. As controls, the sections were incubated with RPMI medium alone. Ten colorectal cancer specimens, likewise fixed in formalin and embedded in paraffin, were used as a positive control for the iNOS antigen in immunohistochemistry. In the corresponding frozen specimens of colorectal tumors, the NOS activity was co-localized by the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase reaction according to Hope and Vincent [8]. After an 18-h incubation period at 4°C, sections were rinsed with Tris buffer and incubated with a secondary, biotinylated goat anti-rabbit antibody (Vectastain, ABC-AP Elite Kit; Vector Laboratories, Burlingame, USA). After incubation with Vectastain ABC-AP Reagent (avidin–biotin complex, alkaline phosphatase, as described by the manufacturer), sections were stained with the Fast Red chromogen system (Coulter-Immunotech.) for 15 min, counterstained with hematoxylin, and mounted with glycerol.

Semiquantitative evaluations of the immunostainings were performed for whole tissue sections. Immunoreactivities and percentages of positive tumor cells in the individual cases were compared with the average immunoreactivities in the samples and classified into four groups: 0, negative; 1, weakly positive; 2, intermediate; 3, strongly positive. The semiquantitative results were compared with the tumor grade and stage using Student's t test. A P value of less than 0.05 was considered significant.

Results

The clinicopathologic data (i.e., grades, stages) of the tumor samples are summarized in Table 1. Positive iNOS immunostaining was detected in all samples of tumor tissue, whereas distant benign bladder regions did not show any iNOS positivity (Fig. 1). In the negative control omitting the primary antiserum, immunoreactivity was completely lacking, while the positive control (colorectal carcinoma) displayed a clear-cut immunopositivity, which was co-localized with NADPH-diaphorase activity of the tumor cells. Our samples of urinary bladder cancer revealed a highly inhomogeneous picture of immunoreactivities. In addition to uniformly stained tumor specimens, we also found markedly iNOS-positive tumor islets in the midst of unstained tumor tissue and scattered individual tumor cells expressing marked staining (Fig. 2). In some cases, areas of tumor tissue showed no or only weak staining intensity, whereas a rather homogeneously moderate immunostaining was seen in many others. In some instances, the superficial epithelial layer of papillary carcinomas was extremely immunoreactive, in other cases it was negative (Fig. 3). The immunoreaction was seen either homogeneously or with a granular pattern in the cytoplasm of the tumor cells (Fig. 4). Sometimes reactions were concentrated in the perinuclear regions. Cell nuclei were completely unstained. In inflammatory areas and in tumor necrosis, tumor cell-associated iNOS immunoreactivity was enhanced and was sometimes also detected in round cells, apparently macrophages, in the direct vicinity of the tumor (Fig. 5). The endothelium of blood vessels was generally free of immunoreaction, whereas vascular smooth muscle cells displayed a low or moderate staining intensity. Somewhat enhanced immunoreactivity was also

Table 1 Expression of inducible nitric oxide synthase (iNOS) immunopositivity (semiquantitative data) and clinicopathologic data of urinary bladder carcinomas included in this study

Tumor stage/grade	iNOS+ (n)	iNOS++ (n)	iNOS+++ (n)	Total (n)
Ta	28	8	0	36
Tis	2	0	0	2
T1	21	9	0	30
T2	11	17	2	31
T3	0	1	0	1
G1	43	11	0	54
G2	10	20	2	32
G3	9	5	0	14

+, low; ++, moderate; +++, strong.

Fig. 1 Homogeneous and strong expression of inducible nitric oxide synthase (iNOS) in invasive bladder carcinoma. Some groups of tumor cells (*arrows*) show somewhat enhanced immunoreactivity. Avidin–biotin complex, alkaline phosphatase, ABC-AP, counterstained with hematoxylin, ×240 ▶

Fig. 2 Inhomogeneous staining pattern of inducible nitric oxide synthase (iNOS) with **a** single immunopositive tumor cells and **b** positive tumor cell groups. ABC-AP, ×240

Fig. 3 Positive inducible nitric oxide synthase (iNOS) immunoreactions of surface cell layers in a papillary carcinoma. ABC-AP, ×240

Fig. 4 Higher magnification of inducible nitric oxide synthase (iNOS) immunoreactions with a granular cytoplasmic staining pattern in tumor cells. ABC-AP, ×460

Fig. 5 Enhanced expression of inducible nitric oxide synthase (iNOS) in the close vicinity of areas displaying tumor necrosis (*thick asterisk*). In contrast, the remaining tumor cells (*thin asterisks*) show only weak or negative immunoreactions. ABC-AP, ×240

Fig. 6 Enhanced expression of inducible nitric oxide synthase (iNOS) in **a** invasive tumor components and **b** islets of tumor cells in the underlying tissue. ABC-AP, ×240

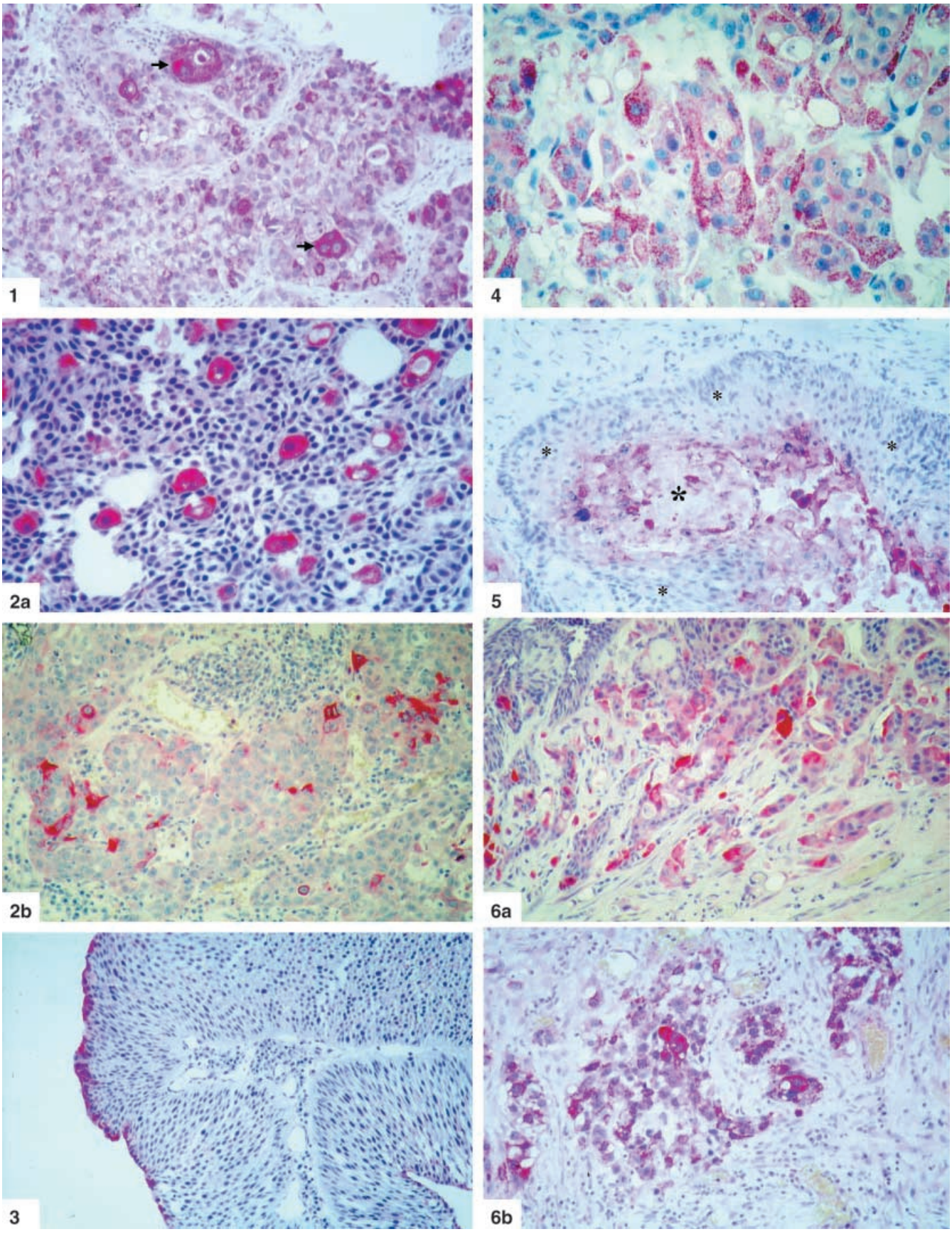

Fig. 1–6 Legend see page 663

found in invasive tumor areas or tumor nests, which display infiltrative growth into the underlining tissues (Fig. 6), whereas immediately adjacent malignant tissue areas with cells of an apparently identical phenotype were almost free of reaction product or showed a very low staining level.

The average intensities of the immunostainings of cancer tissue were semiquantitatively assessed. A comparison of the mean iNOS immunoreactivity with the tumor grades and stages (Table 1) did not show statistically significant differences.

Discussion

The present study demonstrates abundant iNOS immunoreactivity in tumor tissue obtained from a large sample of urinary bladder cancer. Normal bladder tissue was completely negative. So far, only two immunohistochemical studies describing the occurrence of iNOS in bladder cancer have been published [11,26]. Although Jansson and coworkers were able to stain tumor tissue for NOS, their results should be viewed with considerable caution, since, according to the authors, the antibodies used were nonspecific and apparently stained all of the NOS isoforms. The other group [26] reported a specific staining for iNOS in transitional cell carcinomas of the urinary bladder. Similar to our findings based on a large sample size, Swana and coworkers did not find a clear correlation between the degree of iNOS immunoreactivity and tumor grade using a rather small collection of tissue specimens. The lack of a correlation is in contrast to findings in human gynecological cancers, in which Thomson et al. [27,29] have noted a relationship between NOS activity and tumor grade. However, there are several other reports in which such a correlation was not observed either, e.g., in human prostate carcinoma [13].

The biologic role of NO in malignancies is unclear. The supraphysiologic production of NO in malignant tissue by iNOS may cause cytotoxicity; it either supports or inhibits immune defense mechanisms as described [17,18]. Furthermore, NO may increase tumor blood flow and promote angiogenesis [4,19], with NO having both pro- and antitumor activity depending on its concentration [12]. Further investigations of the pathophysiologic and protective roles of NO and the influence of NO inhibitors on tumor growth in carcinoma, especially in bladder tumor, will be necessary.

As seen in the present study, iNOS expression in tumor cells is highly inhomogeneous, and the role of NO must therefore be quite different in terms of the situation within the NO-producing cell and transcellular effects on surrounding tissue. Produced NO may not only attack neighboring cells, but can also be suicidal, with the distribution pattern of iNOS-positive and iNOS-negative tumor cells the result of a selection process. Thus Moochhula et al. [22], for example, observed a loss of the constitutive NOS isoforms and iNOS in colorectal neoplasms and suggested that the aberrant expression of NOS in colon carcinomas might reflect changes in the behavior and aggressiveness of a tumor. Accordingly, expression of iNOS in tumor cells of murine K-1735 melanoma has been reported to be associated with apoptosis, suppression of tumorigenicity, and abrogation of metastases [34]. After incubation with different cytokines or the bacterial lipopolysaccharide, nonmetastatic cells exhibit high levels of iNOS activity and NO production, whereas metastatic cells do not [33]. A correlation between the occurrence of iNOS and the tumor stage should therefore be expected. Moreover, based on histopathologic criteria, we were not able to detect a clear correlation between iNOS positivity and different grades of malignancy. The demonstration of such a correlation may require the use of more sophisticated methods in future studies.

In conclusion, NO plays an important role in recent conceptions of tumor pathogenesis. In the present study, the expression of the inducible isoform of the NO-producing enzyme, iNOS, was able to be distinctly demonstrated in urinary bladder cancer cells. The immunohistochemical iNOS reactions did not show a clear correlation with tumor grade or stage. Further studies using a variety of tumor markers, including molecular genetics techniques, will be necessary to elucidate how and to what extent NO and bladder cancer of different grades and stages are functionally interrelated.

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References

1. Ambs S, Merriam WG, Bennett WP, Felley-Bosco E, Ogunfusika MO, Oser SM, Klein S, Shields PG, Billiar TR, Harris CC (1998) Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. Cancer Res 58:334–341
2. Ambs S, Hussain SP, Harris CC (1997) Interactive effects of nitic oxide and the p53 tumor suppressor gene in carcinogenesis and tumor progression. FASEB J 11:443–448
3. Ambs S, Merriam WG, Ogonfusika MO, Bennett WP, Ishibe N, Hussain SP, Tseng EE, Geller DA, Billiar TR, Harris CC (1998) p53 and vascular endothelial growth factor regulate tumor growth of NOS2-expressing human carcinoma cells. Nature Med 12:1371–1376
4. Andrade SP, Hart ID, Piper PJ (1992) Inhibitors of nitric oxide synthase selectively reduce flow in tumor associated neovasculature. Br J Pharmacol 107:1092–2005
5. Förstermann U, Kleinert H (1995) Nitric oxide synthase expression and expressional control of three isoforms. Arch Pharmacol 352:351–364
6. Förstermann U, Schmidt HHHW, Pollok JS, Sheng H, Mitchell JA, Warner TD (1991) Isoforms of nitric oxide synthase: characterization and purification from different cell types. Biochem Pharmacol 42:1849–1857
7. Fujimoto H, Ando Y, Yamashita T (1997) Nitric oxide synthase activity in human lung cancer. Jpn J Cancer Res 88:1190–1198
8. Hope BT, Vincent SR (1989) Histochemical characteristion of neuronal NADPH-diaphorase. J Histochem Cytochem 37:653–661

9. Janssens MY, van den Berge DL, Verovski VN, Monsaert Ch, Storme GA (1998) Activation of inducible nitric oxide synthase results in nitric oxide-mediated radiosensitization of hypoxic EMT-6 tumor cells. Cancer Res 58:5646–5648
10. Jansson OT, Morcos E, Brundin L, Bergerheim USR, Adolfsson J, Wiklund NP (1998) Nitric oxide synthase activity in human renal cell carcinoma. J Urol 160:556–560
11. Jansson OT, Morcos E, Brundin L, Lundberg JON, Adolfsson J, Söderhäll M, Wiklund NP (1998) The role of nitric oxide in bacillus Calmette-Guerin mediated anti-tumor effects in human bladder cancer. Br J Cancer 78:588–592
12. Jenkins DC, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, Moncada S (1995) Roles of nitric in tumor growth. Proc Natl Acad Sci USA 92:4392–4396
13. Klotz T, Bloch W, Volberg Ch, Engelmann U, Addicks K (1998) Selective expression of inducible nitric oxide synthase in human prostate carcinoma. Cancer 82:1897–1903
14. Knowles RG, Moncada S (1994) Nitric oxide in mammals. Biochem J 298:249–258
15. Kolios G, Rooney N, Murphy CT, Robertson DA, Westwick J (1998) Expression of inducible nitric oxide synthase activity in human colon epithelial cells: modulation by T lymphocyte derived cytokines. Gut 43:56–63
16. Konur A, Krause SW, Rehli M, Kreutz M, Andreesen R (1996) Human monocytes induce a carcinoma cell line to secrete high amounts of nitric oxide. J Immunol 157:2109–2115
17. Kröncke KD, Fehsel K, Kolb-Bachofen V (1997) Nitric oxide: cytotoxicity versus cytoprotection. How, why, when, where? Nitric Oxide 1:107–120
18. Kröncke KD, Fehsel K, Kolb-Bachofen V (1998) Inducible nitric oxide synthase in human diseases. Clin Exp Immunol 113:147–156
19. Leibovich SJ, Polverini PJ, Fong TW, Harbow LA, Koch AE (1994) Production of angiogenetic activity by human monocytes requires an I-arginine/nitric oxide-synthase-dependent effector mechanism. Proc Natl Acad Sci USA 91:4190–5004
20. Macdonald JE (1996) Nitric oxide synthase inhibitors. Ann Rep Med Chem 23:221–231
21. Mayer B, Andrew P (1998) Nitric oxide synthases catalytic function and progress towards selective inhibition. Arch Pharmacol 358:127–133
22. Moochala S, Chhatwal VJS, Ngoi SS, Chia YW, Rauff A (1996) Nitric synthase activity and expression in human colorectal cancer. Carcinogenesis 17:1171–1174
23. Pitzele BS (1997) The modulation of the action of isoforms of nitric oxide synthase. Exp Opin Ther Patents 7:717–727
24. Resink A, Dawson VL, Dawson TW (1996) Nitric oxide synthase inhibitors. Future therapies for CNS disorders? CNS Drugs 6:351–357
25. Suschek C, Bruch D, Kröncke KD, Kolb-Bachofen V (1996) Inducible nitric oxide synthase in human dermal endothelial cells. Eur J Clin Invest 26:A4
26. Swana HS, Smith SD, Perrotta PL, Saito N, Wheeler MA, Weiss RM (1999) Inducible nitric oxide synthase with transitional cell carcinoma of the bladder. J Urol 161:630–634
27. Thomsen LL, Lawton FG, Knowles RG, Beesley JE, Riverros-Moreno V, Moncada S (1994) Nitric oxide synthase activity in human gynecological cancer. Cancer Res 54:1352–1354
28. Thomsen LL, Miles DW (1998) Role of nitric oxide in tumour progression: lesson from human tumours. Cancer Metast Rev 17:107–118
29. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S (1995) Nitric oxide synthase activity in human breast cancer. Br J Cancer 72:41–44
30. Tracey WR, Xue C, Klinghofer V, Barlow J, Pollock JS, Förstermann U, Johns RA (1994) Immunochemical detection of inducible NO synthase in human lung. Am J Physiol 266:722–727
31. Wink DA, Hanbauer I, Grisham BM, Laval F, Nims RW, Laval J, Cook J, Pacelli R, Liebmann J, Krishna M, Ford PC, Mitchell JB (1997) Chemical biology of nitric oxide: regulation and protective and toxic mechanism. Curr Top Cell Regul 34:159–187
32. Wink DA, Vodovotz Y, Cook JA, Krishna MC, Kim S, Coffin D, DeGraff W, Deluca AM, Liebmann J, Mitchell JB (1998) The role of nitric oxide chemistry in cancer treatment. Biochemistry (Mosc) 63:948–957
33. Xie K, Dong Z, Fidler IJ(1996) Activation of nitric oxide synthase gene for inhibition of cancer metastasis. J Leukoc Biol 59:797–803
34. Xie K, Huang S, Dong Z, Juang SH, Gutman M, Xie Q-W, Nathan C, Fiedler U (1995) Transfection with the inducible nitric oxide synthase gene suppresses tumorigenicity and abrogates metastasis by K-1735 murine melanoma cells. J Exp Med 181:1333–1343
35. Yoshioka K, Thompson J, Miller MJS, Fisher JW (1997) Inducible nitric oxide synthase expression and erythropoietin production human hepatocellular carcinoma cells. Biochem Biophys Res Commun 232:702–706