Indian Journal of Pharmacology 1999; 31: 315-318 SHORT COMMUNICATION

EXPRESSION OF INDUCIBLE AND NEURONAL NITRIC OXIDE SYNTHASE IN 20-METHYL CHOLANTHRENE (20-MCA) INDUCED FIBROSARCOMA

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SUMMARY

Objective: Studies have shown that expression and localisation of nitric oxide synthase (NOS) depends on the type and differentiation status of different types of tumours. The present study was conducted to evaluate the expression of NOS in carcinogen induced tumour.

Method: Swiss albino mice were injected with 20-methyl cholanthrene. Seventy six percent of the animals were positive for fibrosarcoma at the end of 100 days following exposure. The tumours were excised when they reached a predetermined size and immunostained for inducible and neuronal NOS. The staining was graded on an increasing intensity scale.

Results: The tumour sections stained very strongly for iNOS as compared to controls (identical sections from tumour negative mice). Weak staining was detected for nNOS in the tumour sections unlike controls.

Conclusions: NO may be involved in carcinogen induced fibrosarcoma as seen from the expression of its synthesising enzymes.

KEY WORDS Nitric oxide nNOS iNOS 20MCA carcinogenesis fibrosarcoma mice

INTRODUCTION

Since the discovery of nitric oxide (NO), an endogenously generated molecule [1], has been implicated in diverse physiological roles including intracellular signalling, as a transcellular messenger or as a cytotoxic agent [2,3]. It is synthesised by the enzyme nitric oxide synthase (NOS) in the arginine to citrulline pathway. Of the three isoforms of NOS, endothelial NOS (eNOS) and neuronal NOS(nNOS) are constitutive calcium dependent enzymes while inducible NOS (iNOS) is expressed in response to immunologic stimuli and is capable of sustained NO release at concentrations which could be cytostatic or cytotoxic[3].

Studies have demonstrated the presence of NOS in human tumours[4]. Cobb's *et al* suggested that NO production could be associated with pathophysiological processes important to tumourogenesis[5]. To the best of our knowledge, no studies have however been conducted to implicate NO in carcinogen induced cancer. The present study was thus conducted to evaluate the involvement of NO in carcinogenesis.

MATERIALS AND METHODS

Swiss albino mice (body wt 15-16g, either sex) were procured from the Central Experimental Animal Facility of AIIMS and housed 5 per cage with food and water available *ad libitum*. They were acclimatised in standard controlled laboratory conditions for 1 week prior to inclusion in the experiment. On the day of the experiment all the mice were injected subcutaneously in the left flank with 20MCA (Sigma, USA) at a dose of 2mg/kg, in DMSO (Sigma, USA) as vehicle. They were then housed in sterile conditions and observed daily until the tumours were palpable. The animals were sacrificed under chloroform anaesthesia when the tumours reached a size of 5 x1000 cu mm and the tumours were immediately excised. Half of the tumour was processed for H&E staining after fixing in 10% formalin. The other half was fixed in 4%

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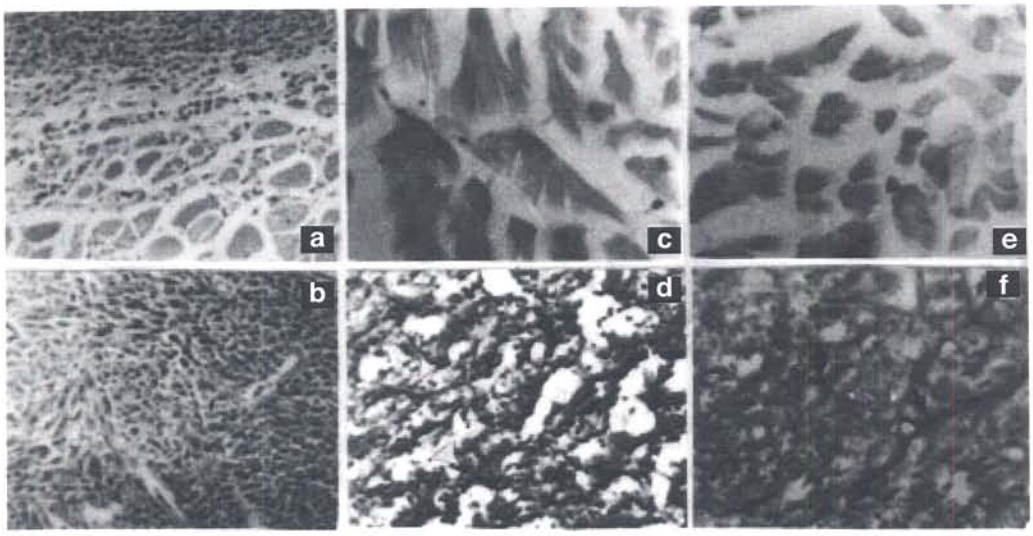
Figure 1. (a) Cross section of the tumour (H&E) at an early stage. The transformed cells can be seen infiltrating the underlying muscles. (b) Fully developed tumour (5000 cu mm) with H&E staining showing classical Herring bone pattern characteristic of fibrosarcoma (4X).(c) Negative control tissue stained for iNOS seen at higher magnification and (d) tumour cross section stained for iNOS with intense staining in the cancer tissues (open arrows). Note the dark stained infiltrated macrophages stained positively for iNOS (closed arrows). (e) Negative control tissue stained for nNOS at higher power vs (f) positive nNOS staining in the tumour sections (open arrows) (4X).



paraformaldehyde in 0.1M phosphate buffer for 2-4 h at 4° C. After several washes in buffer they were cryoprotected in 30% sucrose overnight. Sections were cut at 5-10 μm thickness in a Reichart jung cryostat and stored in vials containing phosphate buffer at 4°C till use for immunohisto-cytochemistry. However, 6 mice did not develop any tumour till day 150 when they were sacrificed and the tissue was excised from the left flank and subjected to the same treatment as above. Of the 25 mice injected with 20-MCA, 6 mice were put in control group since they did not develop tumour and rest 19 were put into the other group considered as tumour group.

H&E staining

Tissue samples were stained with haematoxylin and eosin according to standard protocol following paraffin embedding.

Immuno-histocytochemistry

The sections were treated with 70% methanol containing 0.1% H_2O_2 for a period of 20-30 min to block endogenous peroxidase activity. Sections were washed thoroughly and then treated with 20% normal horse serum (for those to be incubated with mouse monoclonal antibody) and 20% normal goat serum (for sections to be titrated with rabbit polyclonal antibodies) for 3h at 4°C.

The sections were incubated for 3 days at 4°C with primary antibodies against iNOS (1:50 mouse monoclonal, Transduction laboratories, Kentucky, USA) and nNOS(1:250, rabbit polyclonal, Transduction laboratories, Kentucky, USA). The antibodies were diluted in 0.01M phosphate buffer saline containing 0.5% Triton X and 5% normal sera. Following this the sections were washed in buffer and put in secondary

antibodies (1:200) for 5-6h at 4°C. For iNOS the secondary antibody was horse anti-mouse IgG (Vector labs, USA) whereas for nNOS it was goat anti-rabbit IgG (Vector labs , USA). After washing , the sections were treated with avidin-biotin peroxidase complex (Vector Labs, USA) for 2 h at room temperature. For visualisation of the antigen antibody reactions the sections were treated with 0.05M acetate-imidazole buffer (pH 7.4) containing 0.3% nickel sulphate as intensifier, for 15-20 seconds. The sections were finally rinsed in distilled water, mounted on gelatin coated slides and air dried. They were then dehydrated in alcohol and mounted in DPX. The slides were examined by an independent observer and the intensity of staining was graded as - when there was no detectable staining and grades ±, +, ++, +++ were used for weak, moderate, strong and intense staining respectively. The observer was exposed to control slides from untreated sections to establish a baseline zero prior to the sections from negative and positive groups.

RESULTS

Of the 25 animals injected with 20MCA, 19 developed solid tumours characteristic of fibrosarcoma with Herring bone pattern (Figures 1a and b). The tumours reached 200 cu mm between day 90-100 and 5000cu mm within 18±2 days after reaching 200 cu mm, characteristically following a Gompertzian curve. The remaining 6 mice did not develop tumours even after 150 days of administration of 20MCA and served as negative controls. The tumour sections stained very strongly for iNOS (Figure 1d) and was infiltrated with macrophages, which was not detected in control sections (Figure 1c). Neuronal NOS expression was also significantly elevated (Figure 1f) in comparison to controls (Figure 1e). Table 1 gives the gradation of intensity of staining by an independent observer.

Table 1. Staining intensity grades for immunostaining in tumour sections vs control

Tissue	Immunostaining	
	iNOS	nNOS
Control (n=6)	-	-
Tumour (n=19)	+++	++

- no detectable staining , + weak, + moderate, ++ strong, +++ intense staining

DISCUSSION

Nitric oxide (NO) has been implicated in a wide range of biological functions. It is generated enzymatically from terminal guanidinonitrogen of L-Arginine by NOS. Despite intensive investigations, the role of NO, either as a primary product of LArginine/NOS pathway or provided by donors, in carcinogenesis and tumour cell growth remains unclear. NO and its derivatives can cause DNA damage[6] and cell death[7] at higher concentrations and may exert opposite effects at lower concentrations[8]. Growth of solid tumours is regulated by interactions of endothelial cells of tumour vasculature, the tumour cells and the infiltrating immune cells as macrophages[9]. Most of these cellular components have been shown to generate NO *in vitro*[10,11]. Indeed in the present study we found a significant expression of iNOS in macrophages that had infiltrated into the tumour tissue. However as reported in other types of tumours[12,13], in the present study fibrosarcoma induced by carcinogen 20MCA also had a high expression of iNOS unlike the control group which did not develop any tumour. This indicates that NO synthesised by iNOS does play a role in the process of tumourogenesis. Studies have shown that iNOS transfected cells proliferate at a slower rate indicating that NO exerts a cytostatic effect on tumour cells[14] but it has been found to promote solid tumours by promoting angiogenesis[12]. In the present study too a high degree of vascularisation was noted in the tumours; however further experiments are warranted to implicate iNOS synthesised NO in angiogenesis in this tumour type.

The surprising observation was the expression of nNOS in the tumour section. Luck *et al*[15] have shown that nNOS system is of relevance to myogenic differentiation and it is present at an elevated level in inflammatory myopathies[16]. The expression of nNOS in the present system could thus indicate the differentiation, muscle cell death or apoptosis occurring in the tumour. This suggests that both the isoforms of NOS have a significant role in the process of carcino/tumourigenesis. The present study thus opens the door for further pharmacological experiments with selective inhibitors of NOS with the possibility of inhibiting/altering the progress of the pathology. Indeed early results have shown that cancer prevention by

curcumin could be related to inhibition of NOS gene[17] or that administration of NG-nitro-L-arginine-methyl-ester (L-NAME) a NOS inhibitor could reduce the incidence and number of metastasis[18].

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