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In Vitro and *In Vivo* Studies of Nicotinic Acid in an Experimental Metastasis Model of B16F10 Melanoma

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Metastasis is the major impediment to successful eradication of cancer. Therefore, development of antimetastatic agents is of prime importance. The present investigation is a modest step in that direction. Nicotinic acid is one of the products of nicotinamide hydrolysis by proteases and has been shown along with the parent compound (nicotinamide) to possess anticarcinogenic property. Therefore, it was interesting to find out whether it had any antimetastatic properties also. These studies have been done in a well standardised experimental metastasis model viz. B16F10 melanoma. Our results clearly demonstrates that at a dose level of 150 mg/kg on 1 through 9 schedule, there was a significant decrease in lung metastasis. Considering the fact that nicotinic acid is a dietary constituent related to vit. B_3 (nicotinamide), these results suggest that nicotinic acid could be used as an antimetastatic agent alongwith cytotoxic anticancer drug treatment in the therapy of human cancers.

The metastatic cascade comprises of several successive steps which begins with loss of cell-cell adhesion and ends with metastatic spread to distant organs[1]. Despite the evidences for specificity in metastatic development, metastasis is an inefficient process and may depend to some extent on random survival factors associated with traversing the metastatic cascade. Since, metastasis is a multi step process, each of the step provides a target which when manipulated can results in the arrest of the remaining steps of the cascade[2]. Therefore, literature abounds with experimental evidence where inhibition of one of the key step of the metastatic cascade can arrest metastasis[3]. Adhesion of the metastatic cell to the distant organ is one such key step. Our earlier studies on pentoxifylline, a microfilament depolymerising agent with hemorrheological properties using a B16F10 melanoma model showed that metastatic spread could be arrested successfully[4]. Recently dietary constituents such as vitamins have been recommended as a new strategy for cancer control[5,6]. Nicotinamide has been shown to prevent pellagra and esophageal cancers[5]. It has also been well documented as a blood flow modifier[6]. Several reports indicate the role of nicotinamide as tumor radiosensitizer on account of its ability to improve blood perfusion and thus oxygenation[7,8]. Nicotinic acid, the proteolytic product of nicotinamide has also been shown to have anticancer properties. Nicotinamide brings about cell deformability by unknown mechanism, probably related to actin microfilament depolymerization which in turn decreases cellular adhesion to basement membrane as in the case of pentoxifylline[4,9,10].

In the present study, we have investigated the action of nicotinic acid *in vitro* on adhesion and recovery effect and *in vivo* on lung homing of B16F10 melanoma cells in DBA/2 mice.

MATERIALS AND METHODS

Nicotinic acid (Niacin, Pyridine-3-carboxylic acid), anhydrous Mol. wt. 123.1 (No. N-4126) was obtained from Sigma Chemical Co. MO, U.S.A. Iscove's Modified Dulbecco's Medium (IMDM) was reconstituted from

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powdered media from GIBCO, USA. Heat inactivated Fetal Bovine Serum (Mexican) was used from GIBCO, U.S.A. the complete medium of IMDM with 10% serum was prepared containing penicillin and streptomycin for *in vitro* studies.

Animals:

Six to eight weeks old, inbred DBA/2 pathogen free male and female mice were obtained from the animal house, Cancer Research Institute, Mumbai (India).

Enrichment of B16F10 melanoma from B16F1 melanoma cells:

The B16F1 melanoma cell line was obtained from the National Centre for Cell Science, Pune, India. The cells were maintained in IMDM with 10% fetal calf serum. The cells were detached with saline-EDTA in phosphate buffered saline (PBS) and the cell count was adjusted to $1x10^6$ cells/ml in PBS. The cells were inoculated by intravenous route in mice and the mice were sacrificed after 20 days. Pulmonary nodules from lung were picked up aseptically and were cultured *in vitro*. This process of alternate *in vitro* and *in vivo* cultivation was continued till ten passages. Thus enriched population of B16F10 melanoma cells were obtained after ten passages as shown by Fidler[8].

***In vitro* adhesion assay on treatment with nicotinic acid:**

B16F10 melanoma cells were harvested using saline-EDTA. The cells were suspended in plain IMDM and washed free of saline EDTA. Cell count was adjusted to $1x10^4$ per petri dish. The volume of nicotinic acid was adjusted to final concentration 100 μM. The petri dishes were incubated at 37° for 2 h. The cells adhered to surface of plastic surface were carefully fixed with methanol and carefully washed with PBS to remove traces of methanol without losing adhered cells. Cells were stained with haematoxylin and adhered cells were counted under microscope.

Effect of *in vitro* treatment of B16F10 cells with nicotinic acid on the lung homing activity:

B16F10 cells were harvest from culture plates using saline EDTA in PBS. The viability was assessed by Trypan blue exclusion. The cell suspension was adjusted to $1x10^6$ cells in IMDM and nicotinic acid was added to cell suspension to make final concentration to 100 μM. The cells were incubated for 2h and were washed twice with phosphate buffered saline (PBS). The treated cells ($1x10^5$ cells in 0.1 ml of PBS) were implanted in DBA/2 mice by intravenous route. The animals were sacrificed on day 20 and lung nodules on the surface of lung were counted after fixing in Bouin's fluid.

The washed cells which were exposed to NA for 2 h were resuspended in IMDM and recovery study was carried out till 4 h. The animals were inoculated with tumor cells as explained above. The animals were sacrificed and lung colonies were counted as described above[4].

***In vivo* treatment with nicotinic acid on day 1,5,9, day 1 through 9 and on day 1 and 2 and its effect on pulmonary lung metastasis:**

a. The B16F10 cells were harvested using saline EDTA and were suspended in PBS. The cells were injected by intravenous (i.v.) route in DBA/2 mice as described above. The NA treatment of dose (100 mg/kg) was carried out by intraperitoneal (i.p.) route on day 1,5,9 schedule and on day 1 through 9.

b. The higher dose (150 mg/kg) was tested for 1 through 9 schedule. Similarly NA (100 mg/kg) was used for i.v. route administration[4].

The lungs fixed in Bouin's fluid from respective experimental groups were processed for histological examinations. To process for light microscopic studies, 5μm thick paraffin sections were cut and stained with haematoxylin and eosin.

Statistical Analysis:

Statistical analysis was performed by Student's 't' test at level of significance $p \leq 0.01$.

RESULTS AND DISCISSION

An important objective in cancer research is to investigate new ways to control metastases. Recent developments in molecular biology have provided several candidates as targets of therapy such as oncogenes[11], the matrix metalloproteases[12] and adhesion molecules[13]. We have shown earlier that pentoxifylline, a xanthine compound with rheological properties acts via depolymerisation of actin microfilament and brings about significant decrease in metastasis of B16F10 in to lung, in an experimental metastatic model[4]. Nicotinic acid has vasodialatory action and may function similar to pentoxifylline. Grassetti[14] reported that sulphur contain-

Table 1 - Effect of nicotinic acid (100 μM) on adhesion of B16F10 melanoma cells *in vitro*

	Cells plated per plate	Cell Number counted/field [Mean±S.E, n=3]	Percent Cells adhered/10^4 cells with respect to control (100 %)
Untreated control	$1x10^4$	110.7±21.20	—
Treatment with nicotinic acid 100 μM for two hours.	$1x10^4$	116.3±5.26[a]	5.05

a = N.S. (non-significant)

Table 2 - Effect of *in vitro* treatment of B16F10 melanoma cells with nicotinic acid to show percent inhibition in lung metastasis

Group	No. of mice bearing lung colonies/no.of mice tested	No. of lung colonies per $1x10^5$ cells [Mean±S,E, n=3]	Percent decrease in lung nodules with respect to control (100%)
Zero h control.	3/3	81.66±6.58[a]	—
Untreated control (2 h exposure)	3/3	78.00±3.75	4.49
100 μM Nicotinic acid treated cells. (2 h exposure)	3/3	16.07±1.22*[b]	80.41*[b]
Recovery experiment			
Untreated control after 2 hrs. (total 4 h)	3/3	80.66±0.87	—
Nicotinic acid treated recovery after 4 h (total 4 h)	3/3	11.66±2.49*	85.79*

a = Values expressed as Mean±S.E., *b = P<0.01

ing analogs of nicotinamide could retard the pulmonary metastatic nodules by more than five fold in Lewis Lung carcinoma tumor model. Therefore, we were prompted to study the effect of nicotinic acid on B16F10 melanoma metastasis in to lung. Meanwhile, there are many reports in which nicotinic acid, nicotinamide and related aromatic amines have been shown to possess anticarcinogenic property[15]. Defficiency of NA could lead to reduced NAD levels responsible for causing carcinogenesis[16]. Deamidation of nicotinamide to nicotinic acid could stimulate the formation of prostaglandins, causing alteration in lipid metabolism[17].

The results of the adhesion assay demonstrated that nicotinic acid treatment *in vitro* does not affect adhesion of B16F10 melanoma cells to tissue culture plastic surface (Table 1). The concentration used was 100 μM for 2 h, which was non toxic. However, when these cells were transplanted in to mice, intravenously, they failed to form lung metastatic nodules as efficiently in comparison to untreated control. Recovery experiments did not show any reversal (Table 2). Results reported in this paper suggest that the effect of NA on B16F10 is not through cell adhesion mechanism as was shown by us for pentoxyfylline[4]. When nicotinic acid was injected

Table 3 - Effect of nicotinic acid treatment (100 mg/kg) on day 1, 5, 9 and day 1 through day 9 by intraperitonial route administered on lung metastasis

Group	No. of mice bearing lung colonies/no.of mice tested	No. of lung colonies/1 x 10^5 cells[a] [Mean±S.E, n=3]	Percent decrease in lung nodule with respect to control (100%)
Untreated control	4/4	80.00±4.17[a]	—
Nicotinic acid treatment on day 1, 5, 9 schedule.	4/4	65.75±2.51(N.S.)[b]	17.81[b]
Nicotinic acid treatment on day 1 through day 9 schedule	4/4	79.00±6.57(N.S.)[b]	1.25[b]

a = Values expressed as Mean±S.E.

b = N.S. (Non-significant)

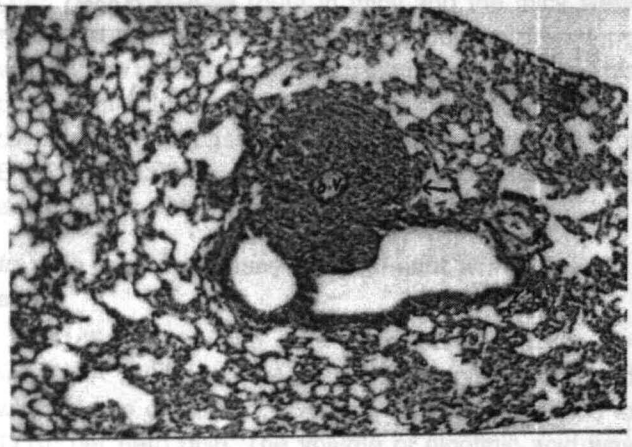

Fig. 1 : Section of lung showing diffuse microtumor with pigment (arrow) around the blood vessel (b.v.) x 78

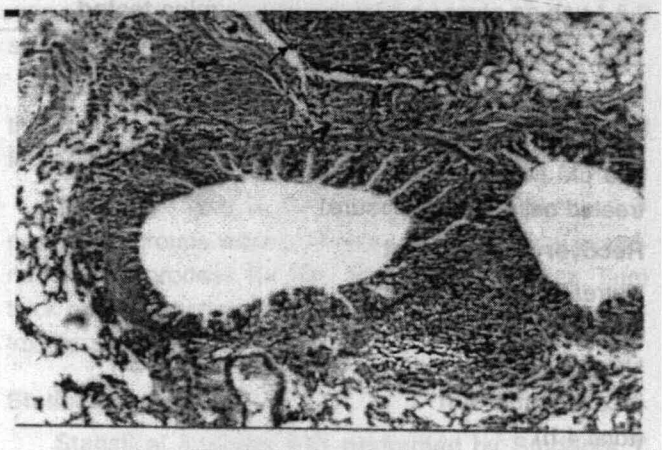

Fig. 2 : Section of lung shows number of small (arrow-head) and large (arrow) tumor islands with pigment x 100

intraperitoneally after the tumor cells were transplanted into mice, in 1, 5, 9 day scheduie, no significant decrease in lung colonies was observed. This suggest that prior exposure of B16F10 cells to nicotinic acid *in vitro* is needed for colony inhibition. This could be due to insufficient level of nicotinic acid in blood when the intraperitoneal route is used (table 3). However, tissue section of lung demonstrated changes in lung histology and ultimately in lung metastatic pattern. When compared to untreated control (Fig. 1), treatment with NA on both the schedules showed increased number of small and large tumor islands with pigment (Fig. 2). However, the only difference was that the infiltration of tumor was found in the entire wall of the large blood vessel but not the endothelium on day 1,5,9 treatment (Fig. 3), while infiltration of tumor could be demonstrated in the bronchiole wall but not the epithelial layer of the bronchiole (Fig. 4.). Treatment with NA at a dose of 150 mg/kg by i.p. route showed similar histological features of lung as described above (Table 3). Treatment with 100 mg/kg by the i.v. route resulted in small tumour islands near the bronchiolar periphery which penetrated the musculature of blood vessle. We found inhibition of pulmonary metastasis in i.v. administration (Table 4) when compared with i.p.

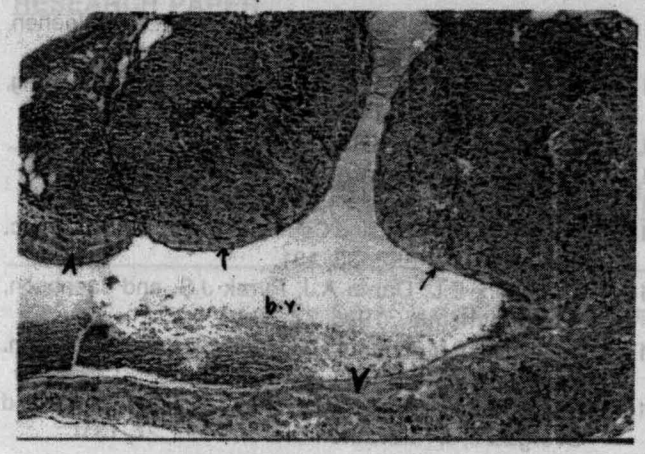

Fig. 3 : Tumor island infiltrated entire wall (arrowhead) of the large blood vessel (b.v.) but not the endothelium (arrow) x 78

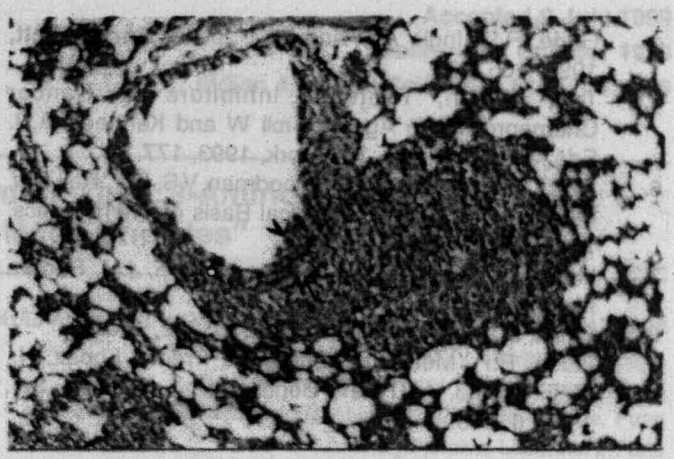

Fig. 4 : Tumor cell infiltration in the bronchiole wall (arrow) but not the epithelial layer (arrowhead) of the bronchiole. x 100

Table 4 - Effect of nicotinic acid treatment on B16F10 lung metastasis

Group	No. of mice bearing lung colonies/no.of mice tested	No. of lung colonies per 1 x 10^5 cells [Mean±S.E, n=3]	Percent decrease in lung nodules with respect to control (100%)
Untreated Control	4/4	150.25±4.72	—
Nicotinic acid (150 mg/kg) treatment by intraperitoneal route on day 1 through 9	4/4	17±12.13	88.69*[a]
Nicotinic acid (100 mg/kg) treatment by intravenous route on day 1 and 2 schedule	4/4	49.75±13.72 (N.S.)	66.89

*a=P<0.01

administration (Table 5). These observations suggest that availability of nicotinic acid in the i.v. route is higher as compared to the i.p. route, which requires absorption through the peritoneal wall. It has been reported that under similar conditions nicotinamide showed plasma half life of 2-3 h. in mouse and peak plasma level within 20 min[18]. Nicotinic acid is a product of deamidation of nicotinamide and has similar kinetics as nicotinamide, resulting in prolonged action on B16F10 cells.

This study suggests the use of NA as an antimetastatic agent due to the fact that it is a dietary constituent. A better understanding of a metabolism of NA in decreasing pulmonary homing of B16F10 melanoma is of particular importance in metastasis. Further experiments will be required to understand the mechanism of lung homing inhibition of B16F10 by nicotinamide.

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