ANTI-INVASIVE AND ANTIMETASTATIC ACTION OF LYSINE OXIDASE FROM *Trichoderma* SP. IN VITRO AND IN VIVO

S. Kh. Khaduev,[1] V. Yu. Umanskii,[3] V. S. Vesa,[4] K. Shinkai,[2] H. Akedo,[2] and T. T. Berezov[1]

UDC 615.277.3.015.4.07

KEY WORDS: lysine oxidase; metastasization; dissemination

The screening of new preparations (especially of natural origin) with antimetastatic activity is a very urgent problem in tumor chemotherapy. The study of the possible mechanisms of invasion and metastasis is a priority trend in current oncology. This trend of research is not only important from the theoretical point of view, by broadening our ideas on the nature of malignant growth, but it also is of great practical importance, for the direct cause of death of most patients is not the primary tumor, but the results of metastasis of the so-called "mother" cancer cells in the affected organism.

The main obstacle in the way of an explanation of the nature of a complex process such as metastasis the absence of any reliable and any adequate models which would enable a detailed study to be made of tumor dissemination.

In 1986 a group of Japanese research workers developed a model which could be used to quantify and analyze individual steps of the first stage of metastasis, namely invasion, by experiments in vitro [6]. Cells of a suspension culture of rat ascites hepatoma (AH-130) were used in the suggested model. During growth of the tumor in the peritoneal cavity of rats, the AH-130 cells adhere to the peritoneum, penetrate into the mesentery, and appear in the blood stream [15].

On implantation of AH-130 cells in the peritoneal cavity, they initially interact with mesothelial cells lining the peritoneal cavity. This interaction is evidently the initial stage of a complex process of invasion of the submesothelial tissue by AH-130 cells, followed by intravasation.

During seeding of AH-130 cells on a monolayer of cells obtained from the rat mesentery, invasive growth of tumor cells can be assessed quantitatively beneath a monolayer of mesothelial cells in culture, and in this case a high degree of correlation with the results of invasion of the rat peritoneal cavity by AH-130 cells is maintained.

In our previous investigations [4, 5] the enzyme L-lysine oxidase (EC 1.4.3.12) was found to have an antitumor action in vitro and in vivo.

The aim of this investigation was to study the effect of the new antitumor enzyme preparation L-lysine α-oxidase (LO) on invasion and metastasis.

EXPERIMENTAL METHOD

A preparation of the enzyme LO from the fungus *Trichoderma* sp. with specific activity of 29 IU/mg, obtained by methods developed in the Department of Biochemistry, Medical Faculty, Patrice Lumumba Peoples' Friendship University

[1]Department of Biochemistry, Medical Faculty, Patrice Lumumba Peoples' Friendship University, Moscow.
[2]Department of Biochemistry of Cancer, Center for Adult Pathology, Osaka, Japan.
[3]Department of Pathogenetic Mechanisms of Metastasis, R. E. Kavetskii Institute for Problems in Oncology, Academy of Sciences of the Ukrainian SSR, Kiev.
[4]All-Union Research Institute of Applied Enzymology, Vilnius, Lithuania.

Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 112, No. 10, pp. 419-422, October, 1991. Original article submitted April 24, 1991.

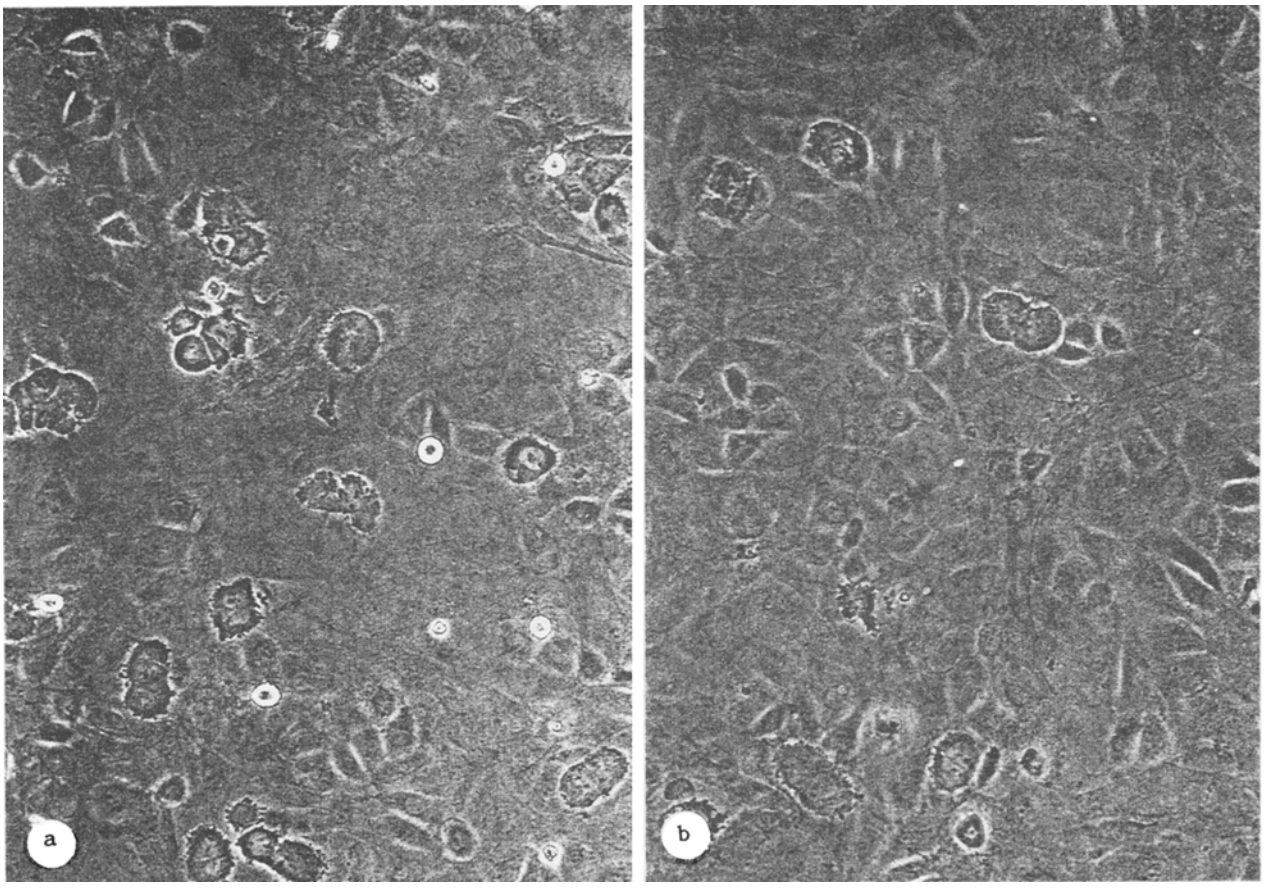

Fig. 1. In vitro invasion of MMI cells through the M layer. Phase-contrast microscopy; 110×. Penetrating single units and colonies marked. MMI cells after preincubation with 2.5 mIU/ml (a) and without preincubation (b) of L-lysine-α-oxidase.

and the All-Union Research Institute of Applied Enzmology, homogeneous as shown by gel-electrophoresis and ultracentrifugation, was used.

The study of the effect of LO on invasion of cells of clone MMI was studied. AH-130 cells were obtained by the method described previously [6]. A subline of LC-AH cells was obtained by serial passages of cells of the maternal AH-130 culture. Clone MMI, which is highly invasive in vitro and in vivo [11], was isolated from cells of LC-AH subline by seeding in soft agar [17].

Invasiveness of cells of the MMI clone was assessed by the method of Akedo and co-workers [1,6]. $2 \cdot 10^5$ MMI cells were seeded on a dish on a monolayer of cells obtained from the mesentery (the M layer) and cultured for 24 h on Eagle's medium with 10% embryonic serum.

The number of penetrating units of MMI cells and the number of colonies formed under the M layer were counted in 60 different visual fields (each measuring 1.13 mm^2) by means of an "Olympus" CK-2 (Japan) phase-contrast microscope (magnification: 110 and 220).

Invasiveness of the MMI cells in vitro was expressed as the number of penetrating single cells and colonies per square centimeter. Since the tumor cells were used in the form of a suspension culture and since each colony was formed from single cells which had penetrated, the total number of penetrating cells and colonies correlated with the number of penetrating tumor cells.

To assess the effect of LO on invasion in vitro, cells of the MMI clone were incubated for 48 h in the presence of different concentrations of LO and of 122 IU/ml catalase. The tumor cells were then washed twice with culture medium without LO and catalase, after which the treated tumor cells ($2 \cdot 10^5$ cells per dish) were seeded on the M layer.

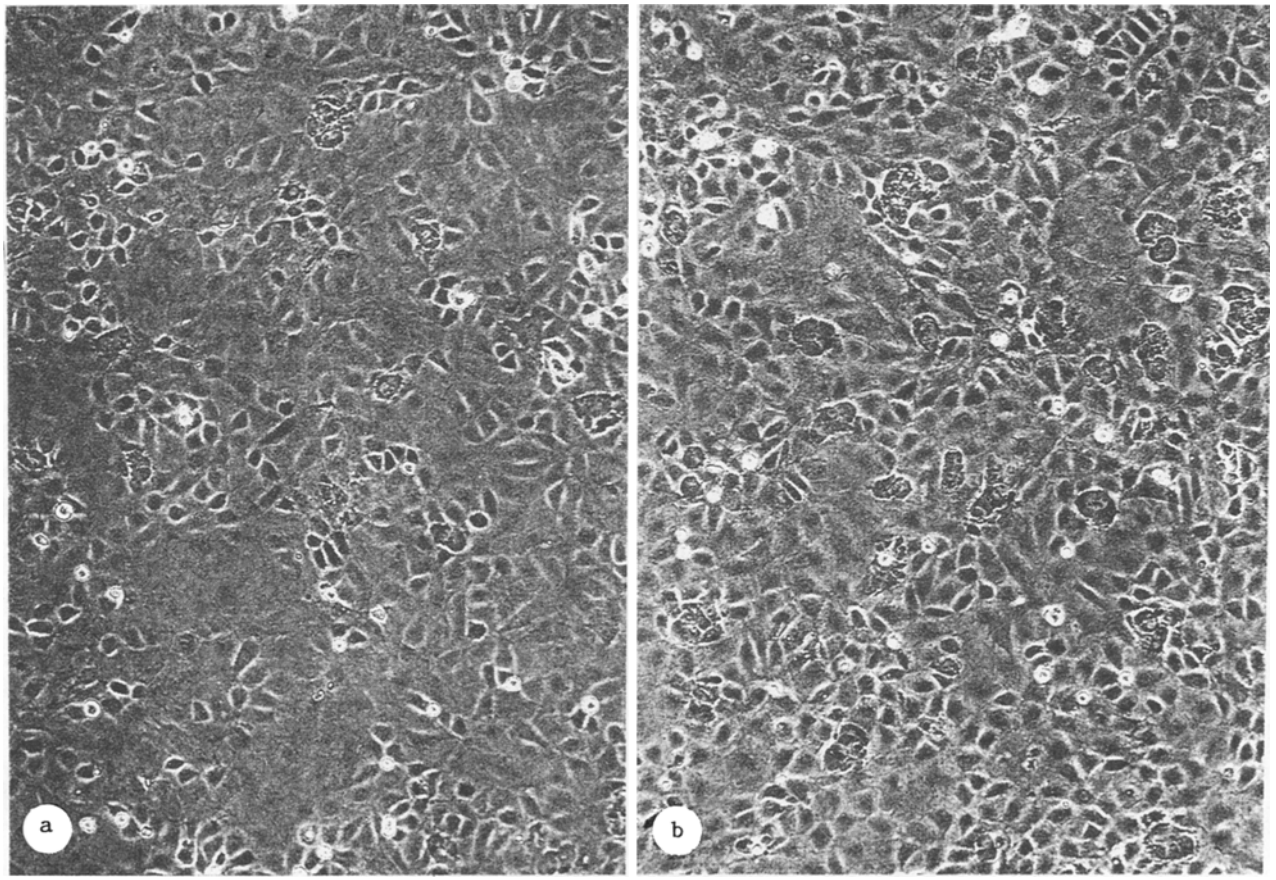

Fig. 2. In vitro invasion of MMI cells through M layer. Phase-contrast microscopy; 220×. Legend as to Fig. 1.

Antimetastatic Action of LO. Experiments were carried out on C57BL/6 mice weighing 20-25 g. Cells of Lewis lung carcinoma (3LL) were injected in 0.05 ml of physiological saline in a dose of $2 \cdot 10^5$ into the footpad. The limb with the tumor was amputated at the knee under hexobarbital anesthesia and with sterile precautions. The volume and number of metastases in the lungs of the mice were determined by the usual method [10]. The enzyme was injected intravenously in 0.2 ml of physiological saline twice a week, six times altogether, starting from the 7th day after transplantation of the tumor cells in doses of 10, 20, and 50 IU/kg.

Alveolar macrophages were isolated by the method in [8]. Adenosine deaminase (AD) and 5′-nucleotidase (5′-N) activity was determined in the lysate with the aid of ^{14}C-adenosine, ^{14}C-AMP, and ascending paper chromatography [3]. Activity of AD was expressed in nanomoles inosine and hypoxanthine per minute Per 10^8 cells; 5′-N activity was determined in nanomoles adenosine, inosine, and hypoxanthine per minute per 10^8 cells.

EXPERIMENTAL RESULTS

Anti-Invasive Effect in Vitro. Table 1 shows that LO inhibits both growth and invasiveness of highly invasive cells of clone MMI. Maximal inhibition was observed after preincubation of the tumor cells with LO in a concentration of 2.5 mIU/ml; preincubation reduced cell growth and invasiveness by 1.9 and 1.6 times, respectively compared with the control. The results are given in Figs. 1 and 2.

Since the invasiveness of the MMI cells was estimated by counting the number of penetrating single units and colonies, inhibition of invasiveness is not simply the result of suppression of cell growth, but it is evidently also the result of direct inhibition of the invasion process as such. Lowering the lysine level in the culture medium probably attenuates the invasiveness of cells of the MMI clone. Preincubation with 0.31 and 1.5 mIU/ml LO also considerably suppressed growth of

TABLE 1. Effect of LO on Invasion of Cells of Clone MMI

L-lysine-α-oxidase, mIU/ml	Catalase, 112 IU/ml	Cell growth (10^5 cells/mho)	Invasiveness of penetrating single cells and colonies, per cm^2
—	—	2,49[a]	1490±53
—	+	2,43[a]	1293±111
0,078	+	2,63[a]	1225±52
0,31	+	1,62[a]	1546±136
1,5	+	1,49[a]	1345±191
2,5	+	1,31[a]	810±95
3,4	+	1,49[b]	n.d.
6,8	+	1,31[b]	n.d.

Legend. a) Cell growth after 48 h, b) after 24 h; n.d.) not determined; cells significantly damaged even after 24 h of preincubation, and their invasiveness was not assessed.

TABLE 2. Effect of LO on Length of Survival of Mice Undergoing Removal of the Primary Tumor (Carcinoma 3LL)

Experimental conditions	Time after transplantation of tumor, days				
	30-	60-	100-	150	200-
Control (without treatment)	12/25	0/25	0/25	0/25	0/25
LO	23/25	17/25	14/25	15/25	12/25

Legend. Numerator gives number of living mice; denominator gives total number of mice in group.

cells of the MMI clone, whereas the invasiveness of the latter was not significantly altered; cell growth may perhaps be more sensitive to a fall of the lysine level than invasiveness.

According to data obtained by Kusakabe and co-workers [9], one product of the reaction of oxidative deamination of lysine, catalyzed by L-lysine-α-oxidase, namely hydrogen peroxide, also effectively inhibits growth of L5178Y lymphatic leukemia cells.

Japanese investigators explain the cytostatic effect of LO on the L5178Y model by a combination of two factors: enzymic lowering of the lysine level in the growth medium and the influence of the H_2O_2 formed.

When studying the anti-invasive effect of LO on the MMI clone cell model we deliberately excluded any effect of H_2O_2 on the tumor cells by adding the enzyme catalase to the medium [16]. On the basis of the results, the enzyme-induced lowering of the lysine level in the medium can be interpreted as the main factor enabling LO to exhibit its anti-invasive effect in vitro.

Antimetastatic action in vivo. The results of determination of the anti-invasive effect in vitro logically supplement our results relative to the antimetastatic action of LO in vivo [2, 7]. We found that intravenous injection of LO into mice with Lewis lung carcinoma (3LL) caused a marked decrease in the number and size of the metastases in the lungs compared with these parameters in untreated animals.

The marked biological action of LO was accompanied by increased activity of AD (EC 3.5.4.4) and a decrease in the activity of 5′-H (EC 3.1.3.5) in alveolar macrophages at different times of observation (on the 15th and 30th days after transplantation of the tumor), evidence of increased functional activity of the macrophages. These enzymes of purine metabolism (AD and 5′-H), which regulate the intracellular adenosine concentration, play an important role in the physiology of macrophages [13, 18]. The changes found in AD and 5′-H activity create the conditions for a fall of the concentration of adenosine — an inhibitor of macrophagal function [12].

However, it remains unclear whether LO acts directly on the macrophagal membrane, as is indicated by the fall of 5′-H activity on the surface of the macrophages [13], or whether trigger mechanisms, whose role may be played by various modulators of cellular activity, are involved in this process.

Changes in the concentrations of polyamines (a decrease in the concentration of putrescine and the putrescine/spermidine ratio) were observed in the erythrocytes of these same mice with 3LL and treated with LO, and the levels of these substances in the serum and blood cells can be regarded as a marker of activity of the tumor process and of the efficacy of antimetastatic therapy [1, 14].

It must emphasized in particular that treatment with LO (50 IU/kg) significantly increases the length of survival of mice after removal of the primary 3LL carcinoma (Table 2).

The use of LO 200 days after transplantation of the tumor led to survival of 50% of the animals in the experimental group.

To sum up the facts described above, LO, an enzyme of natural origin, depresses antimetastatic activity. This fact encourages the hope that its therapeutic use may be promising not only at the stage of the primary tumor, but also in the case of advanced malignant disease, i.e., in the stage of metastasis.

LITERATURE CITED

1. N. K. Berdinskikh and S. P. Zaletok, Polyamines and Tumor Growth [in Russian], Kiev (1987).
2. T. T. Berezov, V. Yu. Umanskii, S. Kh. Khaduev, and K. N. Belitskii, Current Problems in Biochemical and Immunological Diagnosis of Malignant Neoplasms [in Russian], Moscow (1989), pp. 106-107.
3. N. P. Dmitrenko, S. V. Komissarenko, and V. Yu. Umanskii, Dokl. Akad. Nauk SSSR, **251**, No. 1, 251 (1980).
4. S. Kh. Khaduev, O. S. Zhukova, and Ya. V. Dobrynin, Byull. Éksp. Biol. Med., No. 4, 458 (1987).
5. S. Kh. Khaduev, T. Yu. Glazkova, and V. S. Vesa, Byull. Éksp. Biol. Med.. No. 10, 476 (1989).
6. H. Akedo, K. Shinkai, M. Mukai, et al., Cancer Res., **46**, 2416 (1986).
7. T. T. Berezov, S. Kh. Khaduev, and V. Yu. Umanskii (V. Yu. Umansky), FEBS Meeting, 19th: Abstract Book, Rome (1989), p. TH232.
8. I. J. FIdler, A. Ras, W. S. Fogler, et al., Cancer Res., **40**, 4460 (1980).
9. H. Kusakabe, K. Kodama, A. Kuninaka, et al., Agric. Biol. Chem., **44**, No. 2, 387 (1980).
10. B. Luche, C. Breedis, and Z.P. Woo, Cancer Res., **12**, 734 (1952).
11. M. Mukai, K. Shinkai, et al., Cancer Res., **47**, 2167 (1987).
12. A. Ohtani, Y. Kumuzawa, H. Fujisawa, et al., J. Reticuloend. Soc., **32**, 189 (1982).
13. C. E. Pietrangeli, E. Scamene, P. Edelson, et al., Infect. Immun., **32**, 1206 (1981).
14. D. H. Russell, CRC Crit. Rev. Clin. Lab. Sci., **18**, 261 (1983).
15. H. Sato, Acta Pathol. Jpn., 9, Suppl. 685 (1959).
16. K. Shinkai, M. Mukai, H. Akedo, et al., Cancer Lett., **32**, 7 (1986).
17. K. Shinkai, M. Mukai, T. Horai, et al., Jpn. Cancer Res., **80**, 716 (1989).
18. K. Yagawa and J. Okamura, Infect. Immun., **32**, 349 (1981).