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ARTICLE

Angiogenesis – a Putative New Approach in Glutamine Related Therapy

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Angiogenesis or the generation of new blood vessels, is an important factor regarding the growth of a tumor. Hence, it becomes a necessary parameter of any kind in therapeutic studies. Glutamine is an essential nutrient of tumor tissue and glutamine related therapy involves clearance of circulatory glutamine by glutaminase. So, whether this enzyme has any effect on angiogenesis of a tumor or not becomes an obvious question. To address this question, this study has been carried out with different murine tumor models.The results indicate that purified glutaminase reduces tumor volume as well as restricts the generation of new blood vessels. Glutaminase is effective in the case of solid as well as ascites tumor models. In the case of induced cancer, the host exhibits delayed onset of neoplasia following enzyme treatment and tumor host interactions determine the intensity of the neovascularisation process. Therefore, it can be concluded that this enzyme might be an effective agent against cancer metastasis. (Pathology Oncology Research Vol 5, No 4, 309–314, 1999)

Keywords: angiogenesis, glutaminase, murine tumor

Introduction

Angiogenesis is a crucial step in tumor growth and progression. Its quantitation by microvessel counting is of prognostic value in several types of malignancies.[1] Not only in the solid tumors but also in ascitic tumors, the generation of new blood capillaries is an important feature.

Solid tumors are composed of continuously proliferating cells and they need increasing quantities of oxygen and nutrients. Once they reach a diameter of 1–2 mm, the diffusion of oxygen and nutritive substances from the periphery are no longer sufficient and they become dependent on the generation of new blood vessels to maintain their growth.[2,3.]

In the ascites type of malignancy, tumor cells grow as a cell suspension in peritoneal fluid in the apparent absence of vasculature.[4] However, they share similar vasculature characteristics with solid tumor, such as: (a) hyperpermeability of blood vessels (b) extravasation of plasma proteins like fibrinogen (c) clotting of extravasated fibrinogen to form crosslinked fibrin deposits in the peritoneal lining.[5]

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The newly formed blood vessels not only feed the growing tumor cells but also provide an easy way for neoplastic cell travel through the circulation and finally metastasize.[6,7,8] Greenblat and Shubik[9] first demonstrated that tumors produce some diffusible factors which induce the growth of blood vessels. Since then, scientists have shown great interest in several angiogenic and antiangiogenic factors. It has already been known that phosphate activated glutaminase possess antineoplastic properties and in this context bacterial glutaminase has been used clinically.[10] In experimental models, glutaminase purified from mammalian source has also been tried.[11]

Therefore, in our present work, an attempt has been made to observe the effect of purified glutaminase treatment on the generation of new capillary sprouts in different murine tumor models and to observe whether the purified glutaminase plays any role in neovascularisation, or not.

Materials and Methods

Experimental animal models

Six-to-seven-week-old male and female Swiss albino mice of 20–22 gm. body weight were used in these experiments. Ehrlich ascites carcinoma (EAC) and Sarcoma-180 (S-180) were maintained by serial subcutaneous transplantation ($2x10^5$ cells/mouse) for solid tumors. 20-

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Table 1. **Effect of purified glutaminase on growth of murine solid tumor and their neovascularisation**

Group	*Tumor volume (cm^3)*			*Tumor growth inhibition % in treated group*			*No. of blood vessels*			
	day-15	*day-20*	*day-25*	*day-15*	*day-20*	*day-25*	*day-15*	*day-20*	*day-25*	*% of ILS*
MCH	0.36 ± 0.16	0.4 ± 0.16	0.57 ± 0.02				8.0 ± 0.5	7.0 ± 0.8	7.2 ± 0.5	
MCH+glutaminase enzyme	0.25 ± 0.025*	0.15 ± 0.03*	0.17 ± 0.03*	30.6	62.5	70.2	6.0 ± 0.3*	5.2 ± 0.2*	5.0 ± 0.6*	72.7
EAC	0.3 ± 0.05	0.49 ± 0.06	0.5 ± 0.02				7.0 ± 1.1	7.7 ± 0.5	8.0 ± 0.3	
EAC+ glutaminase enzyme	0.23 ± 0.05*	0.24 ± 0.03*	0.22 ± 0.03*	23.3	51.02	56	7.2 ± 0.6*	6.5 ± 0.3*	6.5 ± 0.5*	65.5
S-180	0.4 ± 0.02	0.5 ± 0.02	0.49 ± 0.01				7.5 ± 0.4	6.25 ± 0.05	6.3 ± 0.3	
S-180+ glutaminase enzyme	0.29 ± 0.02*	0.21 ± 0.03*	0.2 ± 0.01*	27.5	58	59.2	6.9 ± 0.1*	6.0 ± 0.8*	6.1 ± 0.5*	66.5

*As compared with respective control $p<0.001$
MCH = Methylcholanthrene , EAC = Ehrlich ascites carcinoma , S-180 = Sarcoma-180, (mean ± SD, n=6)

methylcholanthrene-induced solid tumors [Freund's incomplete adjuvant (Sigma) was used as carrier substance] were also formed by subcutaneous administration of 2 mg of MCH/mouse in the groin region. Ehrlich ascites carcinoma (EAC) were also maintained by serial i. p. transplantation (10^5 cells/mouse) for ascites tumor formation.

Cervical carcinoma was induced by 20-methylcholanthrene (Sigma, USA). Murphy's string method was followed for the induction of cervical cancer. Sterile cotton thread impregnated with bees wax and methylcholanthrene (in a ratio of 1:3 carcinogen:beeswax) was inserted into the canal of the uterine cervix by means of laparotomy under mild ether anaesthesia. This procedure has been undertaken because it ensured continuous exposure of the target tissue to the chemical carcinogen. The thread remained in the cervical canal until the end of the study period.[12,13]

Six mice from each group were sacrificed at a time to study the tumor burden and number of blood vessels. Mice bearing cervical carcinoma, dysplasia and carcinoma in situ were evaluated on the basis of the appearance of abnormal cervical epithelial cells in the smear and that of malignancy by a positive smear. Vagino-cervical smears were taken and fixed in an ether-alcohol solution, and Papanicolaou's (PAP) stain was carried out for the cytological study. Mice were sacrificed routinely and cervical tissues were fixed in Bouin's fluid. Paraffin sections were cut serially and stained with Haematoxylin-Eosin to assess the influence of purified glutaminase.

For solid tumors, tumor volume was measured with the help of a slide caliper and was calculated according to the formula: tumor volume (cm^3) = $\pi/6 \times D_1 \times D_2 \times D_3$, where D_1 is length, D_2 is breadth and D_3 is height of the tumor.

For ascitic tumors, ascitic fluid including tumor cells was aspirated, washed thoroughly with normal saline, and viable cells were counted in a hemocytometer by the trypan blue dye exclusion method.

Quantification of new blood vessels

Tumor bearing mice of both enzyme treated and untreated groups of each tumor type were sacrificed after 5 days, 10 days and 15 days of enzyme treatment. Tumors were first located, and the skin around it was removed. Angiogenesis was quantified by counting the capillaries oriented towards the tumor under a dissecting microscope, according to the method of Danielsen et al.[17] To map the microvasculature of the tissues lining the peritoneal cavity, EAC ascitic tumor bearing mice were injected with a trypan blue dye via their tail vein. (Once the dye reaches the circulation, it automatically maps the microvasculature of the peritoneal lining.) Then, the peritoneal lining was dissected free, fixed in formalin, dehydrated in a sequence of ethyl alcohols and cleared in xylol, for examination under the Wild microscope. The uterus was removed from cervical carcinoma

Table 2. **Effect of purified glutaminase (S-180) on Ehrlich ascites carcinoma growth after 10 days of treatment**

Group	*Tumor cell count ($x10^6$)*	*% of tumor growth inhibition*	*% of ILS*
EAC	375.13 ± 3.2		
EAC+glutaminase enzyme	18.6 ± 1.2*	95	145.6

As compared with respective control $p<0.001$, (mean ± SD, n=6)

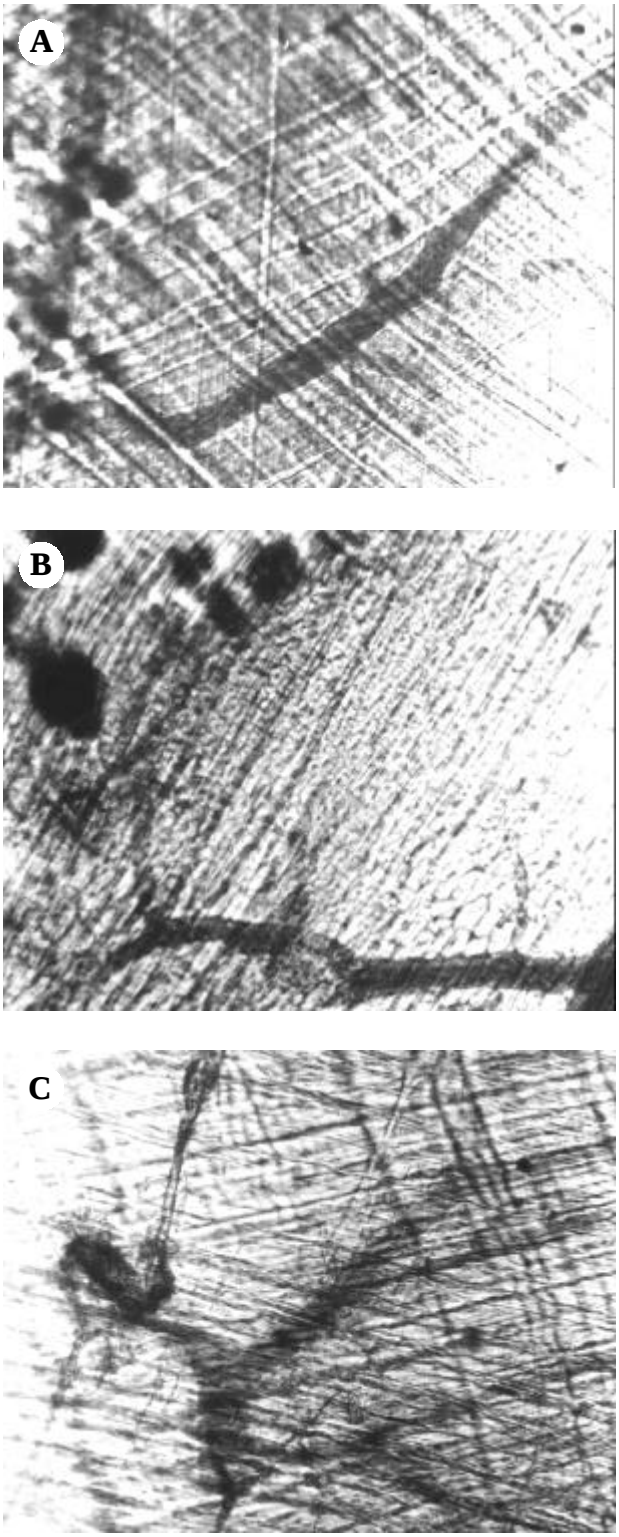

Figure 1. (A) Peritoneal membrane vasculature of normal Swiss albino mice (Trypan blue perfused, x125); (B) Peritoneal membrane vasculature at 10 days in Ehrlich Ascites Carcinoma bearing mice (Trypan blue perfused, x125); (C) Peritoneal membrane vasculature at 10 days in EAC + enzyme treated mice (Trypan blue perfused, x125).

bearing mice. It was then fixed and processed accordingly for light microscopic sections to evaluate blood vessel orientation during cervical carcinoma progression.

Isolation and purification of enzyme

Phosphate dependent glutaminase was purified from highly malignant S-180 cells, according to the method of Quesada et al[14] with some modification. First, tumor cells were suspended from the peritoneal fluid by centrifuging at 2000 rpm. Then, mitochondria were isolated from the tumor cells suspended in media A (35 mM sucrose, 5 mM HEPES, 1 mM EDTA). Cells were then sonicated for 3 min. with 30 sec. pulse and 30 sec. intervals, then centrifuged at 28,000 rpm for 1 hr followed by 40% ammonium sulphate precipitation and dialysis. The samples were then applied to DEAE sepharose columns and finally applied to affinity columns of L-glutamine. An appropriate protein band was visualized, and the glutaminase enzyme was confirmed using anti- rat kidney antibodies (kindly supplied by Dr. N P Curthoys).

Enzyme and protein assay

The enzyme assay was done by endpoint determination of glutamate. Glutamate production was assayed at 340 nm in a spectrophotometer by monitoring NADH formation in the GLDH reaction, according to the method of Lund et al.[15] The protein was measured at 660 nm according to Lowry et al.[16]

Glutamine assay

Glutamine levels were mainly studied in liver and plasma. First, they were deproteinized with 20% perchloric acid, then neutralized with 10% potassium hydroxide, and finally centrifuged and the supernatant was taken for assay. Glutamine was assayed according to Lund's method.[15]

Treatment schedule

The enzyme was injected intraperitoneally at a dose of 1 unit/day/mice for 15 consecutive days into solid tumor bearing mice.The injection regime was started 10 days after tumor transplantation. In thecase of ascites tumor bearing mice (EAC), intraperitoneal injection of enzyme was given 24 hrs. after tumor transplantation. The enzyme was given intraperitoneally for 30 days to the methylcholanthrene- induced cervical carcinoma bearing mice. Control animals received 0.2 ml PBS only.

Results

Table 1 shows the effect of glutaminase on the growth of murine solid tumors and their neovascularisation. Tumor volume showed a marked decrease in all enzyme treated

Table 3. **Tissue glutamine level in EAC (ascites) tumor bearing mice before and after purified glutaminase therapy**

	In liver (mM/g tissue)					*In plasma (mM/ml blood)*				
	0-day	*1-day*	*5-day*	*10-day*	*15-day*	*0-day*	*1-day*	*5-day*	*10-day*	*15-day*
Cont.	0.9 ± 0.06	4.32 ± 0.05	1.08 ± 0.02	2.73 ± 0.06	4.9 ± 0.08	0.26 ± 0.01	0.14 ± 0.05	0.35 ± 0.05	0.31 ± 0.06	0.28 ± 0.07
Exptl.		4.32 ± 0.05*	2.7 ± 0.04*	3.96 ± 0.08*	4.6 ± 0.03*	0.14 ± 0.09*		0.29 ± 0.05*	0.29 ± 0.07*	0.29 ± 0.04*

* As compared with respective control p< 0.001; Cont = Control; Exptl = treated; (mean ± SD, n=6)

groups with respect to their control groups. Among the enzyme treated groups, the methylcholanthrene-induced tumor models showed the highest tumor growth inhibition (70.2%)as well as the highest % of increased life span (72.7%) followed by tumor growth inhibition and % of increased life span in S-180 and EAC tumor models (59.2% and 66.5%, 56% and 65.5%, respectively). Like the tumor volume the number of blood vessels also showed a decrease among all the treated groups when compared with their respective untreated groups. However, no distinct relation was observed here between tumor volume and new blood vessel generation.

Table 2 shows the effect of purified glutaminase on EAC (ascites) bearing mice. Here, tumor cell counts show a significant decrease in treated group with respect to the control group. Tumor growth showed marked inhibition (95%) with an increase in survival time (145.6% of ILS) after 10 days of enzyme treatment.

Figure 1 (A–C) gave the en face macroscopic view of trypan blue perfused peritoneal membrane vasculature. The decreased number of perfused blood vessels in the enzyme treated mice versus the control is remarkable. Most vessels are oriented parallel to the peritoneal surface.

Table 3 shows the profiles of mouse plasma and liver glutamine levels during EAC growth and after treatment with glutaminase enzyme from the S-180 cell. It is noteworthy that a significant increase in glutamine content was observed in liver in such a short time as 24 hrs. after tumor transplantation, indicating that the liver is producing more glutamine in tumor bearing mice than normal. Whereas, in plasma a reverse picture was seen which suggests that tumor cells are taking a huge amount of glutamine from the circulation. However, after 24 hrs. this picture had gradually changed. The plasma glutamine level remained higher for the rest of the life span during EAC growth, but after glutaminase treatment plasma glutamine concentration was decreased showing that glutaminase can prevent the net flux of glutamine from the host tissue to plasma.

Table 4 shows the parameters of cytological and histological evaluation of dysplasia and carcinoma. After 10 days of methylcholanthrene treatment,analysis of the cervical smears reveals early dysplasia. After 20 days of methylcholanthrene treatment, the smears demonstrate late dysplasia. Cervical carcinoma in situ sets in after 30 days of methylcholanthrene treatment. Macroscopic observation showed more vascular isolation of the cervix in MCH as well as enzyme treated group than the normal mice. Fewer blood vessels were found in methylcholanthrene and enzyme treated mice than methylcholanthrene only treated ones. Epithelial hyperplasia or dysplasia was observed in mice treated with methylcholanthrene for 10 days with increased numbers of blood vessels than in the normal mice. (*Figure 2A, 2B*). In situ carcinoma was

Table 4. **Parameters of cytological and histological evaluation of dysplasia and carcinoma (n=6)**

Group	*Days after MHC treatment*	*Smear study*	*Histopathological criteria*
Early dysplasia	10	Abnormality of parabasal and intermediate cells, slight cellular and nuclear enlargement, no cytological change.	Group of cells showing hyperplasia
Dysplasia	20	Intermediate cytoplasmic changes, such as granulation and vacuolation, nucleus showing occassional diffuse translucence.	Group of cells exhibiting atypia and loss of polarity in hyperplastic epithelium.
Cervical carcinoma	30	Cells round to oval with large hyperchromic nuclei. Thickening of nuclear membrane, clump chromatin and multiple nuclei, loss of nuclear chromatin pattern	Carcinoma in the squamous epithelium of uterine cervix

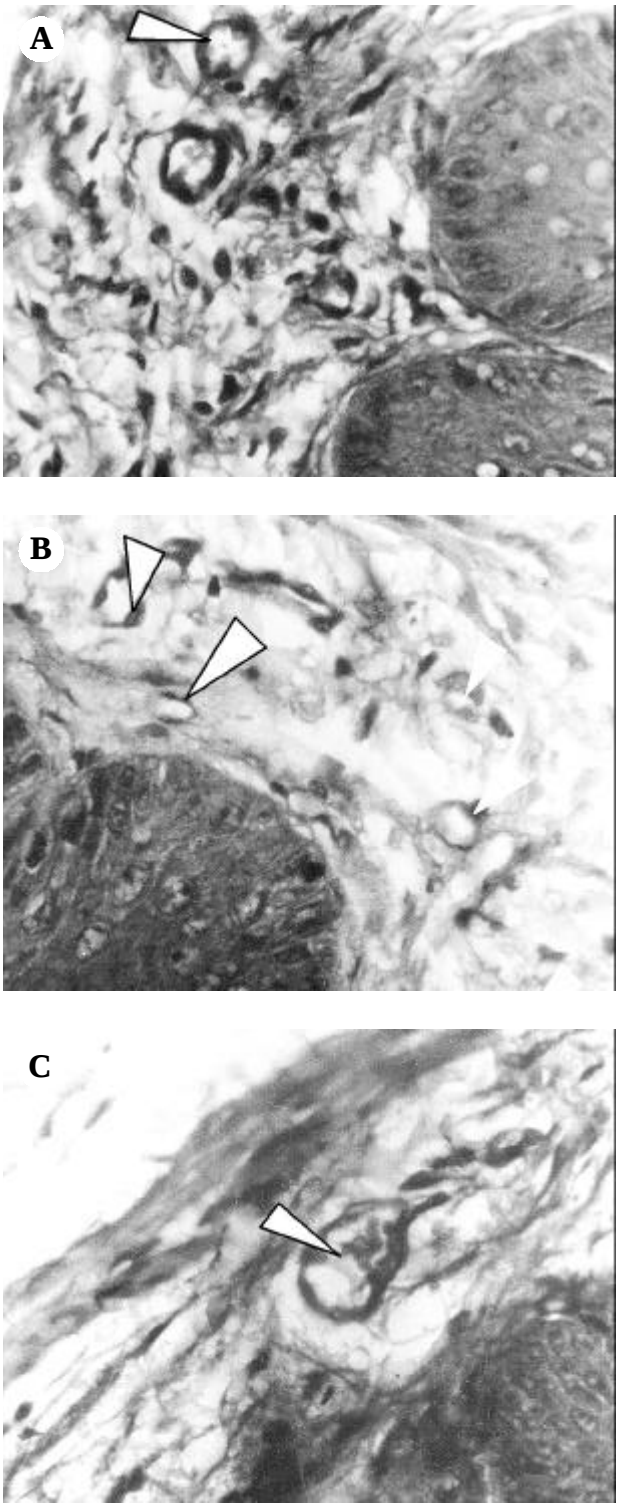

Figure 2. (A) Normal cervix of a female Swiss albino mice showing blood vessels (HE, x500); (B) Dysplastic cervix of a female Swiss albino mice after 10 days of Methylcholanthrene treatment, showing a greater number of blood vessels (HE, x500); (C) In situ carcinoma bearing cervix of a female Swiss albino mice after 30 days of MCH treatment, showing leaky blood vessels (HE, x500).

observed after 30 days of methylcholanthrene treatment with profound leaky blood vessels (*Figure 2C*). Enzyme and methylcholanthrene treatment led to the delayed onset of carcinoma with decreased vasculature.

Discussion

The importance of the neovascularisation process has been already widely accepted.[18,19] The current study shows neovascularisation is invariably present in solid as well as ascitic tumor models. Microvessel counts were found to be increased significantly in solid tumor bearing mice when compared to normal ones. Both induced (methylcholanthrene) and transplanted (EAC, S-180) type solid tumors bearing mice show a greater number of blood vessels than the enzyme treated groups. Simultaneously, a marked reduction in tumor burden has also been observed (*Table 1*). However, the angiogenic response depends upon the host, which is also in agreement with the work of Matar et al.[23] Ascitic tumor bearing mice also displayed a decreased number of new capillary sprouts after receiving the purified glutaminase treatment, compared to their controls (*Figure 1*). The pattern of distribution of blood vessels in our experimental Swiss albino mice was found to be similar with the pattern shown by Nagy et al[5] in their experimental ascitic tumor model. The results suggest that the enzyme inhibits tumor growth and the angiogenic process by reducing plasma glutamine levels (*Table 3*). Therefore, we suggest that increased angiogenesis can be linked with higher glutamine utilization. Further studies with cervical carcinoma bearing mice also supported the hypothesis that treatment with purified glutaminase resulted in delayed onset of carcinoma in situ with decreased microvessel counts. The developing tumor matrix contains an extracellular matrix protein known as fibrin[20] which can induce angiogenesis[21] and it has already been known that histidine and glutamine are both structural components of fibrin. Thus, any kind of depletion of these amino acids may disrupt the formation of the fibrin monomer, which in turn will affect the angiogenesis process. Glutamine donates its amide group to constitute the imidazole nitrogen molecule of histidine,[22] and together they contribute to the fibrin formation. A target of glutaminase therapy is to breakdown the circulatory glutamine, which may affect the process of angiogenesis. Several authors have suggested that the tissue architecture of an organ in which angiogenesis is taking place, has an important impact on the process of angiogenesis and consequently on the structure of the developing neovasculature. According to our preliminary work, experimental glutaminase therapy provides a link to angiogenesis. However, further studies are needed to confirm the mechanistic aspects of this relationship.

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