Neovascularisation offers a new perspective to glutamine related therapy

Putul Maity, Sunil Chakraborty & Pritha Bhattacharya
Department of Metabolic Regulation, Chittaranjan National Cancer Institute, 37, S. P. Mukherjee Road, Calcutta-700 026, India

Received 28 May 1999; revised 15 September 1999

Angiogenesis or the generation of new blood vessels, is an important factor in the growth of a solid tumor. Hence, it becomes a necessary parameter of any kind of therapeutic study. Glutamine is an essential nutrient of tumor tissue and glutamine related therapy involves clearance of circulatory glutamine by glutaminase. Therefore, using different murine solid tumor models, the present study was undertaken to find out whether the S-180 cell glutaminase has any effect on angiogenesis of solid tumor, or not. Result indicates that the purified S-180 cell glutaminase reduces tumor volume and restrict the generation of neo blood vessels. Therefore, it can be concluded that this enzyme may be an effective device against the cancer metastasis.

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. 1,2 As the tumor increases in size new capillaries develop, which is induced either by the tumor itself and/or the host tissues. 3-6 The newly formed blood vessels not only feed the growing tumor cells but also provide an easy way to travel through the circulation and finally metastasize. 7-9 Greenblatt and Shubik 10 first demonstrated that tumor produces some diffusible factors which induces the growth of the blood vessels. Since, then several angiogenic and antiangiogenic factors were found. These promoters and inhibitors maintain a homeostatic equilibrium in normal condition but during the growth of the solid tumor this equilibrium has been broken.

In the present work, effect of purified glutaminase from S-180 cell has been studied on murine solid tumor and also whether it plays any role in neovascularisation, or not.

Animals—Male Swiss albino mice 6-7 week old, weighing 20-22 g. body weight were used.

Tumor strains—Ehrlich ascites carcinoma (EAC) and Sarcoma-180 (S-180) were maintained in Swiss albino mice by serial subcutaneous transplantation (2×10^5 cells/mouse) for solid tumor. Methylcholanthrene induced solid tumors were formed by injecting an emulsion of 1% 20-methylcholanthrene in Freund's incomplete adjuvant (SIGMA) subcutaneously in the groin region of Swiss albino mice, at the dose of 2 mg 20-methylcholanthrene/0.2 ml emulsion/ mouse. The adjuvant served the purpose of the carrier substance.

Isolation and purification of enzyme—Phosphate dependent glutaminase was purified from highly malignant S-180 cells, according to the method of Quesada et al. 11 with some modifications. First, tumor cells were separated from the peritoneal fluid by centrifugation at 2000 r.p.m. Then mitochondria were isolated from the tumor cell suspension in media A (35 mM sucrose, 5mM HEPES, 1mM EDTA). It was then sonicated for 3 min with 30 sec pulse and 30 sec interval and then centrifuged at 28,000 r.p.m for 1hr followed by 40% ammonium sulphate precipitation and dialysis. Dialyzed sample was then applied to DEAE Sepharose column and finally applied to the affinity column of L-glutamine. The purified enzyme gives single band on SDS-PAGE and immunoblot analysis (using anti rat kidney glutaminase antibodies) confirm that it is a kidney type glutaminase.

Enzyme and protein assay—Enzyme assay was done by end point determination of glutamate. Glutamate production was assayed at 340 nm in spectrophotometer by monitoring NADH formation in the GLDH reaction according to the method of Lund et al. 12. Protein was measured at 660 nm according to Lowry et al. 13.
Treatment schedule—The enzyme was injected intraperitoneally at a dose of 1 unit/day/mice for 15 consecutive days into solid tumor bearing mice. Injection was started 10 days after tumor induction. Control animals received equal volume of (0.2 ml) PBS. In each tumor type (methylcholanthrene, S-180, EAC) both control and enzyme treated groups were sacrificed at three different time intervals that is at 15th, 20th and 25th days after tumor induction or in other words at 5th, 10th and 15th days after enzyme treatment.

Measurement of antitumor activity—Tumor volume was measured with the help of a slide caliper and was calculated according to the formula \( V = \frac{4}{3} \pi D_1 \times D_2 \times D_3 \), where \( D_1 \) is length, \( D_2 \) is breadth and \( D_3 \) is height of the tumor.

Quantification of new blood vessels—Tumor bearing mice of both enzyme treated and untreated groups of each tumor type (EAC, S-180 and methylcholanthrene induced) were sacrificed after 15th, 20th and 25th, days of tumor inoculation. Tumors were first located and the skin around it was removed. Angiogenesis was quantified by counting the number of capillaries oriented towards the tumor, under a dissecting microscope according to the method of Danielsen et al.14

Table 1 displayed the effect of purified glutaminase therapy from S-180 cell, on the number of new blood vessels and tumor volume.

In methylcholanthrene induced tumor type, enzyme treated groups showed a significant decrease in number of blood vessels when compared with their respective control groups. Maximum inhibition (30.5%) in neo blood vessel formation has been found on 25 th day after carcinogen treatment followed by 20 th and 15 th day groups. Tumor volume also showed a significant decrease in enzyme treated groups of methylcholanthrene model with highest inhibition (70.2%) in tumor growth on 25th days after tumor induction, in comparison to respective control group.

Ehrlich ascites carcinoma (EAC) showed a decrease in tumor volume as well as blood vessel count in enzyme treated groups with respect to their untreated groups. Generation of new blood vessels were inhibited maximum (18.75%) accompanied with highest (56%) tumor growth inhibition in 15th day enzyme treated group.

Comparing the results obtained from S-180 groups, it was found that there was no direct relation with tumor volume and generation of new blood vessels. But enzyme therapy showed significant inhibition in tumor volume as well as neovascularisation.

Importance of neovascularisation process has been already widely accepted.15,16 Advanced research in this field finds out involvement of stimulators and inhibitors in tumoral angiogenesis.4,5,17,18 Developing tumor matrix contains extracellular matrix proteins, such as fibrin and it induces angiogenesis.19 It is already known that histidine and glutamine both are the structural components of fibrin. So, any kind of depletion of these amino acids may disrupt the formation of fibrin monomer, which will in turn affect angiogenesis. Glutamine donates its amide group to constitute the imidazole nitrogen molecule of histidine and together they contribute to the fibrin formation. Target of glutaminase therapy is to breakdown the circulatory glutamine, and thereby affecting the process of angiogenesis. Results showed that glutaminase therapy reduces the number of neo

### Table 1—Effect of glutaminase therapy on formation of new blood vessels and tumor volume of different murine solid tumor model

<table>
<thead>
<tr>
<th>Group</th>
<th>15-day Number of blood vessel</th>
<th>20-day Number of blood vessel</th>
<th>25-day Number of blood vessel</th>
<th>15-day Tumor volume (cm³)</th>
<th>20-day Tumor volume (cm³)</th>
<th>25-day Tumor volume (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylcholanthrene control</td>
<td>8.0±0.5</td>
<td>7.0±0.8</td>
<td>7.2±0.5</td>
<td>0.36±0.16</td>
<td>0.40±0.05</td>
<td>0.57±0.02</td>
</tr>
<tr>
<td>Methylcholanthrene+enzyme</td>
<td>6.0±0.3*</td>
<td>5.2±0.2*</td>
<td>5.0±0.6*</td>
<td>0.25±0.05*</td>
<td>0.15±0.03*</td>
<td>0.17±0.03*</td>
</tr>
<tr>
<td>EAC control</td>
<td>7.0±2.1</td>
<td>7.7±0.5</td>
<td>8.0±0.3*</td>
<td>0.30±0.25</td>
<td>0.49±0.06</td>
<td>0.50±0.02</td>
</tr>
<tr>
<td>EAC ± enzyme</td>
<td>7.2±0.6*</td>
<td>6.5±0.3*</td>
<td>6.5±0.5*</td>
<td>0.23±0.05*</td>
<td>0.24±0.03*</td>
<td>0.22±0.03*</td>
</tr>
<tr>
<td>S-180 control</td>
<td>7.5±0.4</td>
<td>6.2±0.5</td>
<td>6.3±0.3*</td>
<td>0.40±0.02</td>
<td>0.50±0.02</td>
<td>0.49±0.01</td>
</tr>
<tr>
<td>S-180 ± enzyme</td>
<td>6.9±0.1*</td>
<td>6.0±0.8</td>
<td>6.1±0.5*</td>
<td>0.29±0.02*</td>
<td>0.21±0.03*</td>
<td>0.20±0.01*</td>
</tr>
</tbody>
</table>

* As compared with the respective control (P<0.001)  
EAC=Ehrlich ascites carcinoma, S-180=Sarcoma-180
blood vessels as well as decrease the tumor volume of malignant host. Though the tumor volume does not always showed a direct relation with number of blood vessels, from the present experiment it is evident that glutaminase therapy restricts the generation of new blood vessels. This inhibitory effect varies with tumor types. According to present results glutaminase therapy is much more evident in methylcholanthrene induced tumor type than in the two transplanted tumors and among them EAC type was found to be more prone to therapy than the S-180 type. It has been suggested that tissue architecture of the 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 present results the glutaminase therapy provides a relation with angiogenesis. But, further studies are needed to confirm the mechanistic aspects.

References
7 Miguez M, Davel L & Sacerdote de Lustig E, Invas Metast, 6 (1986) 313.