Alteration of blood-CSF barrier by tumor invasion into the meninges

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Cyclophosphamide and 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) were found to have an equivalent cytostatic effect in rats with subcutaneous transplants of Walker 256 carcinosarcoma. Rats with meningeal carcinomatosis received a single intravenous dose of cyclophosphamide (30 mg/kg) or ACNU (15 mg/kg) at various times after intracisternal inoculation of $1 \times 10^4$ Walker 256 carcinosarcoma cells. Cyclophosphamide, administered 1 day after tumor inoculation, failed to prevent tumor growth in the subarachnoid space. The survival time of these rats was prolonged only 10% to 14% compared to the controls, while ACNU produced a maximum increased survival time of 180%. If administered 2, 3, 4, and 5 days after tumor inoculation, both drugs were effective; cyclophosphamide yielded a maximum increase in median survival time of 109%, 94%, 90%, and 52%, and ACNU 127%, 139%, 240%, and 100%, respectively. These results indicate that the blood-cerebrospinal fluid (CSF) barrier was circumvented in the early stage of subarachnoid tumor growth, although some areas remained where the infiltrating tumor cells were protected from systemically administered drugs by the intact barrier.

KEY WORDS blood-CSF barrier □ meningeal carcinoma □ carcinomatosis □ chemotherapy

To date, no general agreement has been reached on the role of the blood-brain or blood-cerebrospinal fluid (CSF) barrier in the chemotherapy of brain tumors. Although there is a consensus that these barriers do not exist in brain tumors, no systemic approach has yet been devised to determine the time course of the tumor-induced breakdown of these barriers.

With meningeal carcinomatosis as an experimental model, we investigated how the blood-CSF barrier is altered by tumor growth in the subarachnoid space. This alteration was evaluated by the delivery to the tumor of systemically administered lipid- and water-soluble drugs.

Materials and Methods

Drugs

Cyclophosphamide* was dissolved in sterile water to obtain a concentration of 12 mg/ml. The active metabolites of cyclophosphamide are water-soluble and are known to cross the blood-brain barrier poorly.

We dissolved 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU)† in sterile water to obtain a concentration of 6 mg/ml. This drug is lipophilic (octanol/water partition coefficient = 0.92) and crosses the blood-brain barrier easily.

Subcutaneous Tumor Model

Walker 256 carcinosarcoma was obtained from the Research Institute of Microbial Diseases of Osaka University and maintained in our laboratory by serial subcutaneous transplantation. The tumor was aseptically removed 9 days after the last transplantation and minced with fine scissors in Earle's basic medium. Adult female Sprague-Dawley rats were anesthetized with ether and a tumor pellet, approximately 1 cm in volume, was transplanted subcutaneously into the abdominal wall. When the tumor reached about 1 cm in volume, the animals were randomly divided

* Cyclophosphamide supplied by Shionogi Seiyaku Co., Osaka, Japan.

† ACNU supplied by Sankyo Co., Tokyo, Japan.
into four groups consisting of nine or ten rats, and one group each was treated with a single intravenous dose of 30 mg/kg cyclophosphamide, or 15 or 30 mg/kg ACNU, or left untreated (control). The length, width, and height of the tumors were measured every other day, their volume was calculated based on the assumption that they were hemi-ellipsoid, and their growth curves were compared.

Meningeal Carcinomatosis Model

Details regarding the present model have been described previously. Minced Walker 256 carcinosarcoma was aseptically passed first through a 40-mesh and then an 80-mesh stainless steel screen. Using the trypan blue exclusion test, inocula of $1 \times 10^4$ viable cells per 0.1 ml of Earle's medium were obtained.

Female Sprague-Dawley rats weighing approximately 150 gm were intraperitoneally anesthetized with sodium pentobarbital (20 mg/kg), and $1 \times 10^4$ viable tumor cells were injected percutaneously into the cisterna magna, using a No. 26 needle. On the 1st, 2nd, 3rd, 4th, or 5th day after inoculation, the rats were randomly divided into three groups consisting of eight to ten rats, and one group each was treated with a single intravenous dose of 30 mg/kg cyclophosphamide, 15 mg/kg ACNU, or left untreated (control). All animals were checked daily and the day of death was recorded. The life span of the treated and untreated animals was compared and analyzed by the Wilcoxon rank sum test. All experiments were performed in duplicate.

Results

Subcutaneous Tumor Model

Figure 1 shows the growth curves of subcutaneous transplants of Walker 256 carcinosarcoma. In the untreated controls, the tumors grew rapidly, reaching a volume approximately 10 times greater than the implant within 4 or 5 days. In the treated animals, inhibition of tumor growth was observed; the cytostatic effect of cyclophosphamide (30 mg/kg) and ACNU (15 mg/kg) was equivalent.

![Graph](image-url)
Alteration of blood-CSF barrier

**Meningeal Carcinomatosis Model**

When administered 1 day after tumor inoculation, cyclophosphamide failed to prevent tumor growth in the subarachnoid space. The survival time of these rats was prolonged only 10% to 14% compared to the controls. In ACNU-treated rats, on the other hand, survival time was increased 71% to 180% (Table 1). The survival curve of cyclophosphamide-treated rats was very similar to the controls (Fig. 2).

When administered 2, 3, 4, or 5 days after tumor inoculation, both drugs markedly prolonged survival time compared to the controls. In all experiments, ACNU was more effective than cyclophosphamide. Among rats treated 3 days after tumor inoculation, those injected with cyclophosphamide lived almost twice as long as the controls; however, ACNU was markedly more effective in prolonging survival time (Fig. 3).

**Discussion**

In the present experimental model of meningeal carcinomatosis, rats were percutaneously inoculated in the cisterna magna with Walker 256 carcinosarcoma cells. The resulting histopathological pattern was similar to that seen in diffuse leptomeningeal involvement of human systemic cancer. Using this model, we previously tested the effect of various chemotherapeutic agents and found that intravenously administered water-soluble agents (cyclophosphamide, methotrexate, and bleomycin), which are thought not to cross the blood-CSF barrier, effectively prolonged the survival time of rats with meningeal carcinomatosis when administered 5 days after tumor inoculation. Those findings suggested that tumor growth in the subarachnoid space circumvents the blood-CSF barrier.

In the present study, we investigated the time course and extent of blood-CSF barrier breakdown by tumor invasion into the meninges. Alteration of the barrier was evaluated by the effects of systemically administered lipid- or water-soluble agents against the tumor in the subarachnoid space. We chose ACNU and cyclophosphamide because the Walker 256 carcinosarcoma used in this experiment is sensitive to both drugs, and because ACNU crosses and cyclophosphamide does not cross the blood-brain barrier. First we looked for a drug dose which would produce an equivalent cytostatic effect on subcutaneously transplanted Walker 256 carcinosarcoma (Fig. 1). We then used this dose to treat tumors in the subarachnoid space. We reasoned that if the blood-CSF barrier was intact, ACNU should be effective, and cyclophosphamide ineffective, whereas both drugs should be ineffective.

**TABLE 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Median Day of Death</th>
<th>ILS* (% control)</th>
<th>p Value†</th>
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<td>Treated</td>
<td>Control</td>
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<tr>
<td>Day of Treatment After Inoculation</td>
<td>Experiment No.</td>
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<td>cyclophosphamide</td>
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<td>NS 0 9</td>
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<tr>
<td></td>
<td></td>
<td>ACNU</td>
<td>28 10 180</td>
<td>&lt; 0.001 2 9</td>
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<td>NS 1 9</td>
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<td>&lt; 0.001 4 10</td>
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* ILS = increased life span.
† Significance computed by the Wilcoxon test. NS = not significant.

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Fig. 2. Survival curves of rats with meningeal carcinomatosis treated once intravenously with cyclophosphamide or ACNU 1 day after intracisternal inoculation of $1 \times 10^4$ Walker 256 carcinosarcoma cells. $N =$ number of rats in each group.

Our results indicate that the blood-CSF barrier was intact 1 day after tumor inoculation; however, its circumvention was noted as early as the 2nd day. Our experiments did not identify the exact nature of the alterations of the blood-CSF barrier by leptomeningeal tumor invasion. It has been suggested that, in solid tumors, such alterations are due to neovascularization by blood vessels that lack the barrier. However, it is unlikely that the alteration we observed at such an early period of tumor growth is the result of neovascularization. We previously reported that, 1 day after tumor inoculation, tumor deposition and proliferation occurred; two to three tumor cell layers were formed in the subarachnoid space over the medial and ventral surface of the cerebral hemispheres. By 2 days after inoculation, the tumor formed a maximum of five to six layered sheets over the cerebral and cerebellar hemispheres, and no neovascularization was observed in the tumor. Although by 2 days, the tumor had infiltrated the brain along the perivascular space of penetrating blood vessels, and infiltration of the overlying meninges proceeded, there was no invasion into the brain parenchyma.

Based on these observations, the alteration of the blood-CSF barrier at the early stage of meningeal tumor invasion may be ascribable to the breakdown of the barrier proper, which exists between the meningeal capillaries and CSF, due to tumor deposition and growth over the leptomeninges. In the advanced stage of meningeal carcinomatosis, when the interstices of the subarachnoid space are filled with solid tumor masses or when the brain parenchyma, spinal cord, and nerve roots are infiltrated, neovascularization may play a role in the alteration of the blood-CSF barrier.

In all of the present experiments, ACNU exceeded cyclophosphamide in efficacy. This suggests that the barrier was not completely circumvented and, in some areas, the tumor cells were protected from the systemically administered water-soluble drug by the intact barrier. Our previous histopathological findings had indicated that, even in the far-advanced stage of meningeal carcinomatosis, there are some areas with only one- or two-layered tumor cells spread in sheets in the subarachnoid space. In these areas, the blood-CSF barrier might remain intact, as was suggested by our results shown in Fig. 2.

Our findings suggest that systemic chemotherapy of...
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meningeal carcinomatosis should include an agent that crosses the blood-brain barrier, although the agent of first choice should be one to which the original tumor is highly sensitive.

Acknowledgment
We thank Miss R. Fujita for her help in this investigation.

References

Fig. 3. Survival curves of rats with meningeal carcinomatosis treated once intravenously with cyclophosphamide or ACNU 3 days after intracisternal inoculation of 1 x 10^4 Walker 256 carcinosarcoma cells. N = number of rats in each group.

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