MECHANISM OF CYCLOPHOSPHAMIDE ACTIVATION*1

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Synopsis

Activation of cyclophosphamide took place not only in the liver of mice but also in the bone marrow of rabbits or in the kidney of mice and can thus be considered to be activated in all the organs in the body to a various extent. The activation was also demonstrated in human organs, such as the liver or bone marrow. Activated substance appeared in a short period. Solid Yoshida sarcoma or Ehrlich carcinoma activated cyclophosphamide but ascites form did not. This difference may be due to the presence of interstitial tissue of solid tumor. Various human carcinomas obtained at surgery activated cyclophosphamide in their solid form and, therefore, the administration of cyclophosphamide by means of regional perfusion might designate clinical significance. The action of bone marrow will also be helpful for the evaluation of its effect.

INTRODUCTION

Cyclophosphamide developed by Arnold1) in 1958 is an inactive “transport form” in vitro but is effective in vivo after being transformed into an “active form.” The mechanism of its activation from “transport form” to “active form” has been investigated by several workers.2, 4) Among them, Foley7) stated that the activation took place in the liver, Brocks5) mentioned it in the lung and adrenal glands, recently the cortex of kidney was also one of the places,8) and Sakurai9) postulated that the drug was activated in the liver and transformed form in the blood stream would show antitumor activity. On the other hand, Tasaka et al.10) investigated the activation of cyclophosphamide by various kinds of organ emulsions and could not observe the effect in the liver and kidney of mice. Thus, there is no established theory on the activation of cyclophosphamide in the body. We have investigated, therefore, the organ specificity of activation and the activation in the tumor by means of tissue culture method. HeLa cells were cultured in a monolayer with various kinds of organ slices. Cell culture and organ culture were performed in the same bottle simultaneously and a definite concentration of cyclophosphamide was added to see the activation of the drug at special sites of body by means of a growth inhibition rate of HeLa cells.

MATERIALS

Cyclophosphamide Endoxan (Shionogi & Co., Osaka), 100 mg/vial, was injected to animals as 20% glucose solution and as YLE or YLEG (YLE with 0.45% glucose) solution in tissue culture study.

*1 This study was presented at the 27th Annual Meeting of the Japanese Cancer Association in Tokyo, 1968.

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Experimental Animals and Tumors  The liver, spleen, or kidney was removed from Swiss strain mice, the bone marrow was obtained from the femur of a rabbit. As human material, surgical specimens of liver or bone marrow from ribs was used. Fifty mg of the wet tissue was applied for the experiments.

Golden Hamster: Golden hamster, 60 g in body weight, was used to transplant HeLa cells.

Yoshida Sarcoma and Ehrlich Carcinoma: Subcutaneous or ascites tumor was transplanted in Donryu rats or Swiss mice.

Cultured Cell Strain

HeLa Cells: The monolayer cultured HeLa cells were stripped off from the glass surface by treatment with 0.2% trypsin solution. After washing, the cell suspension of 1~3×10⁵/ml was subcultured in TD-15 culture bottle at 37°C. The medium used was YLEG with 20% calf serum.

Human Embryo Fibroblast: Human embryo cultured primarily in our laboratory and subcultured in YLE medium with 20% calf serum.

METHOD AND RESULTS

Sensitivity of HeLa Cells (in vitro and in vivo) to Cyclophosphamide

For the first experiment, the sensitivity of HeLa cells to cyclophosphamide either in vitro or in vivo was investigated. For the sensitivity test in vivo, 4×10⁵ HeLa cells were transplanted into the cheek pouch of a golden hamster and injected subcutaneously with 5 mg of cyclophosphamide 4 times, and its effect on tumor growth was observed to find out the effect of cyclophosphamide. In the treated group, histologically, the destruction of tumor cells was observed by poor staining of nucleus and vacuolization of cell plasma. For the in vitro test the effect of 50, 100, or 200μ/ml of cyclophosphamide was examined. There were some effects observed with 100 and 200μ/ml but no effect with 50μ/ml. Therefore, the final concentration of 50μ/ml was used in vitro in the following experiment.

Activation of Cyclophosphamide by Various Organs

Twenty-four hours after the beginning of HeLa cell culture, when the cells settled on the glass surface, a small glass plate was inserted into the bottle with 50 mg of an organ specimen on it in order to avoid direct contact of the two materials (Photo 1). The culture medium containing 50μ/ml of cyclophosphamide was used for this culture, the cell culture and organ culture being performed simultaneously. HeLa cells stained with Trypan Blue solution were counted to see the effect of the drug. The growth inhibition percentage was calculated with the number of treated and untreated cells. The growth inhibition of HeLa cells on the 8th day after beginning the culture was 75% with mouse liver, 50% with rabbit bone marrow, and 28% with mouse kidney, but there was no inhibition of HeLa cell growth with mouse spleen. With human materials, the growth inhibition of HeLa cells was 62% with the liver and 65% with the bone marrow of ribs. In order to see the production of activating substance from the liver of a mouse from time to time, the minced liver and 1.0 mg/ml of cyclophosphamide were spinner cultured in a bottle and then the supernatant was added to the cultured HeLa cells. Inhibition of 52% was observed with the supernatant of spinner culture of 15 min, 58% with that of 30 min, and 60% with that of 60 and 120 min.
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Activation by Experimental Tumor Tissue

A subcutaneous solid Yoshida sarcoma,\textsuperscript{11,12} which was previously proved to be effective \textit{in vivo}, was used in the same experiment, and 38\% growth inhibition was observed.

![Photo 1. After beginning the HeLa cell culture, when the cells settled on the glass surface, a small glass plate was inserted into the bottle with a 50 mg of organ specimen on it in order to avoid direct contact of these materials and to effect cell culture and organ culture simultaneously.](image)

![Fig. 1. Effect of cyclophosphamide on HeLa cells incubated with Yoshida sarcoma (solid).](image)
observed (Fig. 1). After $10^6$/ml of an ascites form of Yoshida sarcoma cells was spinner cultured with 1.0 mg/ml of cyclophosphamide, 0.1 ml of the supernatant was added to HeLa cells but there was no effect (Fig. 2). Next, a subcutaneous solid Ehrlich carcinoma was investigated to see the activation of cyclophosphamide by its effect on HeLa cells and it showed 14.6% inhibition of HeLa cell growth in the culture experiment. However, in its ascites form in the experiment of spinner culture, incubating cyclophosphamide and Ehrlich free cell to see the effect of supernatant on HeLa cell growth, the drug did not show any activation by free tumor cells. From these experiments with Yoshida sarcoma and Ehrlich ascites carcinoma, cyclophosphamide was found to be activated by solid tumors, but not by free tumor cells.

**Activation of Cyclophosphamide by Fibroblasts**

From the above facts, we tried to find out the reason why only the solid tumor had been effective to cyclophosphamide. L cells, which are known to originate from subcutaneous fibroblasts of C3H mice, were cultured in $3 \times 10^5$/ml concentration, in a medium containing cyclophosphamide for 24 and 48 hr, and the supernatant was examined for its effect on HeLa cells. The L cells effected 52~20% inhibition. Next, the human embryo fibroblast was used in the same way for spinner culture. The supernatant effected inhibition of 60% in 24 hr and 21% in 48 hr. When the spinner culture was made at 4°, the fluid did not show any effect (Fig. 3).

Fig. 2. Effect of supernatant fluid of Yoshida sarcoma (ascites) incubated with cyclophosphamide on HeLa cells
Activation of Cyclophosphamide by Human Solid Tumor

Surgical specimens of human solid tumor were examined for its activation of cyclophosphamide. Thirteen specimens including 6 gastric cancer, 1 rectal cancer, 1 malignant mixed tumor, 1 fibrosarcoma, and 1 breast cancer were examined and all showed activation of 55~37% (Table I).

**Table I. Inhibition of HeLa Cell Growth by Cyclophosphamide incubated with Various Human Tumor Tissues (inhibition rate on the 3rd day)**

<table>
<thead>
<tr>
<th>Name</th>
<th>Kind of tumor</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W. B.</td>
<td>Gastric cancer</td>
<td>65</td>
</tr>
<tr>
<td>O. T.</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>K. T.</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>H. B.</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>S. K.</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Y. D.</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>S. I.</td>
<td>Rectal cancer</td>
<td>37</td>
</tr>
<tr>
<td>K. T.</td>
<td>Malignant mixed tumor</td>
<td>50</td>
</tr>
<tr>
<td>A. G.</td>
<td>Fibrosarcoma</td>
<td>59</td>
</tr>
<tr>
<td>H. G.</td>
<td>Breast cancer</td>
<td>39</td>
</tr>
</tbody>
</table>
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DISCUSSION

There are various reports on the activation of cyclophosphamide in vivo.\(^5\)\(^-\)\(^10\) In our experiments, the activation was observed not only with liver but also with bone marrow or kidney tissue. There might be some direct cytotoxic influence of inserted minced organ or of the pH decrease in long-term culture, but the difference from control means the activation of cyclophosphamide by inserted organs which results in the growth inhibition of HeLa cells. There was no activation observed with spleen but spleen contains a large amount of blood cells and there might be some influence on it which requires further investigation. We believe that the activation of cyclophosphamide in the body of a human or animals might takes place in all sites of the organs to a various extent and not in a special organ selectively. Foley \textit{et al.}\(^7\) stated that the active form was observed in the serum only 30 min after the injection of cyclophosphamide and, from our results of spinner culture of liver minces, a large part of cyclophosphamide was transformed in a short period. Ebina \textit{et al.}\(^6\) observed that the intraperitoneal injection of cyclophosphamide into Ehrlich ascites carcinoma was ineffective, whereas remarkable effect was observed when it was given subcutaneously, and Tasaka \textit{et al.}\(^10\) stated that after incubation of the supernatant of Ehrlich ascites tumor with 500 γ/ml cyclophosphamide at 37° for 1 hr, no activation was observed. Bolt\(^3\) injected cyclophosphamide labeled with tritium in moribund cancer patients and found high concentration of it in the primary tumor and metastases. In our experiment with Yoshida sarcoma and Ehrlich carcinoma, there was no activity in ascites form but had activity in subcutaneous tumor. As the reason why there is a great difference between solid and ascites forms, we can consider the presence of interstitial tissue. To see the possibility of activation by the interstitial tissue we used L cells and human embryo fibroblasts and clarified that cyclophosphamide can be activated by normal fibroblasts. One of the reasons for effectiveness of cyclophosphamide to solid tumor of human or experimental animals might be the presence of interstitial tissue. Cyclophosphamide is presumed to be activated by enzymic hydrolysis because no activation was observed at 4° in the present experiment.

In general, cyclophosphamide is thought to be activated only by liver tissue and, therefore, ineffective to local treatment but we have experienced a case of malignant melanoma to whom 500 mg of cyclophosphamide was given by regional perfusion which resulted in remarkable effect and without recidivism for more than 3 years. As can be seen from this case, if the drug is activated by tumor tissue itself, it is useful to apply cyclophosphamide as much dose as possible by regional perfusion or local injection effectively without side effects. As shown in Expt. 4, the growth of HeLa cells decreased to 1/3 in 24–hr group compared to that in 48–hr group, the active form seems to be unstable and therefore the drug should be given continuously to be more effective. In case of regional perfusion, the activation of cyclophosphamide by bone marrow may be another element for effectiveness.

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REFERENCES