Cartilage-derived Antitumor Factor (CATF): A High Molecular Weight Fraction in Cartilage Extract Inhibits Solid Tumor Growth

Fujio SUZUKI*, Masaharu TAKIGAWA*, Yuji HIRAKI*, Yukio KATO*, Keisuke FUKUO*, Tsuyoshi SHIIO and Yasumi YUGARI**

ABSTRACT

A high molecular weight factor(s) was extracted from fetal bovine cartilage with 1 M guanidine hydrochloride. This factor inhibited neither the proliferation of sarcoma 180 cells in culture nor the growth of their ascites form. However, it strongly inhibited the growth of solid sarcoma 180 in vivo.

Cartilage is known to be normally avascular and resistant to invasion by neoplasms or inflammatory processes1,2). After extraction with guanidine hydrochloride (GuHCl), the tissue loses this resistance5). These facts have let some investigators try to extract and purify a factor(s) from cartilage which inhibits neovascularization and tumor invasion. For example, Kuettner and his coworkers4–6) have in a series of papers reported extractable protease inhibitors from bovine cartilage and aorta which inhibited tumor growth in mice. Folkman and his collaborators partially purified angiogenesis inhibitors from cartilage7) and showed their inhibitory activity on tumor growth8). These factors have been known to be of low molecular weight, but they have neither been completely purified nor characterized yet.

On the other hand, we purified cartilage-derived factor(s), (CDF), which is a somatomedin-like growth factor and of low molecular weight, from GuHCl extract of fetal bovine cartilage8,10). In the present study, we report cartilage-derived antitumor factor (CATF) obtained from a high-molecular-weight fraction of GuHCl extract from fetal bovine cartilage, after removal of CDF.

Fetal calves were obtained from a public slaughter house. Within 10 h of sacrifice, avascular cartilage was excised from their acapulae, ribs and limbs. Adherent soft tissue was removed completely and then the cartilage was sliced and homogenized in a Polytron (Kinematica) with 10 volumes of 1 M GuHCl containing 0.02 M 2-(N-morpholino) ethanesulfonic acid (MES), pH 6.0 at 0°C. The homogenate was stirred at 4°C for 48 h and then centrifuged at 10,000×g for 20 min. The resulting supernatant was mixed

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with acetone at a final concentration of 45% (V/V). The insoluble material was removed by centrifugation, and acetone was added further to the supernatant to a final concentration of 65% (V/V). The insoluble material was collected by centrifugation (Acetone 45-65%) and dissolved in 80 volumes of 4 M GuHCl containing 0.1 M 6-amino-n-caproic acid, 0.01 M EDTA, 0.02 M MES and 1 M NaCl. The solution was filtered through an Amicon XM300 filter, which excludes molecules of more than $3 \times 10^5$ daltons. The filtrate was concentrated by an Toyo UP20 filter, which excludes molecules of more than $2 \times 10^4$ daltons. The fraction concentrated by UP20 was dialyzed against distilled water and lyophilized (UP20-XM300).

Sarcoma 180 cells were purchased from Dainippon Pharmaceutical Co. Inc. The tumor cells were maintained by being cultured in Eagle’s minimum essential medium (MEM) containing 20% fetal bovine serum (FBS). For in vivo experiments, cell suspension containing $1 \times 10^6$ cells of sarcoma 180 was inoculated in ICR/CRJ mice subcutaneously on their loins. Two mg of cartilage-extract were injected 2 or 4 times around the inoculated tumors, at 2-day intervals started on the next day of the inoculation. The same volume of saline was injected into control mice. The sizes of solid tumors were measured 3 and 5 weeks after the inoculation. Same number of tumor cells were inoculated into peritoneal cavity to have mice bearing ascites tumor. In these mice, survival time was determined. For in vitro experiments, $2 \times 10^4$ cells were placed in microwells (16 mm) of 24-well plates in 1 ml of MEM containing 20% FBS. After the cells attached to the bottom (about 5 h after plating), cartilage-extract (UP20-XM300) was added to the cultures at a final concentration of 200 µg/ml. Phosphate-buffered saline (PBS) was added to the control cultures. The medium was refreshed every other day and the same concentration of cartilage-extract was added at the same time.

Table 1 shows the effect of cartilage-extract on the growth of solid sarcoma 180. In control mice, the tumors grew slowly in the first week, then grew rapidly. Even when 2 mg of acetone-fractionated cartilage extract were injected 2 times in the first week, the growth of the tumor was not inhibited. However, when 2 mg of the further purified fraction, UP20-XM300 were injected 2 times in the first week, the tumor growth was inhibited by 60.7% and 56% after 3 and 5 weeks, respectively. In experiment II, the tumor growth was inhibited by 86% and 77% after 3 and 5 weeks, respectively. Moreover, in experiment II, the effect was striking, because visible solid tumors did not appear in 43% of the mice in the treated group. To clarify whether or not the cartilage-extract (UP20-XM300), named the cartilage-derived antitumor factor (CATF), affects sarcoma 180 cells directly, two experiments were performed. When mice were treated with intraperitoneal inoculation of sarcoma 180 cells followed by intraperitoneal injection of the same dose of CATF, no difference was observed in the survival time between treated and the control animals. Fifty percent of the mice died 17 days after the tumor inoculation in both the control group and the CATF-treated group.

When CATF was added to sarcoma 180 cultures at a concentration of 200 µg/ml, the factor did not significantly inhibit the proliferation of sarcoma 180 cells (Fig. 1). Furthermore, no significant effect on the viability of the cells was observed using trypan blue exclusion test. These results suggest that the inhibition of CATF on the growth of solid sarcoma 180 is not due to a direct effect on
Table 1 Effect of CATF on the growth of solid sarcoma 180

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Tumor size ($\times 10^3$ mm$^3$)</th>
<th>No. of mice without tumor after 3 weeks</th>
<th>No. of mice without tumor after 5 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3 weeks after inoculation</td>
<td>5 weeks after inoculation</td>
<td></td>
</tr>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>1.63±0.86</td>
<td>6.70±2.12</td>
<td>0</td>
</tr>
<tr>
<td>Acetone (45-65%)</td>
<td>7</td>
<td>2.49±2.67</td>
<td>6.87±3.62</td>
<td>0</td>
</tr>
<tr>
<td>UP20-XM300</td>
<td>7</td>
<td>0.64±0.41</td>
<td>2.95±2.09</td>
<td>1</td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>1.24±0.82</td>
<td>5.72±3.44</td>
<td>0</td>
</tr>
<tr>
<td>UP20-XM300</td>
<td>7</td>
<td>0.17±0.19</td>
<td>1.29±1.58</td>
<td>3</td>
</tr>
</tbody>
</table>

a) Around the inoculated tumors, 2 mg of cartilage-extract dissolved in saline or saline alone was injected twice in experiment I and 4 times in experiment II at 2-day intervals starting 1 day after the inoculation.

b) Sarcoma 180 ($1\times10^6$ cells) was inoculated into the loin of mice.

c) Tumor size was determined in two dimensions by slide calipers and calculated as $\frac{a^2+b}{2} \times 10^3$ mm$^3$ (a : short axis, b : long axis).

d) Numbers in parentheses : Percentage of inhibition of tumor growth defined as:

$$\frac{\text{Control} - \text{CATF-treated}}{\text{Control}} \times 100$$

*P<0.05
**P<0.01
***P<0.005

![Graph](image)

Fig. 1 Effect of CATF on proliferation of sarcoma 180 cells in culture. Sarcoma 180 ($2\times10^4$ cells) was placed in microwells (16 mm) of 24-well plates and cultured in 1 ml of MEM containing 20% FBS. After the cells attached to the bottom (about 6 h after inoculation), CATF was added at a final concentration of 200 μg/ml (●). PBS was added to control cultures (○). The medium was refreshed every other day and CATF was added at each medium change. Points are means for two wells, which varied less than 15% from the mean. Similar results were obtained in repeated experiments.
sarcoma 180 cells.

As shown in Table 1, cartilage-extract (UP20-XM300) had a marked inhibitory effect on solid tumor growth, whereas crude extract (Acetone 45-65%) had virtually no effect. This reason in not known, but it is likely that a high level of low molecular weight CDF in the crude extract\(^\text{8,10}\) suppressed the inhibitory activity on tumor growth. It is unknown how CATF inhibits the growth of solid sarcoma 180. The effect of CATF seems to be localized because CATF was not active on ascites tumors of sarcoma 180. Langer et al.\(^\text{7,8}\) showed the presence of inhibitors of tumor angiogenesis in bovine cartilage, which was obtained by 1 M GuHCl extraction followed by trypsin-affinity column chromatography. It is possible that the CATF has anti-angiogenic properties there by inhibiting the growth of solid tumors. Langer et al. also reported that the molecular weight of the major active protein was about 16,000, although the active material contained several different proteins\(^\text{7}\). Very recently, Langer and Murray\(^\text{11}\) tried to purify further this material by high pressure liquid chromatography and suggested that a higher molecular weight fraction of nearly 30,000 daltons displayed anti-angiogenic activity. Although UP20-XM300 fraction was used in our study, almost all activity was concentrated and recovered by filtration of 100,000 molecular weight cut-off membrane (data not shown). Therefore, the molecular weight of their angiogenesis inhibitor seems to be smaller than that of our CATF. From these findings, it is suggested that CATF is different from the inhibitor reported by Langer et al., even if CATF possesses inhibitory activity against tumor angiogenesis.

Kuettner et al.\(^\text{6}\) obtained collagenase inhibitors by ultrafiltration of a GuHCl extract of bovine cartilage and aorta followed by affinity chromatography on insoluble trypsin. The collagenase inhibitors from cartilage inhibited the activity of collagenase secreted by tumor cells in cultures\(^\text{9}\). The inhibitor from bovine aorta inhibited the growth of a transplantable mammary tumor and a fibrosarcoma in vivo\(^\text{6}\). From these findings, they have hypothesized that protease inhibitors in cartilage inhibit proteolytic activities elaborated by invasive cells such as vascular endothelial cells or malignant tumor cells. They also reported that the inhibitor has anti-growth activity on the fibrosarcoma cells in culture. Our CATF (UP20-XM300) also contains collagenase inhibitors (data not shown). However, the molecular weight of the inhibitors reported by Kuettner\(^\text{4,6}\) is 11,000 or less and hence, it is much lower than that of CATF. Moreover, CATF had no effect on the proliferation of B16 cells in culture.

We are now purifying and characterizing CATF. Recently, Langer and Murray\(^\text{11}\) reported dissociation of anti-angiogenic activity and protease activity in cartilage extract. Trasylol, which is identified as a trypsin inhibitor contained in cartilage extract, did not inhibit the growth of solid tumors\(^\text{10}\). Therefore, we should determine the activity of the growth inhibition on solid tumor in vivo, with the anti-angiogenic activity in vitro to purify the CATF.

REFERENCES


