EFFECT OF CONAGENIN IN TUMOR BEARING MICE
ANTITUMOR ACTIVITY, GENERATION OF EFFECTOR CELLS
AND CYTOKINE PRODUCTION

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Antitumor effects and function of T cells in tumor bearing mice given conagenin (CNG), a low molecular immunomodulator, were investigated. The administration of CNG, once a week for 4 weeks, was the most effective schedule in inhibiting growth of IMC carcinoma, a syngeneic tumor. In this regimen, cytotoxic T lymphocytes and natural killer activities in spleens of CNG treated mice were maintained at higher levels than those of non-treated mice. Lymphokine production by splenic T cells was also enhanced in cultures, whereas monokine production by macrophages, which was increased in accordance with tumor growth, was reduced by CNG administration.

The antitumor effect of CNG was not observed in mice given anti-asialo GM1 serum and in athymic mice.

Results shown in this report suggest that CNG exerts its antitumor effects through activation of T cells and enhancement of generation of antitumor effector cells.

It is known that immunomodulators such as ubenimex1), forphenicinol2) and MDP3) stimulate T cells through activation of macrophages. They have shown antitumor effects on murine transplantable tumor models4~7). Polysaccharides such as lentinan8~10), sizofilan11,12) and PS-K13) activate macrophages and T cells. However, it can be considered that the activation of macrophages induces non-specific augmentation in the host immune system, and high molecular substances such as β-glucan and LPS can show side-effects by their inflammatory activities.

Thus, we have sought immunomodulators which act on activated T cells exclusively and found conagenin (CNG), a low molecular immunomodulator, in cultured broth of Streptomyces roseosporus14). In this paper, we report antitumor effects of CNG and modulation of T cell functions in tumor bearing mice given CNG.

Materials and Methods

Mice
CDF1 mice, 6 weeks old, were purchased from Charles River Japan Inc. (Kanagawa, Japan), and were maintained under specific pathogen-free conditions at 23±1°C and 55±5% humidity. BALB/c nu/nu(−) mice, 6 weeks old, purchased from Japan SLC Inc. (Shizuoka, Japan), were kept in a clean rack with the above conditions. These mice were employed for experiments at 9~11 weeks of age.

Conagenin
Conagenin (CNG) was prepared according to the procedures reported14) by KANEKA Co. Ltd. (Osaka, Japan). For experiments, CNG was dissolved in sterilized saline.
Antitumor Activity

IMC carcinoma cells were maintained in CDF1 mice by weekly intraperitoneal transfer. Cells (1 \times 10^6 cells) were inoculated sc to the inguinal region of CDF1 mice. CNG was administered ip on various schedules starting at day 1 after tumor inoculation.

Antitumor activity was determined by measuring tumor volume (mm^3) at weekly intervals and by weighing at day 35 after the inoculation. The tumor volume was determined by the following formula:

\[ \text{Tumor volume (mm}^3\text{)} = \text{length} \times \text{width}^2 \times 0.5. \]

The percentage of inhibition of tumor weight was calculated as follows:

\[ \text{Inhibition(\%)} = \left( \frac{\text{Mean tumor weight of treated group}}{\text{Mean tumor weight of control group}} \right) \times 100. \]

Antitumor activity of CNG in immunocompromised mice was also tested. CDF1 mice were injected with 5 µl of anti-asialo GM1 serum (Wako Chemicals Co., Ltd., Tokyo, Japan) 2 days before, and 4, 10 and 16 days after inoculation of IMC carcinoma cells. These mice were inoculated sc with 1 \times 10^6 IMC carcinoma cells and given CNG once a week for 4 weeks starting at day 1 after tumor inoculation.

In another experiment, BALB/c nu/nu(−) mice were inoculated sc with 5 \times 10^5 IMC carcinoma cells and were given CNG on the same schedule as above.

Cytotoxic T Lymphocytes and Natural Killer Activities

Cytotoxic T lymphocyte (CTL) activity in splenic T cells and natural killer (NK) activity in unfractionated spleen cells prepared from CDF1 mice were determined against IMC carcinoma cells and YAC-1 cells, respectively. Nylon wool-passed spleen cells were used as T cells. 51Cr (Na251CrO4, sp.act. 14.3 GBq/mg, NEZ-030, New England Nuclear, Boston, U.S.A.) labeled IMC carcinoma cells and YAC-1 cells (2 \times 10^5 cells/ml) were incubated with effector cells at ratios of 100:1 for 16 and 4 hours, respectively. After incubation, the supernatants were collected and 51Cr radioactivity was counted in a gamma counter (ARC-300, ALOKA, Tokyo, Japan). After disruption by 1% SDS the maximum counts in target cells were determined. Triplicate determinations were made. The mean percentage of specific cytotoxicity was calculated as follows:

\[ \% \text{cytotoxicity} = \left( \frac{\text{Test count} - \text{Spontaneous count}}{\text{Maximum count} - \text{Spontaneous count}} \right) \times 100. \]

Statistical Analysis

Statistical significance was analyzed by Student's t-test.
Results

Antitumor effects of CNG at 0.5 or 5 mg/kg, doses which are effective in enhancing T cell activities in mice, were examined against IMC carcinoma in various schedules. As shown in Table 1, although administration of CNG daily at 0.5 mg/kg and every 3rd day at 5 mg/kg had significant antitumor effects of 56 and 40% inhibition, respectively, weekly administration at 0.5 to 5 mg/kg inhibited the tumor growth by 47 to 66%. From these results, the antitumor effect of CNG was hereafter examined by weekly administration. As shown in Fig. 1, CNG at 0.05 to 5 mg/kg exhibited significant antitumor effect but not at 50 mg/kg did. The tumor growth during CNG therapy is shown in Fig. 2. CNG at 5 mg/kg significantly inhibited tumor growth on 21 to 35 days after tumor inoculation.

The antitumor effect of CNG was examined in mice treated with anti-asialo GM1 serum and in athymic mice. As shown in Table 2, in normal mice CNG showed antitumor effects but did not in those immunocompromised mice.

Table 1. Antitumor effect of conagenin (CNG) in different schedules on IMC carcinoma.

<table>
<thead>
<tr>
<th>CNG (mg/kg)</th>
<th>Therapy on days (days) and tumor weight (g±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1~21</td>
</tr>
<tr>
<td>0</td>
<td>2.87±1.08 (0)*</td>
</tr>
<tr>
<td>0.5</td>
<td>1.27±0.39** (56)</td>
</tr>
<tr>
<td>5</td>
<td>1.98±1.12 (31)</td>
</tr>
</tbody>
</table>

1×10^6 IMC carcinoma cells were inoculated sc to CDF mice on day 0. CNG was administered ip on days indicated. Mean tumor weights were determined on day 35 after the inoculation. Each group consisted of 10 mice. * Inhibition rate (%) * P<0.05, ** P<0.01 and *** P<0.001 in comparison with control group.

Fig. 1. Antitumor effect of conagenin (CNG) in various doses on IMC carcinoma.

Fig. 2. Inhibitory effect of conagenin (CNG) on growth of IMC carcinoma.

- Control, ■ CNG.

1×10^6 IMC carcinoma cells were inoculated sc to CDF mice on day 0. CNG (5 mg/kg) was administered ip on days 1, 8, 15 and 22. Tumor volume was measured on days indicated. Each group consisted of 20 mice. *** P<0.001 in comparison with control group.
Table 2. Reduction of antitumor activity of conagenin (CNG) in immunocompromised mice.

<table>
<thead>
<tr>
<th>CNG (mg/kg)</th>
<th>CDF1 mice</th>
<th>CDF1 mice treated with a-ASGM1 serum</th>
<th>BALB/c nu/nu(-) mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.77±0.75</td>
<td>5.89±2.12</td>
<td>3.66±1.65</td>
</tr>
<tr>
<td>0.5</td>
<td>1.03±0.23**</td>
<td>6.44±1.13</td>
<td>2.60±1.60</td>
</tr>
<tr>
<td>5</td>
<td>1.20±0.76*</td>
<td>6.26±1.15</td>
<td>3.02±1.33</td>
</tr>
</tbody>
</table>

1×10⁶ IMC carcinoma cells were inoculated sc to CDF1 mice and anti-asialo GM1 treated CDF1 mice on day 0. 5×10⁵ cells were inoculated sc to BALB/c nu/nu(-) mice. CNG was administered ip on days 1, 8, 15 and 22 after the inoculation of tumor cells. Mean tumor weights were determined on day 35. Each group consisted of 5 mice. *P<0.05 and **P<0.01 in comparison with control group.

In the course of CNG therapy, CTL and NK activities, and cytokine production in tumor bearing mice were monitored every week for 4 weeks. As shown in Fig. 3, CTL and NK activities of antitumor effector cells in tumor bearing mice were reduced in accordance with tumor growth, whereas those effector activities in mice given CNG were maintained at normal levels. These activities were significant in late stages (21 to 28 days after tumor inoculation) of tumor growth.

Production of lymphokines by splenic T cells was determined by [³H]TdR incorporation into cytokine dependent cell lines, CTLL-2 and IC-2 cells. As shown in Fig. 4, the culture supernatants markedly enhanced the incorporation of [³H]TdR into IL-2 dependent CTLL-2 and IL-3 dependent IC-2 cells in 21 and 28 days after tumor inoculation.

On the other hand, monokine production by PEC taken from tumor-bearing mice without CNG increased gradually in accordance with tumor growth whereas it remained in the normal range in mice treated with CNG at 7, 14 and 21 days except at 28 days after tumor inoculation (7 days after the last administration of CNG).

Discussion

The influence of CNG on tumor growth, generation of antitumor effectors and cytokine production in tumor-bearing mice was investigated. Although the administration of CNG daily or on every 3rd day inhibited tumor growth significantly, the most effective schedule in inhibiting tumor growth was weekly administration starting at day 1 or day 8 after tumor inoculation which exhibited a bell-shaped dose response (Fig. 1). Since CNG does not show cytotoxicity to murine (IMC carcinoma, EL-4 thymoma,
CNG (5 mg/kg) was administered ip to IMC carcinoma bearing CDF1 mice on days 1, 8, 15 and 22 after the inoculation of tumor cells. Adherent peritoneal exudate cells were prepared on days indicated and the culture supernatants were provided to measure \[^{3}H\]TdR incorporation into cytokine dependent cell line, D10.G4.1 cells. Each group consisted of 5 mice. *P<0.05, **P<0.01 and *** P<0.001 in comparison with control group.
prevents chronic inflammatory responses caused by macrophages, although CNG does not modulate monokine production by macrophages in vitro.

In this study, it is shown that a low molecular weight immunomodulator CNG which stimulates activated T cells inhibits the growth of a syngeneic solid tumor in mice. CNG therapy, weekly for 4 times starting 1 day after tumor inoculation, enhanced lymphokine production and generation of antitumor effector cells on days 21 and 28 in tumor-bearing mice. It prevented increase of monokine production by macrophages in accordance with tumor growth. Elsewhere, it will be reported that CNG stimulated T cells in bone marrow to produce megakaryocytes and improves the reduced platelet counts in peripheral blood of mice given cyclophosphamide. A low molecular weight immunomodulator like CNG which stimulates activated T cells may be useful for cancer treatment.

Acknowledgment

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References