Modulation of Macrophage Activity in Tumor Bearing Mice by Cytogenin

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Cytogenin recovered the reduced mitogenic response to Con A of spleen cells of tumor bearing mice *in vitro*. The suppressive factor(s) was detected in adherent cell population in spleen cells. The reduced antitumor effector activity of spleen cells taken from tumor bearing mice was also augmented by the treatment with cytogenin *in vitro*. The effect of cytogenin was neutralized by the treatment with anti-Mac 1 serum. Administration of cytogenin inhibited the production of nitric oxide by macrophages which is known as one of suppressor factors. Results indicate that one possible action of cytogenin exhibiting antitumor activity in tumor bearing mice may be due to modulation of Mac 1 positive cells.

It was reported that cytogenin exerts antitumor activity through activation of antitumor effector cells such as macrophages and cytotoxic T cells in tumor bearing mice[1]. It was shown that cytogenin activates primarily macrophages to produce monokines such as interleukin 1α (IL-1α) but not tumor necrosis factor α (TNFα) and the activation leads to stimulation of proliferation and/or differentiation of T cells from normal mice and to augment production of lymphokines such as interferon γ (IFNγ) and granulocyte-macrophage colony-stimulating factor (GM-CSF)[2,3].

In this paper, we report the effect of cytogenin on mitogenic response to Con A and antitumor effector activity of spleen cells, and nitric oxide production by macrophages in tumor bearing mice.

Materials and Methods

Mice

Specific pathogen-free female CDF_1 and ICR mice were purchased from Charles Liver Japan, Inc. (Kanagawa, Japan) and were maintained under specific pathogen-free conditions at 23 ± 1°C and 55 ± 5% humidity. They were used at 7 to 10 weeks old.

Tumors

IMC carcinoma and Ehrlich carcinoma were maintained in CDF_1 mice and ICR mice by weekly intraperitoneal injection, respectively. IMC carcinoma (1×10^6 cells/mouse) and Ehrlich carcinoma (2×10^6 cells/mouse) were inoculated sc.

Cytogenin and Other Reagents

Cytogenin was prepared by Mercian Co. Ltd. (Tokyo, Japan) as reported previously[3]. Concanavalin A (Con A) was purchased from Pharmacia (Sweden). Anti-Mac1 serum[4] were purchased from Bender MedSystems (Austria). Rabbit complement, which was absorbed with agarose and mouse tissues, was purchased from Cedarlane Laboratories Ltd. (U.S.A.). [6-^{3}H]Thymidine ([^{3}H]TdR, specific activity 555KBq/mol, NET-355) was purchased from New England Nuclear (Boston, U.S.A.).

Preparation of Spleen Cells and Proliferation Assay

Spleen cells collected from normal or IMC carcinoma bearing mice at 14 days after tumor inoculation were prepared at 5×10^6 cells/ml in RPMI1640 medium (Nissui Seiyaku Co. Ltd,. Tokyo, Japan) supplemented with 10% FCS. Spleen cells were treated with or without cytogenin for 4 hours in 5% CO_2 air. After washing, cells were prepared at 2×10^5 cells/well in RPMI1640 medium supplemented with 1% FCS, 1 mM of sodium pyrubate, non-essential amino acids, 50 μM of 2-mercaptoethanol, 50 units/ml of penicillin and 50 μg/ml of streptomycin. Non-adherent cells were prepared as follows: Spleen cells were treated with or without cytogenin as above. Non-adherent cells were collected by washing thoroughly and prepared at 2×10^5 cells/well in RPMI1640 medium supplemented with 1% FCS. Those cells were cultured with Con A at 0.5 μg/ml for 48 hours, and were pulsed with [^{3}H]TdR (7.4 KBq/well) for 18 hours. The proliferation of those cells was determined by incorporation of [^{3}H]TdR into cells.

Antitumor Effector Activity of Spleen Cells Taken from IMC Carcinoma Bearing Mice and Treatment with Cytogenin *In Vitro*

Antitumor activity of spleen cells was assessed by the method reported by WINN[5]. Spleen cells collected from IMC carcinoma bearing mice were taken at 7, 14, 21 and 28 days after tumor inoculation. Spleen cells were mixed with 5×10^5 IMC carcinoma cells at a ratio of 20 : 1 and the mixture in 0.1 ml was inoculated sc to CDF_1 mice. Antitumor activity was determined on day 35 by measuring tumor weights and compared with the

antitumor activity of spleen cells collected from normal mice.

The effect of cytogenin on antitumor effector activity of spleen cells taken from IMC carcinoma bearing mice was assessed as follows: Spleen cells taken from IMC carcinoma bearing mice on day 14 after tumor inoculation were cultured with or without cytogenin for 4 hours. After washing, the antitumor activity of cytogenin-treated cells was determined as same as above.

Neutralization of Antitumor Effector Activity

Spleen cells taken from IMC carcinoma bearing mice were cultured with or without cytogenin for 4 hours, and then cytogenin was washed out by centrifugation. To remove macrophages, spleen cells were incubated with a diluted (40 folds) anti-Mac1 serum at 4°C for 30 minutes. After washing, cells were incubated with diluted (20 folds) complement at 37°C for 60 minutes. Then, cells were washed and antitumor activity of spleen cells treated with anti-Mac 1 serum was determined as mentioned above.

Production of Nitric Oxide by Macrophages

Ehrlich carcinoma cells were inoculated sc to ICR mice. Cytogenin at 100mg/kg was administered po to mice from day 1, daily for 20 days. Peritoneal exudate cells (PEC) were collected from the mice on days 7, 14, and 21 after tumor inoculation, and prepared at 1×10^6 cells/ml in RPMI1640 medium supplemented with 10% FCS. After incubation for 1 hour at 37°C, non-adherent cells were removed and adherent cells as macrophages were collected. Macrophages (5×10^4 cells/well) were cultured with 5 μg/ml of lipopolysaccharide (LPS) for 2 days.

In next, effect of cytogenin on nitric oxide production *in vitro* was examined. Macrophages collected from tumor bearing mice at 21 days after tumor inoculation were cultured with LPS and cytogenin at 0.001 to 10 μg/ml for 2 days.

Concentration of nitric oxide in the culture supernatants was measured by the method reported by KELLER *et al.*[6]. Briefly, 100 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, 2.5% H_3PO_4) was mixed with 100 μl of the culture supernatants and incubated for 10 minutes at room temperature. The absorbance at 540 nm was measured by a microplate reader (Titertech Multiscan, Flow Laboratories Inc., U.S.A.). Nitric oxide concentration was calculated from a $NaNO_2$ standard curve.

Statistical Analysis

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

Results

Recovery of Mitogenic Response to Con A of Spleen Cells Taken from Tumor Bearing Mice by Cytogenin

It is known that mitogenic response to Con A of lymphocytes taken from tumor bearing mice is reduced in the presence of the suppressor factor induced by tumor growth[7]. As shown in Table 1, the marked reduction in mitogenic response to Con A of spleen cells taken from IMC carcinoma bearing mice was observed on day 14 after implantation of tumor cells. In this test system, the reduced mitogenic response of spleen cells treated with cytogenin for 4 hours at 0.001 to 1 μg/ml *in vitro* were recovered to more than normal level. The successive treatment of spleen cells with cytogenin for 3 days was not effective in restoring the reduction at 0.001 to 10 μg/ml(data not shown). In this conditions, cytogenin showed no cytotoxicity against spleen cells.

To clarify which cell population, lymphocytes or macrophages, in spleen cells causes to suppress the mitogenic response to Con A, non-adherent cells were prepared from spleen cells of tumor bearing mice and the mitogenic response to Con A of non-adherent cells was determined. As shown in Table 1, although the response to Con A of whole spleen cells from tumor bearing mice was reduced markedly, the mitogenic response to Con A of non-adherent cells were not affected and cytogenin did not show any effect. It indicates that adherent cells produce suppressor factors and cytogenin acts on adherent cells to inhibit the reduction.

Table 1. Effect of cytogenin on Con A induced mitogenic response to spleen cells taken from IMC carcinoma bearing mice.

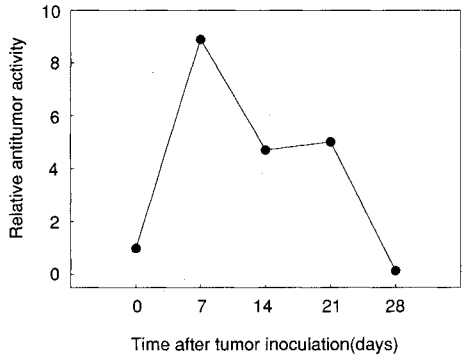
Spleen cells from	Cytogenin (μg/ml)	Whole spleen cells		Non-adherent spleen cells	
		cpm ± SD	T/C (%)	cpm ± SD	T/C (%)
Normal mice	0	18,937 ± 932		19,150 ± 969	
Tumor bearing mice	0	6,948 ± 1,850	100	18,063 ± 945	100
	0.001	30,535 ± 2,109***	440	19,011 ± 1,420	105
	0.01	28,312 ± 867***	408	18,969 ± 702	105
	0.1	33,882 ± 1,164***	448	18,352 ± 972	102
	1	25,949 ± 1,220***	374	17,904 ± 258	99
	10	12,409 ± 5,053	179	18,610 ± 253	103

*** $P < 0.001$ against non-treated tumor bearing group.

Recovery of Antitumor Effector Activity of Spleen Cells Taken from IMC Carcinoma Bearing Mice by Cytogenin

Antitumor effector activity of spleen cells taken from IMC carcinoma bearing mice after implantation of tumor cells was monitored by WINN assay. As shown in Fig. 1, the highest effector activity was observed on day 7, it was reduced in accordance with tumor growth and disappeared on day 28. Thus, the effect of cytogenin on antitumor effector activity of spleen cells was examined. Spleen cells taken from tumor bearing mice on day 14 were treated with cytogenin at 0.01 to 0.1 μg/ml *in vitro* and the antitumor effector activity was examined. As shown in Table 2, cytogenin at either concentration recovered the antitumor activity of spleen cells and the number of tumor-free mice was increased. As reported[8,9], antitumor effector cells sensitive to IMC carcinoma cells have been shown to be cytotoxic T lymphocyte (CTL) and macrophages. It is possible that cytogenin might modulate macrophage function in spleen cells to be effector cells in this test system. In order to determine whether macrophages are required for the effect of cytogenin, spleen cells were treated with anti-Mac 1 serum and cytogenin, and the antitumor activity was examined. As shown in Table 3, the effect of cytogenin disappeared after the treatment of spleen cells with anti-Mac 1 serum.

Fig. 1. Antitumor activity of spleen cells taken from IMC carcinoma bearing mice.



$$\text{Relative antitumor activity} = \frac{\text{Inhibition rate of spleen cells taken from tumor bearing mice against IMC carcinoma}}{\text{Inhibition rate of spleen cells taken from normal mice against IMC carcinoma}}$$

Table 2. Activation of antitumor effector cells in spleen cells taken from IMC carcinoma bearing mice by cytogenin.

Spleen cells from	Cytogenin (μg/ml)	Tumor weight (mg±SE)	Tumor free-mice
—	0	1,987±333	0/10
Tumor bearing mice	0	2,079±837	0/5
	0.01	178	4/5
	0.1	118± 95	3/5

Inhibitory Effect of Cytogenin on Nitric Oxide Production by Macrophages

As mentioned above, it is obvious that cytogenin modulate functions of macrophages as a suppressor factor in tumor bearing mice to restore the reduced response to Con A of splenic lymphocytes and the reduction of antitumor effector activity. For this reason, the production of nitric oxide, one of the chemical suppressor factors produced by macrophages, was monitored in tumor bearing mice given cytogenin. Mice were inoculated with Ehrlich carcinoma sc and given cytogenin at 100 mg/kg po, starting day 1 and daily for 20 days and the production of nitric oxide by peritoneal macrophages was monitored. As shown in Fig. 2, the production of nitric oxide was increased in accordance with tumor growth and the administration of cytogenin inhibited the production on days 14 and 21 significantly. Then, we investigated the inhibitory effect of cytogenin against nitric oxide production by macrophages *in vitro*.

As shown in Table 4, cytogenin was inhibited nitric oxide production of macrophages taken from tumor

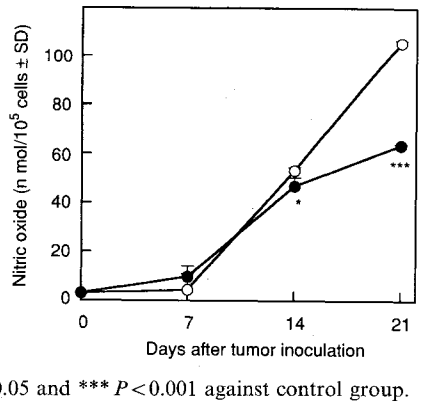
Table 3. Reduction of cytogenin-activated antitumor effector activity by anti-Mac 1 serum.

Spleen cells from	Cytogenin (μg/ml)	anti-Mac 1	Tumor weight (mg±SE)	Tumor free-mice
—	0	−	2,421± 320	0/10
Tumor bearing mice	0	−	1,023± 218	0/7
	0.01	−	340± 141*	2/5
	0	+	1,836± 546	0/5
	0.01	+	2,501±1,290	0/5

* $P<0.05$ against cytogenin non-treated group.

Fig. 2. Nitric oxide production of macrophages taken from Ehrlich carcinoma bearing mice given cytogenin.

○ Control, ● cytogenin.



* $P<0.05$ and *** $P<0.001$ against control group.

Table 4. Effect of cytogenin on nitric oxide production of macrophages taken from tumor bearing mice *in vitro*.

Cytogenin (μg/ml)	Nitric oxide (nmol/10^5 cells$\pm$SD)	T/C (%)
0	66.5$\pm$5.5	100
0.001	65.4$\pm$4.7	99.8
0.01	55.6$\pm$5.1*	84.9
0.1	54.3$\pm$7.3*	82.9
1	51.2$\pm$3.3**	78.2
10	45.5$\pm$3.1***	69.5

* $P<0.05$, ** $P<0.01$ and *** $P<0.001$ against control group.

bearing mice *in vitro* at 0.01 to 10 μg/ml significantly, although inhibitory ratio was weak (15 to 30%) compared with the inhibition *in vivo*.

Discussion

As reported previously[2,3], it has been shown that cytogenin activates macrophages to produce monokine such as IL-1α. The activation stimulates production of lymphokines such as IFNγ and GM-CSF, and generation of antitumor effector cells in tumor bearing mice. In the course of studies on the action of cytogenin, we found that it restored the reduced mitogenic response to Con A of splenic lymphocytes taken from tumor bearing mice. It is known that the suppressor factors induced in tumor bearing mice are derived from macrophages[10,11] and T cells[12,13]. In IMC carcinoma bearing mice, it was determined that the mitogenic response to Con A of spleen cells was affected by adherent cells and cytogenin restored the reduced mitogenic response to Con A of spleen cells to normal level.

These results led us to examine the effect of cytogenin on activation of antitumor effector cells reduced in tumor bearing mice. We found that cytogenin restored the reduced effector activity. The effect of cytogenin disappeared in spleen cells treated with anti-Mac 1 serum. These results suggest that cytogenin modulates macrophages to remove the suppressor activity in spleen cells and to restore their antitumor effector activity.

It is known that chemical mediators produced by macrophages, such as prostaglandins[14] and nitric oxide[15,16] *etc.* can be suppressor factors. Then the production of nitric oxide by macrophages from tumor bearing mice given cytogenin was examined. Although the production of nitric oxide was increased in accordance with tumor growth, the administration of cytogenin inhibited the production on days 14 and 21 after tumor inoculation. As reported[2], the administration of cytogenin augments the production of superoxide anion and IL-1α by macrophages from normal mice and it does not inhibit prostaglandin synthesis. Though it is known that production of nitric oxide by macrophages is enhanced by inflammatory mediators, IL-1 and TNF do not enhance nitric oxide production but oxygen radicals by macrophages[17]. For this reason, it can be thought that one of chemical suppressor factors which are inhibited by administration of cytogenin is nitric oxide. Since cytogenin inhibited only 15 to 30% of nitric oxide production *in vitro* test system, the activity of cytogenin on activation of antitumor effector in tumor bearing host is due to not only nitric oxide but also other factors which are mediated by some cytokines, such as IL-10 and IL-12.

We found that cytogenin modulate macrophage function and restores the reduced proliferative and antitumor effector activities of spleen cells in tumor bearing mice. The activity of cytogenin may useful for the treatment of cancer and inflammatory diseases. The mechanism of action of cytogenin is now under study.

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References

1) KUMAGAI, H.; T. MASUDA, M. ISHIZUKA & T. TAKEUCHI: Antitumor activity of cytogenin. J. Antibiotics 48: 175~178, 1995
2) KUMAGAI, H.; M. OSONO, M. SAKASHITA, M. ISHIZUKA & T. TAKEUCHI: Action of cytogenin on lymphoid cells and their cytokine production. J. Antibiotics 48: 317~320, 1995
3) KUMAGAI, H.; T. MASUDA, M. OSONO, S. HATTORI, H. NAGANAWA, T. SAWA, M. ISHIZUKA & T. TAKEUCHI: Cytogenin, a novel antitumor substance. J. Antibiotics 43: 1505~1507, 1990
4) MILLER, L. J.; R. SCHWARTING & T. A. SPRINGER: Regulated expression of Mac-1, LFA-1, p150,95 glycoprotein family during leukocyte differentiation. J. Immunol. 137: 2891~2900, 1986
5) WINN, H. J.: Immune mechanisms in homotransplantation. II. Quantitative assay of the immunologic activity of lymphoid cells stimulated by tumor homografts. J. Immunol. 86: 228-239, 1961
6) KELLER, R.; M. GEIGES & R. KEIST: L-Arginine-dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. Cancer Res. 50: 1421~1425, 1990
7) LEJEUNE, P.; P. LAGADEC, N. ONIER, D. PINARD, H. OHSHIMA & J. F. JEANNIN: Nitric oxide involvement in tumor-induced immunosuppression. J. Immunol. 152: 5077~5083, 1994
8) KAWATSU, M.; T. YAMASHITA, M. OSONO, T. MASUDA, M. ISHIZUKA & T. TAKEUCHI: Effect of conagenin in tumor bearing mice. Antitumor activity, generation of effector cells and cytokine production. J. Antibiotics 46: 1692~1698, 1993
9) ISHIZUKA, M.; T. MASUDA, S. MIZUTANI, T. TAKEUCHI & H. UMEZAWA: Antitumor cells found in tumor-bearing mice given ubenimex. J. Antibiotics 40: 697~701, 1987
10) FUJII, T.; T. IGARASHI & S. KISHIMOTO: Significance of suppressor macrophages for immunosurveillance of

tumor-bearing mice. J. Natl. Cancer Inst. 78: 509~517, 1987

11) ALLEVA, D. G.; C. J. BURGER & K. D. ELGERT: Interferon-γ reduces tumor induced Ia$^-$ macrophage-mediated suppression: Role of prostaglandin E2, Ia and tumor necrosis factor-α. Immunopharmacol. 25: 215~227, 1993

12) BLOOM, B. R.; P. SALGAME & B. DIAMOND: Revisiting and revising suppressor T cells. Immunol. Today 13: 131~135, 1992

13) GEER, J. M. & W. J. HALLIDAY: Comparison of T suppressor factors from tumor-bearing mice and immunized with a monoclonal anti-idiotypic antibody. Cancer Immunol. Immunother. 31: 151~156, 1990

14) GOODWIN, J. S. & J. CEUPPENS: Regulation of the immune response by prostaglandins. J. Clin. Immunol. 3: 295~315, 1983

15) MILLS, C. D.: Molecular basis of "suppressor" macrophages.: Arginine metabolism via the nitric oxide synthetase pathway. J. Immunol. 146: 2719~2723, 1991

16) ALLEVA, D. G.; C. J. BURGER & K. D. ELGERT: Tumor-induced regulation of suppressor macrophage nitric oxide and TNF-α production: Role of tumor-derived IL-10, TGF-β, and prostaglandin E2. J. Immunol. 153: 1674~1686, 1994

17) DING, A. H.; C. F. NATHAN & D. J. STUEHR: Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: Comparison of activating cytokines and evidence for independent production. J. Immunol. 141: 2407~2412, 1988