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ALTERATION OF NATURAL KILLER (NK) ACTIVITY WITH GABEXATE MESILATE (FOY) AND ITS METABOLITE, ε-GUANIDINOCAPROIC ACID (GCA)

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For the development of an effective immunotherapy for cancer patients, I studied the effect of gabexate mesilate (FOY) and its metabolite, ε-guanidinocaproic acid (GCA), on natural killer (NK) activity in vitro, and found that the pretreatment with GCA of the peripheral blood mononuclear cells (PBMC) of both healthy volunteers and gastric cancer patients resulted in the augmentation of NK activity, whereas that with FOY did not. Further investigations revealed that the same pretreatment of PBMC with GCA had little effect on the production of either IL-2 or IL-4, or on the expression of IL-2 receptor. Therefore, the effect of these agents on NK activity may be independent of the effect of these cytokines.

These results indicate that FOY, which is widely used in postoperative cancer patients, and especially its metabolite, GCA, are theoretically useful for the treatment of cancer patients.

Introduction

Only a few studies have been carried out on the possible antineoplastic activities of gabexate mesilate (FOY)[1)2)]. Yet no study in tumor-bearing humans has come to the author's knowledge, and the detailed mechanism of this agent against growing tumors is still obscure. Such mechanism may comprise direct or indirect effects. On the other hand, our clinical experiences seem to suggest that FOY may alter the effects of various immunocompetent cells, including the activity of the natural killer (NK) cells. Since FOY is widely used in Japan and in Europe for cancer patients, such as for those with postoperative acute pancreatitis or disseminated intravascular coagulation, clarification of the antineoplastic activity of this agent seems important. Simultanelously, it is also well documented[3)4)] that FOY administered is rapidly metabolized to ε-guanidinocaproic acid (GCA) in vivo.

The purpose of the present study was to examine the effects of FOY and its metabolite, GCA, on the activity of NK cells, as well as to investigate the production of various cytokines and the expression of their receptors in the exertion of NK activity.

Materials and Methods

Reagents used in this study

Medium: Unless otherwise stated, all assays were performed in complete medium (CM), consisting of RPMI 1640 (Gibco) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS) (Gibco).

FOY (MW=417.48) and its metabolite, GCA, as sulfonate (MW=269.31) were supplied by Ono Pharmaceutical Co., Osaka, Japan.

Peripheral blood mononuclear cells (PBMC)

Twenty gastric cancer patients undergoing surgery at our Department and 20 healthy vol-

unteers were examined in this study. All the patients who donated their blood were diagnosed pathologically as having gastric carcinoma. Healthy volunteers were used as controls.

PBMC obtained from both patients and healthy volunteers were purified by the Ficoll-Hypaque (specific gravity=1.077) density sedimentation technique and adjusted with RPMI supplemented with 10% FCS and FOY or GCA to final concentrations.

Pretreatment of PBMC with FOY or GCA

To determine the optimal pretreatment condition, PBMC from healthy volunteers were cultured in Corning 12-well culture plates with various concentrations of FOY or GCA for 6 hours to 4 days. PBMC from gastric cancer patients were cultured for 24 hours at 1.0×10^6 cells/ml with 0.5 mM GCA in CM, using Corning 12-well culture plates, in a humidified environment with 5% CO_2 at 37°C.

NK activity

NK activity was measured using a standard 4-hour chromium release assay with erythroleukemic cell line K562 as target. Labeling of K562 was performed by incubation of cells with chromium 51 (^{51}Cr, Japan Atomic Energy Research Institute, Tokyo, Japan) for 90 min. The cells were then washed with phosphate buffer solution (PBS), centrifuged at 1,000 rpm for 5 min, washed again, counted, and adjusted to 5.0×10^3 cells/ml in CM. A mixture of 1.0×10^5 effector and 5.0×10^3 target cells (E:T=20:1) was incubated in a humidified environment with 5% CO_2 at 37°C for 4 hours on a 96-well Linbro® tissue culture plate. Chromium 51 released in the supernatant was harvested using a Titertek® supernatant collection system (press, fork, rack, harvesting frame, and transfer tube strips). Then the radioactivity was measured with a gamma counter (Aloka Auto Well Gamma System ARC-500, NaI scintillation counter).

Percent-specific ^{51}Cr release was calculated according to the following formula:

$(E-S)/(M-S) \times 100$ (%),

where E is the ^{51}Cr release in the presence of effector cells, S is the spontaneous ^{51}Cr release in the presence of medium, and M is the maximum release obtained by the addition of 7% 7X® (Flow Laboratories) into targets.

All assays were done in triplicate. Viability of the cells was determined by the trypan blue (0.5%) exclusion test.

Measurement of IL-2, IL-4, and IL-2 receptor

PBMC from healthy volunteers were cultured in CM with final concentrations of 0.003 mM to 3 mM of GCA for 2 days at 37°C under 5% CO_2 as pretreatment on Corning 24-well tissue culture plates at 2×10^6 cells/500 μl/well. These cells were then washed and resuspended in CM containing 5 μg/ml concanavalin A (Sigma Laboratories) and cultured for 2 more days.

After the 48-hour cultivation, the cells and the supernatant were isolated by centrifugation. The cells were washed with Hank's balanced salt solution 3 times, and were then used for the measurement of IL-2 receptor. The supernatant was used for that of IL-2 and IL-4.

1) Measurement of IL-2

The supernatant obtained from the in vitro culture of PBMC with concanavalin A was diluted with CM 1, 2, 4, 8, 16, and 32 times. The diluted supernatant was added into a Corning 96-well culture plate, 100 μl per well. Recombinant IL-2 (Takeda Pharmaceutical Co., Osaka, Japan) was adjusted to 15 ng/ml (ca. 1,260 U/ml) and was used as a standard.

One hundred μl of CTLL-2 suspension, adjusted to 1.0×10^5 cells/ml, was added to each well, and the wells were cultured for 24 hours in a CO_2 incubator at 37°C, with or without the culture supernatant. After 24 hours of incubation, the plate was centrifuged (2,000 rpm × 2 min, at 25°C) and the supernatant was removed. RPMI medium containing 0.5 mg/ml of MTT (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolum bromide; Dojindo Laboratories, Kumamoto, Japan) was added, and incubation was further carried out for 3 hours at 37°C under 5% CO_2.

After this incubation, the plate was again cen-

trifuged (2,000 rpm × 2 min at 25°C) to remove the supernatant, and 200 μl of ethanol was added to each well and allowed to stand for 5 min. The optical density (OD) was measured with a microplate optical densitometer (sample, 570 nm; reference, 690 nm). The average OD for each sample was divided by that of the negative control. This value for each sample was defined as its titer for IL-2.

2) Measurement of IL-4

Monoclonal antibody for human IL-4, No. 380-1 (murine $IgG_1\kappa$, Ono Pharmaceutical Co., Osaka, Japan) was adsorbed to 96-well EIA plates for 24 hours. After washing, 300 μl/well of blocking solution (Behringer No. 1112589) was added and made to react for 4 hours. After removing the blocking solution, the plate was washed and the supernatant collected after stimulation with concanavalin A was added at a quantity of 100 μl/well and left overnight. In this assay, recombinant human IL-4 (Ono Pharmaceutical Co., Osaka, Japan) prepared in 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 0.0 pg/ml was used as a standard. After being left overnight, 100 μ/well of polyclonal rabbit IgG anti-human IL-4 antibody (Ono Pharmaceutical Co., Osaka, Japan) was added and left for 2 hours. After the supernatant was removed, anti-rabbit IgG labeled with peroxidase (goat IgG; Zymed No. 62-610-2) was added at 100 μg/well and left to react for 3 hours. Ortho-phenylenediamine and hydrogen peroxide were added and left to react for 10 min at room temperature. OD at 450 nm was measured with ELISA. IL-4 production was calculated with the standard curve of "standard" IL-4.

3) Measurement of cell surface receptors for IL-2

Fifty μl of monoclonal antibody for the α-chain of human IL-2 receptor, labeled with FITC (MOC Co., IT-0201), was added to 2×10^6 pretreated PBMC and incubated for 30 min at 0°C. Excess antibody was removed by washing the cells with 2 ml of Hank's balanced solution 3 times and the cells were suspended in 300 μl of the same solution. Fluorescence of FITC was measured by flow cytometry (Beckton Dickinson Immunocytometry System).

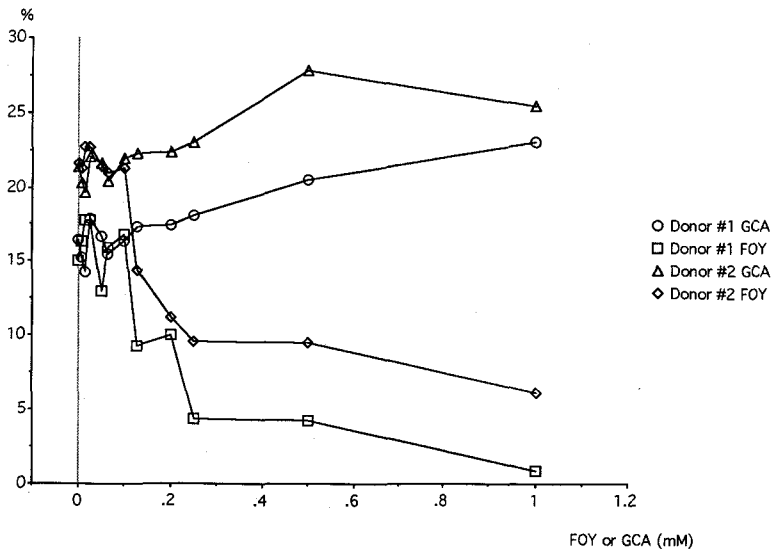

Fig. 1 NK Activity after Pretreatment
NK activities after pretreatment of PBMC from healthy volunteers with FOY or GCA are shown. The PBMC were incubated with FOY or GCA for 48 hours here. Note that the pretreatment with GCA at the concentration of 0.5 mM augmented NK activity. The same treatment with FOY actually suppressed NK activity at the same concentration.

Statistical analysis

The paired t-test was used to compare the results. Calculation was done on an Apple Macintosh® IIfx. Microsoft Excel® and StatView II® were used.

Results

The effect of FOY and GCA on NK activity

To examine the effects of FOY and GCA on NK activity, I pretreated PBMC obtained from healthy volunteers with various doses of these agents and subsequently examined their cytotoxicities against NK-sensitive K562. The effects of FOY and GCA on NK activity are represented in Fig. 1. Regarding the effect of FOY, I found that at any dose used in this study, FOY significantly reduced NK activity. On the other hand, GCA augmented NK activity when PBMC were pretreated with 0.5 mM GCA. However, higher doses of GCA suppressed NK activity.

To examine more precisely the augmenting effect of GCA on NK activity, I examined whether different pretreatment periods, in this case 12 and 24 hours, with GCA of PBMC would affect NK activity differently. As shown in Fig. 2, the 12-hour pretreatment had little effect on NK activity (9.4 ± 2.9 vs. 6.6 ± 2.5; n.s.), whereas the 24-hour pretreatment significantly augmented NK activity (13.8 ± 4.3 vs. 8.7 ± 3.0; $p<0.05$ with the paired t-test).

Similar studies were performed by using PBMC obtained from 20 healthy volunteers and 20 gastric cancer patients. Their results are represented in Fig. 3. In both studies, the pretreatment of these cells with 0.5 mM GCA significantly ($p<0.01$ with the paired t-test) enhanced their NK activities (30.1 ± 10.8 vs. 23.2 ± 8.2 in healthy volunteers and 17.4 ± 11.4 vs. 14.0 ± 10.3 in gastric cancer patients; $p<0.01$ in both studies).

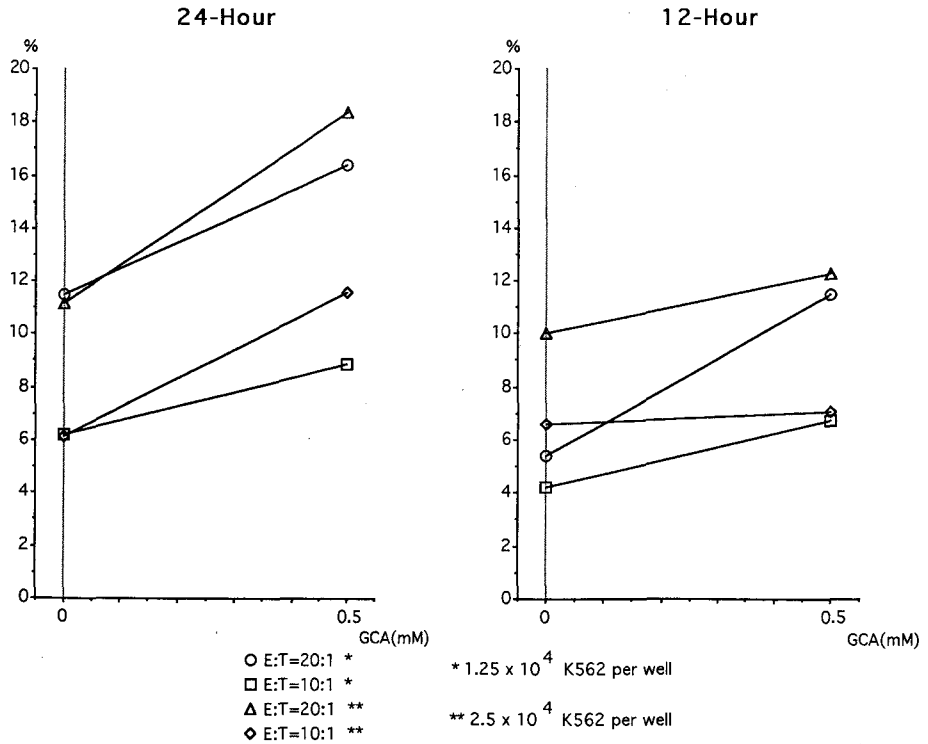

Fig. 2 NK Activity after Pretreatment
NK activities after pretreatment of PBMC with GCA for 12 and 24 hours are shown. The 24-hour treatment (left) significantly augmented NK activity, whereas that for 12 hours (right) did not.

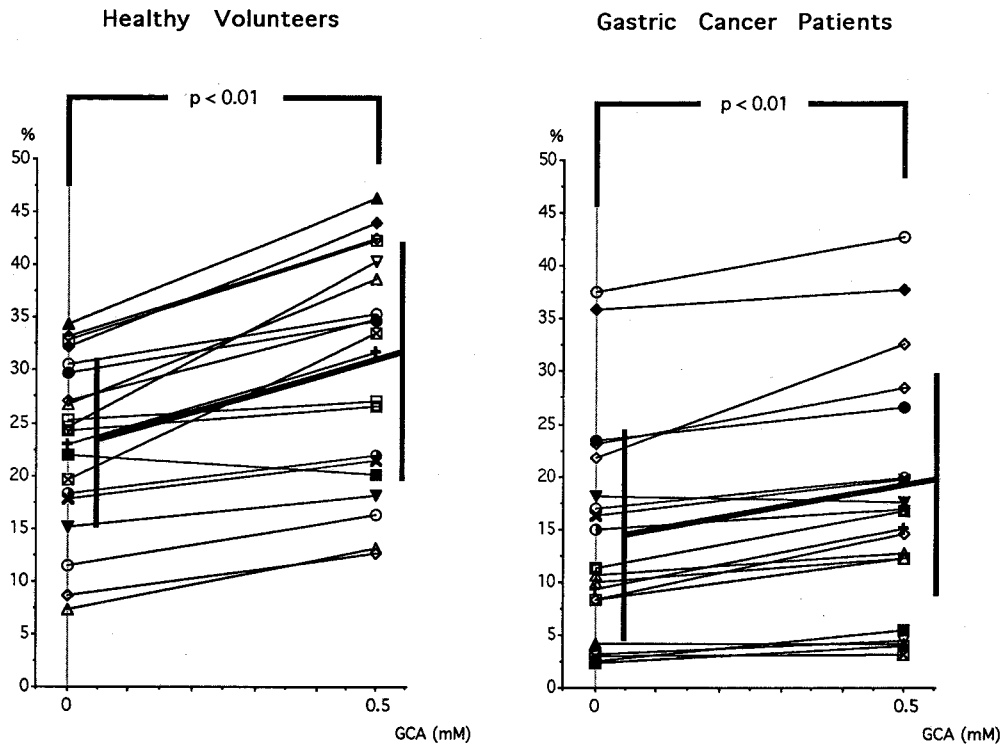

Fig. 3 NK Activity after Pretreatment
NK activities of PBMC from healthy volunteers and gastric cancer patients after pretreatment with 0.5 mM GCA for 24 hours are shown. NK activity was significantly augmented in both healthy volunteers (left) and gastric cancer patients (right).

Table Effect of GCA on IL-2 Receptor Expression, IL-2 Production, and IL-4 Production

Group	IL-2 receptor expression		IL-2 production		IL-4 production	
	(%)	r.v.(%)	(U/ml)	r.v.(%)	(pg/ml)	r.v.(%)
Blank	3.8±2.5		0.3±0.6		0.0±0.0	
Control	47.4±25.0	100±0	54.7±49.0	100±0	18.0±17.5	100±0
GCA 0.003 mM	47.8±25.0	105.3±9.9	55.0±51.2	99.3±9.1	21.5±19.9	125.7±10.6
GCA 0.01 mM	46.7±29.0	99.7±28.1	61.3±56.2	108.3±47.1	23.3±25.6	104.7±51.7
GCA 0.03 mM	42.4±35.9	81.7±45.7	55.3±69.3	75.0±65.4	20.6±23.0	85.3±67.9
GCA 0.1 mM	47.4±21.0	107.3±23.2	65.7±59.2	117.0±17.7	23.8±26.0	111.0±40.4
GCA 0.3 mM	45.6±21.9	102.0±20.7	55.7±54.6	101.7±49.6	22.7±27.1	121.7±50.4
GCA 1 mM	47.6±25.9	104.3±34.8	72.7±39.7	162.7±75.4	25.5±27.8	125.7±25.5
GCA 3 mM	37.4±28.7	74.7±10.0	55.3±35.3	116.3±38.6	18.2±18.5	107.0±29.1

r.v.; relative value
The effect of pretreatment of PBMC with various concentrations of GCA is shown. In this table, the pretreatment time was 48 hours. No statistically significant difference was observed. The effect of GCA on NK activity did not seem to depend on these cytokines. "Blank" was cultured under the same conditions without IL-2 or CTLL-2, and "Control" was similarly cultured without IL-2 but with CTLL-2.

The effect of FOY and GCA on cytokines and on the expression of cell surface receptors

To analyze the mechanism of GCA on the immune response at the molecular level, I attempted to examine its effect on cytokines and on the cell surface expression of IL-2 receptor. PBMC

obtained from healthy volunteers were pretreated with various amounts of GCA, and subsequently their IL-2 and IL-4 productions and IL-2R expression were examined. As shown in Table, no significant elevation was observed.

Discussion

It is well documented[5] that various immune responses are impaired in cancer patients. For example, many of them have a defective autologous mixed lymphocyte reaction, a low level of NK activity[6][7] and the induction of LAK cells is also defective in many cancer patients[8][9]. These impaired immune responses are believed to be among the important factors for the growth of tumor cells. In fact, deletion of NK cells by the treatment with anti-asialomonoganglioside (anti-asialo-GM_1) results in a successful inoculation of tumor cells into the mouse[10]. Therefore, one might expect that the repair of these impaired immune responses would be an important factor for the development of effective therapies for cancer patients. For this reason, several recent reports have focused on the augmenting effect of various agents, including some traditional Chinese and Japanese herbal drugs[11][12] and biological response modifiers[13][14] on immune responses. As shown in this study, FOY in vitro could not augment NK activity. However, this drug is rapidly metabolized to GCA in vivo[15]~[17] and hence similar studies with GCA might be useful for a precise analysis of the effect of FOY on the NK activity. As shown in Fig. 1, GCA augmented NK activity. The same results were obtained from both the PBMC of gastric cancer patients and healthy volunteers.

The chemical structure of GCA is similar to that of arginine, which is α-amino-δ-guanidinovaleric acid. Arginine has been well documented as having immunopotentiative and antineoplastic effects[18]~[20]. The detailed mechanism of the immunopotentiative activity of arginine is still obscure. However, it was reported[21] that polyamines might play an important role in the augmenting effect of arginine on NK activity, suggesting that the effect of arginine is not related to the action of cytokines. Consequently the present study was directed at an attempt to determine the effect of GCA on the production of either IL-2 or IL-4 and/or the expression of IL-2 receptors. No remarkable change in the production of IL-2 or IL-4, or expression of IL-2 receptors on PBMC was observed by the pretreatment of PBMC with GCA alone (data not shown). In addition, the production of either of these cytokines or the expression of IL-2 receptors on ConA-stimulated PBMC was not altered by the pretreatment with GCA, suggesting that GCA did not form activated T-cell. So I found that GCA had no significant relation with these immunological parameters. Therefore, taken together, I think that the effect of GCA on NK activity is not dependent on the actions of these cytokines. I think that GCA has direct effect on NK activity, and this may be related to that of arginine or other substances such as leukotrienes.

In conclusion, it was determined that GCA, a metabolite of FOY, augmented NK activity without the participation of IL-2 or IL-4. Although there may be some direct antineoplastic effect of FOY, the eventual effectiveness of FOY in the treatment of cancer patients must be due to the positive effect of its metabolite, GCA, which was shown to augment the NK activity in the present study. This means that administration of GCA may be clinically useful.

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メシル酸ガベキサート（FOY）とその代謝産物 ε-グアニジノカプロン酸（GCA）の NK 活性に及ぼす影響

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近年免疫療法が癌の治療法として注目されているが，今回メシル酸ガベキサート（FOY）と，その代謝産物 ε-グアニジノカプロン酸（GCA）の NK 活性に及ぼす影響を in vitro で検討した．

その結果，健常人および胃癌患者にて，末梢単核細胞を GCA で前処理すると，NK 活性の上昇が認められたが，FOY では却って低下した．この系でインターロイキン 2 および 4，またインターロイキン 2 受容体を測定したが，有意な差を認めず，これらの関与はないと考えられた．

以上より，胃癌術後患者にしばしば用いられる FOY とその代謝産物 GCA は，理論的に癌患者の治療に有用であると考えられた．