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IN VITRO AUGMENTATION OF THE CYTOTOXIC ACTIVITY OF PERIPHERAL BLOOD MONONUCLEAR CELLS AND TUMOR-INFILTRATING LYMPHOCYTES BY FAMOTIDINE IN CANCER PATIENTS

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Abstract — We investigated the *in vitro* effects of famotidine on the cytotoxic activity of peripheral blood mononuclear cells (PBMC) and tumor-infiltrating lymphocytes (TILs). The cytotoxic activity of PBMC was augmented by famotidine at a concentration of 10 ng/ml, which is equivalent to the serum level achieved by the intravenous administration of a dose of 20 mg. This response to famotidine was seen only in cancer patients. Both the cytotoxic activity and DNA synthesis of activated TILs were increased by the combination of interleukin-2 and 1 μg/ml of famotidine. Augmentation of cytotoxic activity by famotidine occurred independently of any decrease in the population of suppressor T-cells.

Thus, famotidine may have the potential to be used in adoptive immunotherapy with TILs for cancer patients.

Histamine has a variety of suppressive effects on lymphocyte function, including the suppression of cytotoxic T-lymphocytes (Bourne, Lichtenstein, Melmon, Henney, Weinstein & Shearer, 1974), the down-regulation of some cytokines (Bourne *et al.*, 1974; Rigal, Monier & Souweine, 1979), the suppression of lymphocyte proliferation (Plaut, Lichtenstein & Henney, 1975), and the activation of suppressor T-cells by histamine-induced suppressor factor (Rocklin, 1977; Rocklin, Greineder & Melmon, 1979). Histamine acts on lymphocytes via the histamine-2(H_2) receptor (Black, Duncan, Durant, Genellin & Parsons, 1972; Rocklin, 1976), so H_2 receptor antagonists can block the suppression of lymphocyte function induced by histamine (Cavagnaro & Osband, 1980; Osband, Carpinito, Levine, Hamilton & Krane, 1989). It has been suggested that the inhibition of various suppressor factors such as suppressor T-cells may be important in the performance of successful adoptive immunotherapy, and it has been shown that suppressor T-cells express H_2 receptors on the cell surface (Rocklin, Greineder, Littman & Melmon, 1978; Rocklin, Beard, Gupta, Good & Melmon, 1980; Khan, Sansoni, Engleman & Melmon, 1985; Melmon & Insel, 1977). In this study, we investigated the effects of famotidine, a H_2 receptor antagonist, on the cytotoxic activity of peripheral blood mononuclear cells (PBMC) and tumor-infiltrating lymphocytes (TILs).

EXPERIMENTAL PROCEDURES

Materials

Famotidine, 3-{{{2-{(diaminomethylene)amino}-4-thiazolyl}methyl}thio}-*N*^{2}-sulfamoylpropionamidine, was supplied by Yamanouchi Pharmaceutical Co. (Japan). Recombinant human interleukin-2 (rhIL-2) was obtained from Shionogi Pharmaceutical Co. (Japan). RPMI 1640 medium (Gibco, Grand Island, New York, U.S.A.) was supplemented with 10% heat-inactivated human AB serum obtained from healthy donors, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 mmol/L-glutamine to produce complete medium.

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Tumor cell lines

The following established tumor cell lines were used: K562, a natural killer (NK)-sensitive cell line established from human erythroleukemia cells; Daudi, an NK-resistant cell line established from human Burkitt's lymphoma; and KATO-III, an NK-resistant cell line established from human signet ring cell carcinoma.

Separation of PBMC, TILs, and autologous tumor cells

PBMC were collected from 21 patients with gastrointestinal tract cancer and 13 healthy subjects by layering blood samples onto Ficoll – Hypaque gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and performing centrifugation at 400 ***g*** for 30 min at 20°C.

Surgical specimens were obtained from 15 patients with gastrointestinal tract cancer. Tumor masses were obtained directly from the operating room and dissected under sterile conditions. The tumor tissues were cut into 2-mm^2 pieces in complete medium using a scalpel and scissors. For enzymatic digestion, the medium was supplemented with collagenase (2 mg/ml, type V – S; Sigma), hyaluronidase (10 units/ml, type VI – S; Sigma), and DNase I (0.4 mg/ml; Sigma). After a 40-min incubation at room temperature with gentle stirring, the enzymatically digested suspensions were harvested and passed through a stainless steel mesh to remove undigested tissue and cell clumps (Yamaue, Tanimura, Tsunoda, Iwahashi, Tani, Tamai & Inoue, 1990; Itoh, Tilden & Balch, 1986). The cells were then washed twice in complete medium, centrifuged at 400 ***g*** for 5 min, and suspended again in complete medium. Cells were then layered onto Ficoll – Hypaque gradients and centrifuged at 400 ***g*** for 30 min at 20°C. The fraction depleted of erythrocytes, polymorphonuclear cells, and aggregated tumor cells was collected from the interface, washed, suspended again in complete medium, and layered on discontinuous gradients of 70 and 100% Ficoll – Hypaque. After centrifugation at 400 ***g*** for 30 min, the TILs were concentrated at the 100% interface, and the tumor cells were concentrated at the 70% interface. The purity of lymphocytes in the TIL-rich fraction was 60 – 85%, and this fraction was used as the source of TILs. The tumor cell-rich fraction was contaminated by mesothelial cells and mononuclear cells. To eliminate these cells, the fraction was layered onto discontinuous gradients composed of 4 ml each of 20, 15 and 10% Percoll (Pharmacia) in complete medium in 15-ml plastic tubes, and then centrifuged at 25 ***g*** for 7 min at 20°C (Yamaue *et al.*, 1990). Tumor cells depleted of lymphocytes were collected from the bottom of each tube, washed, and resuspended in complete medium. The purity of the tumor cells was usually more than 90% after this extra centrifugation procedure. Freshly isolated tumor cells were over 95% viable according to the trypan blue dye exclusion test, and only cell fractions having less than 10% contamination by nonmalignant cells were accepted for use.

Characteristics of patients

Table 1 showed the characteristics of patients. In PBMC, the cases of stage IV were 52.4% (11 cases) and in TILs, these were 53.3% (8 cases) in this study.

Effector cells

Both PBMC and the TIL-rich cell fractions were suspended in complete medium. PBMC were incubated with famotidine alone (1 ng/ml ~100 μg/ml) for 4 days, and TILs were incubated with rhIL-2 (1500 IU/ml) and/or famotidine (1 ng/ml ~ 100 μg/ml) in 24-well microtiter plates (Corning No. 25820) or 96-well microtiter plates (Corning No. 25860) for 10 – 40 days.

Cytotoxic assay

A 4-h ^{51}Cr-release assay was performed. Target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ and then washed three times. The ^{51}Cr-labeled target cells (100 μl; 1×10^5/ml) were then cultured in triplicate with 100-μl aliquots of effector cell suspensions (the effector-to-target ratio was fixed at 15 : 1) in round-bottomed microtiter plates (Corning No. 25850). After a 4-h incubation at 37°C, the radioactivity of the supernatants was determined using a gamma counter. Spontaneous release did not exceed 10% for K562 cells, 15% for Daudi cells, and 25% for autologous tumor cells of the maximum release that was obtained with 1 N HCl. The percentage cytotoxicity was calculated as follows (all ^{51}Cr values in counts/min):

$$\frac{\text{Test } {}^{51}\text{Cr release} - \text{sponteneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100.$$

DNA synthesis

TILs (1×10^6/ml) were cultured in triplicate in round-bottomed microtiter plates with 1500 IU/ml of rhIL-2 and/or various concentrations of famotidine at 37°C. After 80 h, 1 μCi of ^3H-thymidine (^3H-TdR, New England Nuclear, Boston, U.S.A.)

Table 1. Characteristics of patients enrolled in this study

PBMC (n = 21)				TILS (n = 15)			
Disease		TNM classification		Disease		TNM classification	
Gastric carcinoma	9	Stage I	2	Gastric carcinoma	8	Stage II	1
		Stage II	2			Stage III	3
		Stage III	1			Stage IV	4
		Stage IV	4				
Pancreatic carcinoma	6	Stage II	2	Colorectal carcinoma	4	Stage III	1
		Stage III	2			Stage IV	3
		Stage IV	2				
Esophageal carcinoma	3	Stage IV	3	Thyroid carcinoma	2	Stage II	1
						Stage III	1
Non-Hodgkin's lymphoma	2	Stage II	1				
		Stage IV	1				
Colorectal carcinoma	1	Stage IV	1	Ovarian carcinoma	1	Stage II	1

was added to each well and culture was continued for an additional 16 h. Samples were harvested using a cell harvester (Cambridge Technology Inc., Cambridge, MA) and the amount of incorporated radioactivity was measured using a scintillation counter.

Phenotypic analysis of activated TILs

Phenotypic analysis of the activated TILs was performed by flow cytometry. Activated TILs were suspended in 0.5% bovine serum albumin-phosphate-buffered saline (PBS) at a concentration of 1×10^7/ml, monoclonal antibody (100 μl/ml) was added, and incubation was performed at 4°C for 30 min. The antibodies used were FITC-conjugated anti-CD2, CD3, CD4, CD8, CD16 and CD56 antibodies (Becton Dickinson, U.S.A.), and PE-conjugated anti-CD11 antibody (Becton Dickinson). Cells were washed and resuspended in buffer for flow cytometry (FACScan, Becton Dickinson). A phenotypic analysis was also performed by the negative selection method. The activated TILs were suspended at 1×10^7/ml and incubated with monoclonal antibodies (100 μl/ml) for 60 min at 4°C. Then, after being washed twice, the effector cells were treated with complement (low-tox-H Rabbit Complement; Cedarlane Laboratories, Ontario, Canada). Using these effector cells, a 4 h ^{51}Cr-release assay was performed as described above. The percent inhibition was determined as follows:

$$\left(1 - \frac{\text{Complement monoclonal antibody}}{\text{Complement alone}}\right) \times 100\ (\%).$$

Determination of the internalization of rhIL-2

Cells (2×10^6) were incubated with 200 pM ^{125}I-rhIL-2 in RPMI – HEPES at 4°C on ice for 30 min. Then they were washed three times, resuspended in 1.0 ml RPMI – HEPES, and incubated at 37°C for the indicated times. The suspension was centrifuged, and the cell pellet was then treated for 10 min at 4°C with chilled 0.2 M glycine – HCl buffer (pH 2.8). The radioactivity of the nonacid-eluted fraction was then counted with a gamma counter and taken to represent the labeled rhIL-2 internationalized by TILs (Fujii, Sugamura, Sano, Nakai, Sugita & Hinuma, 1986).

RESULTS

Dose titration of famotidine for the activation of in vitro *cytotoxicity*

PBMC from cancer patients were incubated with famotidine (1 ng/ml ~ 100 μg/ml) for 4 days, and then washed and tested for cytotoxicity (Fig. 1). Augmentation of cytotoxic activity was observed when PBMC from cancer patients were treated with a dose of between 1 ng/ml and 10 μg/ml of famotidine. In this concentration range, famotidine significantly augmented PBMC cytotoxic activity for

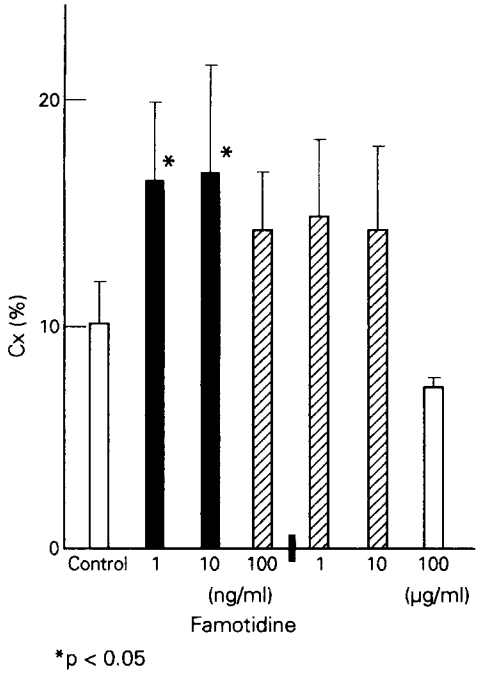

Fig. 1. PBMC were incubated with famotidine (1 ng/ml ~ 100 μg/ml) for 4 days, and tested for cytotoxicity against K562 cells in order to determine the optimal concentration of famotidine. Augmentation of cytotoxic activity was observed at doses of 1 ng/ml – 10 μg/ml ($P<0.05$), and a dose of 10 ng/ml was used for subsequent studies.

Table 2. Augmentation of the cytotoxic activity of PBMC from cancer patients by famotidine

	% Cx	
Target cell	(−)	Famotidine (10 ng/ml)
Autologous tumor cells	0.4 ± 0.8	6.0 ± 3.6*
K562 cells	10 ± 1.7	17 ± 3.8*
Daudi cells	9.0 ± 4.0	12 ± 3.9

*$P<0.05$ vs without famotidine.
Famotidine (10 mg/ml) increased the cytotoxicity of PBMC from cancer patients for autologous tumor cells from 0.4 to 6.0% and that for K562 cells from 10 to 17%.

K562 cells ($P<0.05$). Therefore, a dose of 10 ng/ml, close to the mean of this dosage range, was used for the subsequent studies.

Effect of famotidine on the cytotoxic activity of PBMC from cancer patients

Table 2 shows the augmentation of PBMC from cancer patients cytotoxic activity by famotidine

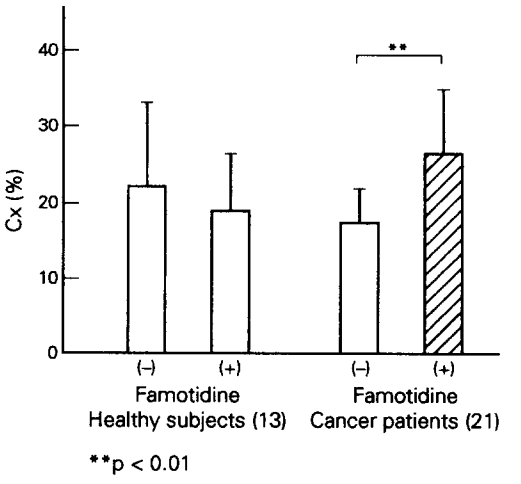

Fig. 2. Famotidine (10 ng/ml) augmented the cytotoxic activity for K562 cells of PBMC from cancer patients ($P<0.01$), but not that of PBMC from healthy subjects. Cancer patient PBMC cultured with famotidine had cytotoxic activity as well as PBMC from healthy subjects.

alone. At 10 ng/ml, famotidine increased the cytotoxicity of PBMC for autologous tumor cells from 0.4 to 6.0% ($P<0.05$), that for K562 cells from 10 to 17% ($P<0.05$), and that for Daudi cells from 9.0 to 12%.

Different responses to famotidine in healthy subjects and cancer patients

When the effects of famotidine (10 ng/ml) were investigated, in cancer patients and healthy subjects, it was found that this drug significantly increased the cytotoxic activity of PBMC for K562 cells from 17 to 26% in 21 cancer patients ($P<0.01$), but did not produce an increase in PBMC cytotoxicity in 13 healthy subjects (Fig. 2). The cytotoxic activity of cancer patient effector cells cultured with famotidine was compared with that of untreated effector cells from healthy subjects. There was no significant difference between untreated and famotidine-treated PBMC from healthy subjects (data not shown).

Effects of famotidine on the cytotoxic activity of activated TILs

After incubation with the combination of rhIL-2 and famotidine, the cytotoxic activity of TILs against K562 cells was measured. With 1500 IU/ml of IL-2 plus 1 μg/ml of famotidine, the cytotoxicity was 76%, whereas it was only 63% after treatment with IL-2 alone ($P<0.05$, Fig. 3). Therefore, a

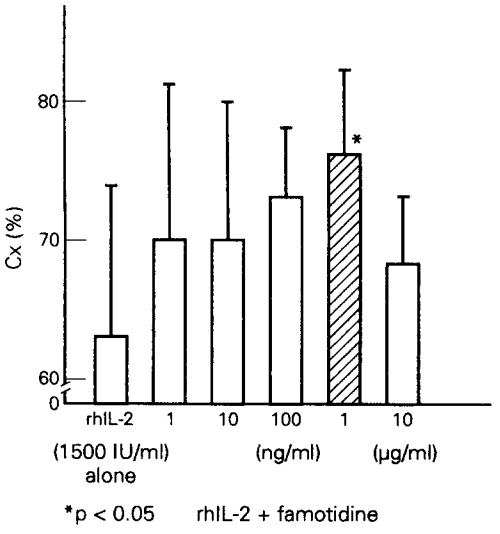

Fig. 3. Famotidine (1 μg/ml) augmented the cytotoxic activity of rhIL-2-activated TILs against K562 cells ($P<0.05$). The concentration of IL-2 was fixed at 1500 IU/ml.

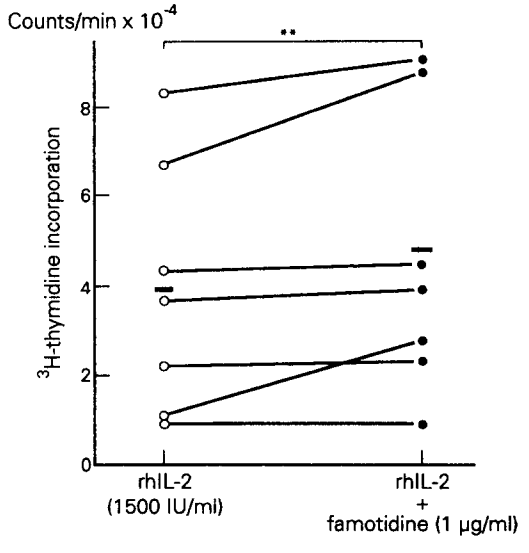

Fig. 4. Famotidine (1 μg/ml) accelerated the ^{3}H-thymidine incorporation of rhIL-2-activated TILs ($P<0.01$). In six out of seven cases, DNA synthesis was increased by famotidine.

concentration of 1 μg/ml of famotidine was used in the subsequent studies of TILs.

Effects of famotidine on DNA synthesis by activated TILs

Figure 4 shows that DNA synthesis by activated TILs was enhanced by culture with IL-2 (1500 IU/ml) and famotidine (1 μg/ml). In six out of seven cases, DNA synthesis was significantly increased by the addition of famotidine to the cultures ($P<0.01$).

Effects of famotidine on the surface phenotype of activated TILs

The negative selection method indicated that CD4-positive or CD8-positive cytotoxic T-cells and CD56-positive lymphocytes were markedly induced by incubation with famotidine ($P<0.05$, Table 3). However, analysis of cell surface phenotypes by flow cytometry indicated that antigen expression by effector cells was not changed by the addition of famotidine (1 μg/ml) to IL-2 (1500 IU/ml). Moreover, the number of CD8-positive and CD11-positive cells (suppressor T-cells) also did not change in the two-color analysis.

Internalization of IL-2

Internalization of IL-2 by TILs cultured with IL-2 (1500 IU/ml) and famotidine (1 μg/ml) was accelerated from early in the incubation period (Fig. 5). After 60 min, the radioactivity of TILs incubated with famotidine was 73,000 counts/min and that of TILs incubated with IL-2 alone was 61,000 counts/min. A representative result of three independent experiments is illustrated.

DISCUSSION

In cancer therapy, an important aspect to consider is the reduction of suppressor factors. It is known that OKT8-positive suppressor T-cells have H_2 receptors (Rocklin, Greineder, Littman & Melmon, 1978; Rocklin *et al.*, 1980; Khan *et al.*, 1985; Melmon *et al.*, 1977), and that H_2 receptor antagonists can inhibit their suppressor activity (Cavagnaro *et al.*, 1980; Osband *et al.*, 1989). Famotidine is generally used as a potent inhibitor of gastric acid secretion, and it is recognized to be a stronger H_2 receptor antagonist than the other commonly available H_2 blockers. In this study, we investigated the *in vitro* augmentation of the cytotoxic activities of PBMC and TILs by famotidine.

Famotidine (10 ng/ml) augmented the cytotoxic activity of PBMC from cancer patients against both autologous tumor cells and established tumor cell lines. The concentration of 10 ng/ml is equivalent to the serum level achieved by the intravenous administration of single dose of 20 mg of famotidine (unpublished data). Interestingly, such a response to famotidine was only seen in cancer patients. This

Table 3. Cell surface phenotype of activated TILs with famotidine

1. Negative selection method	Target cell: K562					
	Inhibition (%)					
In vitro treatment	CD2	CD3	CD4	CD8	CD16	CD56
IL-2 alone	21 ± 6	5 ± 3*	4 ± 2*	6 ± 2*	12 ± 5	13 ± 7*
IL-2 + famotidine	21 ± 5	16 ± 2	16 ± 6	14 ± 2	13 ± 4	25 ± 5

2. Flow cytometry								
In vitro treatment	CD2	CD3	CD4	CD8	CD16	CD56	$CD8^+CD11^-$	$CD8^+CD11^+$
IL-2 alone	95 ± 7	85 ± 7	10 ± 2	73 ± 10	4 ± 1	17 ± 2	56 ± 4	10 ± 1
IL-2 + famotidine	96 ± 10	86 ± 8	10 ± 1	73 ± 9	2 ± 1	17 ± 4	54 ± 1	11 ± 2

*$P<0.05$.

Phenotypic analysis of activated TILs was performed by the negative selection method and by flow cytometry. Negative selection indicated that CD4-positive or CD8-positive cytotoxic T-cells and CD56-positive lymphocytes were markedly induced by famotidine. However, the cytometry showed no change of the cell surface phenotype of activated TILs.

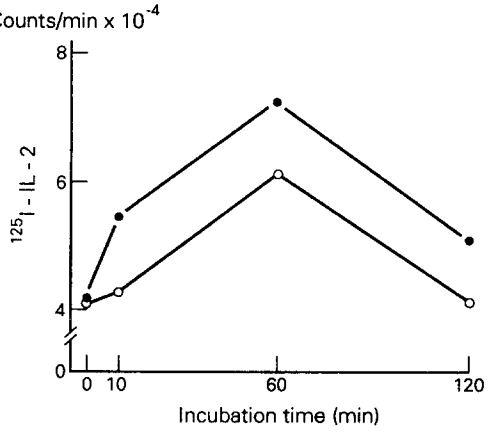

Fig. 5. Solid circles (●) show the radioactivity of the nonacid-eluted fraction of TILs cultured with ^{125}I-labeled rhIL-2 (1500 IU/ml) and famotidine (1 μg/ml). Open circles (○) show the radioactivity of TILs cultured with labeled rhIL-2 (1500 IU/ml) alone. The internalization of ^{125}I-labeled rhIL-2 into TILs incubated with famotidine was accelerated from early in the incubation period. After 60 min, TILs incubated with famotidine had internalized 73,000 counts/min and TILs incubated with IL-2 alone had internalized only 61,000 counts/min. A representative result of three independent experiments is shown here.

suggested that famotidine may have acted to inhibit suppressor T-cell activity which is enhanced in cancer patients, and thus augmented PBMC cytotoxic activity. From the point of the response of famotidine, we analysed the difference of augmentation of the cytotoxic activity in various diseases and stages. There was a tendency to augment PBMC with extent of disease. However, there was not a statistical significance among diseases and stages of cancer.

In adoptive immunotherapy using activated TILs and LAK cells, the concentration of IL-2 that gives maximum enhancement of cytotoxic activity against autologous tumor cells and the maximum proliferation of activated TILs has been found to be 1500 IU/ml (Yamaue *et al.*, 1990). However, even when this optimal IL-2 concentration was provided, famotidine (1 μg/ml) further augmented the cytotoxicity and accelerated the proliferation of IL-2-activated TILs. Moreover, famotidine also augmented the generation of LAK cells from PBMC (unpublished data). It was recently reported that IL-2 could induce suppressor cells against LAK cells (Ebihara, Koyama, Fukao & Osuga, 1989). Therefore, it appears that famotidine inhibited suppressor cell activity, while augmenting the cytotoxic activity and proliferation of activated TILs and LAK cells cultured with IL-2. The discrepancy of the optimal concentration of famotidine for PBMC and activated TILs may cause to the suppressor T-cells to be modified by IL-2.

To clarify the mechanism of augmentation of the cytotoxic activity of activated TILs by famotidine, we made a phenotypic analysis of effector cells. The negative selection method showed that activated TILs cultured with IL-2 and famotidine showed an increased proportion of CD4-positive and CD8-positive cytotoxic T-lymphocytes and CD56-positive lymphocytes. However, flow cytometric analysis showed that the number of effector cells was not altered by famotidine and two-color analysis

indicated that famotidine did not decrease the number of CD8-positive and CD11-positive cells, which are considered to be suppressor T-cells (Takeuchi, Dimaggio, Levine, Schlossman & Morimoto, 1988). These results suggested that famotidine might change the functional characteristics of the activated TILs rather than antigen expression.

It has also been reported that H_2 receptor antagonists increase IL-2 production by T-cells and make T-cells more sensitive to the effect of IL-2 (Gifford & Tilberg, 1987). We analysed the expression of the IL-2 receptor (Tac antigen) by activated TILs and found that famotidine did not increase the number of Tac-positive cells, although it augmented the internalization of IL-2 by activated TILs. This suggested that the high-affinity IL-2 receptor (β-chain or α- and β-chain complex) was related to the mechanism by which famotidine augmented TIL cytotoxicity.

Famotidine was effective in increasing the cytotoxic activity of TILs, and it may have the potential to be used in adoptive immunotherapy for cancer patients.

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