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ORIGINAL ARTICLE

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Markedly induced asialoGM1+CD8+ T cell production and enhancement of antimetastatic activity by interferon β with folic or folinic acid

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Abstract Either folic or folinic acid enhanced the antimetastatic activity of recombinant murine interferon β (rMuIFNβ) toward highly metastatic colon carcinoma 26 (Co 26Lu). Folinic acid administered with rMuIFNβ markedly increased asialoGM1+CD4+ and asialoGM1+CD8+ T cell production in the peritoneal cavity but not in the thymus and spleen. Peritoneal cells expressed killing activity toward Co 26Lu cells in vitro. In athymic nude mice, the above combination produced many asialoGM1+CD4+ and few asialoGM1+CD8+ T cells in the peritoneal cavity, but did not decrease lung metastatic colonies. AsialoGM1+CD4+ T cells would thus appear to have no or only very weak killing activity toward these tumor cells. The antimetastatic activity of folinic acid with rMuIFNβ was significantly decreased with anti-asialoGM1 and anti-CD8 antibodies. Inactivated CD8+ and asialoGM1+ cells cease to have killing activity toward Co 26Lu cells as shown by Winn's assay. AsialoGM1+CD8+ cell production was markedly induced in the peritoneal cavity by treatment with rMuIFNβ and folinic acid. AsialoGM1+CD8+ T cells may be inhibiting lung metastasis of Co 26Lu. Folinic acid and interferon are used in combination therapy with 5-fluorouracil for biochemical modulation. Folinic acid with interferon, as adjuvant therapy, may promote the induction of CD8+ T cell production with consequent prevention of metastasis.

Key words Colon carcinoma 26 · Antimetastatic activity · Interferon · Folinic acid · AsialoGM1+CD8+ T cells

Introduction

Interferons are glycoproteins with strong antiviral, immunomodulatory and antiproliferative effects [2] and are essential for lymphocyte functions such as maturation. In interferon-stimulated lymphocytes, DNA synthesis and the cell cycle occur. Interferons induce extrathymic CD4+ T lymphocyte production in athymic nude mice [8]. Folic acid is required for maintaining nucleotide precursor pools and DNA synthesis. 5,10-Methylenetetrahydrofolate is produced from folic acid and a cofactor needed to produce the active one-carbon unit for purines and pyrimidines. Folinic acid (leucovorin), an external source of 5,10-methylenetetrahydrofolate, strongly stimulates progenitor cells circulating in peripheral blood even after chemotherapy [5]. Folic and folinic acids may promote the synthesis of DNA in interferon-stimulated lymphocytes. In this study, the effects of folic and folinic acids on the induction of lymphocyte production and antitumor activity of rMuIFNβ were examined, using the highly metastatic potential colon carcinoma 26 established at this laboratory.

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Materials and methods

Mice

BALB/c athymic nude and euthymic BALB/c mice, 5 weeks old, were obtained from Charles River Japan (Atsugi, Japan) and maintained under specific-pathogen-free conditions at this laboratory. All experiments were initiated when the mice became 6 weeks old.

Tumor

Highly metastatic colon carcinoma 26 (Co 26Lu, 1×10^5 cells/mouse), which was established by the authors [4], was implanted s.c. into the right hind thigh of the mice.

Antibodies

Anti-asialoGM1 rabbit serum was purchased from Wako Pure Chemicals (Osaka, Japan). Fluorescein-isothiocyanate (FITC)- or phycoerythrin-conjugated antibodies, anti-CD4 (RM.4-5), anti-CD8a (53.6.72) and TcRαβ (H57-597), were obtained from PharMingen (San Diego, Calif.).

Cytokines and treatment

Recombinant murine interferon β (rMuIFNβ: specific activity, 5×10^7 IU/mg protein) was from Toray Industries Inc. (Kamakura, Japan). (–)-Leucovorin was kindly supplied by Lederle (Japan) Ltd.,

Tokyo. Folic acid was purchased from Sigma, Mo. rMuIFNβ (1×10^5 IU/mouse), and folic (50 mg/kg) and folinic acids (50 or 100 mg/kg) were administered i.p. daily from days 10 to 27, five times a week. Diluted anti-asialoGM1 antibody (1:8) or anti-CD8 antibody (1:4) was injected i.p. (0.2 ml/mouse) on days 14 and 17 to mice administered rMuIFNβ and folinic acid.

The longest (*a*) and shortest (*b*) diameters of the primary tumor were determined twice a week by caliper, and the volume was calculated using the formula: $ab^2/2$ (mm^3). All surviving animals were killed on day 28. The lungs were removed, rinsed in 0.9% NaCl solution containing heparin and fixed for 1 or more days in acetone to determine the number of macroscopic lung metastases.

Lymphocytes and fluorescence-activated cell sorting (FACS) analysis

Lymphocytes were collected from the peritoneal cavity following daily i.p. injections of rMuIFNβ (1×10^5 IU/mouse) and/or folinic acid (50 mg/kg) for 6 days (five mice per group). The mice were sacrificed 12 h after the final injection. An 8-ml sample of 0.9% saline solution was injected i.p. and, after gentle lavaging, the peritoneal cavity contents of five mice were pooled, washed to remove cell debris, suspended in 5 ml RPMI-1640 medium (10% Fetal bovine serum, Immuno-Biological Laboratories, Fujioka, Japan) and stored on ice for flow-cytometry assay.

Immunofluorescent staining was conducted as described previously [8]. Briefly, the lymphocytes were allowed to react with anti-asialoGM1 antibody and, after being washed, with the second antibody, FITC-conjugated goat anti-[rabbit Ig(G+L)] at 4 °C for 30 min. This was followed by staining with anti-CD4-conjugated phycoerythrin (PE-anti-CD4) or PE-anti-CD8. For the other two-color stainings, FITC-anti-CD4 and PE-anti-TcRαβ were used. After being washed three times with RPMI-1640 medium, the stained cells were subjected to two-color analysis by FACScan (Becton, Dickinson, Mountain View, Calif.).

Winn's assay

The peritoneal cells in tumor-bearing BALB/c mice were harvested 12 h after the last injection of rMuIFNβ and folinic acid (six injections). The unfractionated peritoneal cells were washed three times in RPMI-1640 medium containing 10% Fetal bovine serum. To obtain nonadhering peritoneal cells, the unfractionated peritoneal cells were incubated in a collagen-type-1-coated dish (Iwaki Glass, Tokyo) for 1 h at 37 °C. Cells remaining in the supernatant following the second incubation were designated as nonadhering peritoneal cells. For pretreatment of peritoneal cells with anti-CD8 antibody and Low-Tox-M rabbit complement (Cedarlane Laboratories Ltd.) or anti-asialoGM1 antibody and Low-Tox-M guinea-pig complement, the peritoneal cells were suspended in diluted anti-CD8 antibody (1:50) or anti-asialoGM1 antibody (1:25) and allowed to stand at 4 °C for 60 min. The cells were then washed twice, suspended in complement-containing medium (a dilution of rabbit and guinea-pig complements, 1:15 and 1:10 respectively) and incubated for 40 min at 37 °C. The peritoneal cells were then washed twice. Co 26Lu cells were adjusted to 1×10^6 cells/ml. Mixed lymphocytes and tumor cells were implanted s.c. in the mice. Tumor weight was determined 14 days after tumor impantation.

Data analysis

The results were averages of data obtained at least in duplicate under identical conditions. The Turkey-Kramer HSD test was carried out to determine statistical significance of differences in tumor volume or weight. The number of the lung metastases was evaluated by the Mann-Whitney *U*-test.

Results

Effects of folic and folinic acids on the antimetastatic activity of rMuIFNβ in s.c. implanted Co 26Lu

At a primary tumor volume of 100 mm^3 or more (day 14), microscopic metastasis in the lungs was noted in all animals. Many macroscopic lung metastatic nodules could be seen in all mice on day 25 in the untreated control and in the groups treated with folic and folinic acid. Lung metastatic nodule formation in rMuIFNβ -treated mice was slightly inhibited. With rMuIFNβ plus folic acid or folinic acid (Table 1) metastases in the lungs markedly decreased compared with the untreated control ($P < 0.01$) and treatment with rMuIFNβ alone ($P < 0.05$); though rMuIFNβ and folinic acid in combination significantly inhibited tumor growth, this was not the case with rMuIFNβ plus folic acid. rMuIFNβ and folinic acid together at a high dose (100 mg/kg) showed a greater antimetastatic effect than did rMuIFNβ and folinic acid at a low dose (50 mg/kg) in combination (Table3).

In tumor-bearing athymic nude mice (BALB/c origin) given rMuIFNβ with folinic acid, tumor growth and the numbers of lung metastatic nodules were the same as in the untreated control group (data not shown).

Folinic acid did not enhance the cytotoxicity of rMuIFNβ in Co 26Lu cells in vitro (data not shown).

Table 1 Effects of recombinant murine interferon β (*rMuIFNβ*) and folic or folinic acid on lung metastatic nodules of Co 26Lu. Drugs were administered i.p. daily from day 10 (five times a week). Tumor volumes were determined on day 24; lung metastases were counted on day 28

Treatment	No. of mice	Tumor volume, mean ± SE (mm^3)	No. of lung metastatic nodules/mouse median (range)	
Folic acid				
Untreated control	13	1127 ± 163	48	(13–80)
rMuIFNβ (1×10^5 IU)	9	1315 ± 206	29*	(15–49)
Folic acid (50 mg/kg)	9	1170 ± 199	43.5	(25–84)
rMuIFNβ + folic acid	10	938 ± 145	15.5**, ***	(12–35)
Folinic acid				
Untreated control	9	1562 ± 131	44.5	(18–70)
rMuIFNβ (1×10^5 IU)	10	1559 ± 143	22.5	(2–78)
Folinic acid (50 mg/kg)	10	1381 ± 151	37	(8–84)
rMuIFNβ + folinic acid	11	982 ± 153*, ***	12**	(0–50)

* $P < 0.05$ compared with untreated control
** $P < 0.01$ compared with untreated control
*** $P < 0.05$ compared with rMuIFNβ

Table 2 Effects of rMuIFNβ and folinic acid on asialoGM1+ CD4+ and asialoGM1+CD8+ cell subpopulations in the peritoneal cavity of BALB/c mice. *LV* folinic acid (leucovorin), *GM1* asialoGM1. rMuIFNβ (1×10^5 IU/mouse) and/or folinic acid (50 mg/kg) were administered i.p. daily from day 10 for 6 days. Peritoneal cells from four mice were collected 12 h following the final injection. Values are means of two experiments. Rabbit and rat controls, which were stained by secondary fluorescein-isothiocyanate(FITC)-labeled goat anti-(rabbit Ig) antibodies and FITC-labeled anti-(rat Ig) antibodies respectively, were less than 3%

Subsets	Composition (%)					
	Tumor-bearing mice				Nontumor-bearing mice	
	None	rMuIFNβ	LV	rMuIFNβ+LV	rMuIFNβ	rMuIFNβ+LV
GM1+CD4+	2.7	40.7	21.6	54.9	17.6	10.9
GM1+CD4−	3.8	38.7	41.8	30.7	42.3	33.8
GM1−CD4+	19.5	2.9	3.5	3.1	3.0	1.4
GM1−CD4−	74.0	17.7	33.1	11.3	37.1	53.9
GM1+CD8+	3.3	15.0	8.9	29.8	7.7	4.6
GM1+CD8−	4.2	61.7	39.6	47.4	44.4	39.1
GM1−CD8+	13.9	0.6	0.6	0.9	0.6	0.5
GM1−CD8−	78.6	22.7	50.9	21.9	47.3	55.8

Table 3 Effects of anti-asialoGM1 and anti-CD8 antibodies on the formation of metastases by Co 26Lu cells treated with rMuIFNβ and folinic acid. Co 26Lu cells (1×10^5 cells) were injected s.c. on day 0. rMuIFNβ (1×10^5 IU) and folinic acid (100 mg/kg) were administered i.p. daily on days 10–27 (five times a week). Anti-asialoGM1 (1:8, 0.2 ml) and anti-CD8 (1:4, 0.2 ml) antibodies were administered i.p. on days 14 and 17. Lung metastases were counted on day 25

Group	Treatment	No. of mice	No. of lung metastatic nodules/mouse median	(range)
1	Untreated control	14	37	(5– 65)
2	Anti-asialoGM1 antibody	13	35	(15–129)
3	Anti-CD8 antibody	13	46	(16–126)
4	Anti-asialoGM1 antibody + anti CD8 antibody	6	56	(25– 73)
5	rMuIFNβ (1×10^5 IU)	10	19*1	(10– 46)
6	Folinic acid + rMuIFNβ	10	5.5*2	(1– 41)
7	Folinic acid + rMuIFNβ + anti-asialoGM1 antibody	10	10.5*3	(3– 24)
8	Folinic acid + rMuIFNβ + anti-CD8 antibody	10	25*4	(14– 39)
9	Folinic acid + rMuIFNβ + anti-asialoGM1 antibody + anti-CD8 antibody	8	25.5*5	(7– 42)

*1 $P<0.05$ compared with group 1
*2 $P<0.001$ and $P<0.01$ compared with groups 1 and 5 respectively
*3 $P<0.001$ and $P<0.01$ compared with groups 1 and 2 respectively
*4 $P<0.01$ and $P<0.001$ compared with groups 6 and 3 respectively
*5 $P<0.01$ compared with groups 6 and 4

Induction of asialoGM1+CD4+ and asialoGM1+CD8+ T cell production in the peritoneal cavity of athymic nude or BALB/c mice following i.p. rMuIFNβ and folinic acid injection

Most peritoneal cells following treatment with rMuIFNβ were lymphocytes (more than 95%). A few macrophages and neutrophils were present. The combination of rMuIFNβ and folinic acid markedly increased the number of large granular lymphocytes in the peritoneal cavity compared to the case with folinic acid alone. The peritoneal cells were analyzed by FACS to determine the numbers of asialoGM1+CD4+ and asialoGM1+CD8+ T cells. Lymphocytes in the peritoneal cavity of athymic nude mice treated with rMuIFNβ were mainly asialoGM1+CD4+ TcRαβ+ T cells (35% of all peritoneal cells), and a few asialoGM1+CD8+ T cells. When rMuIFNβ and folinic acid were administered i.p. to Co-26Lu-bearing athymic nude mice, many more asialoGM1+CD4+ T cells (44%) were produced in the peritoneal cavity than in the untreated control or in the group receiving folinic acid alone (data not shown), and few asialoGM1+CD8+ T cells were present.

There were significantly more peritoneal cells in BALB/c mice treated with rMuIFNβ and folinic acid together than in the untreated control mice and those receiving folinic acid treatment ($P<0.01$) or rMuIFNβ ($P<0.05$) [mean ± SE; untreated control: $(2.2\pm0.2)\times10^6$ cells/mouse; folinic acid: $(2.5\pm0.4)\times10^6$ cells/mouse; rMuIFNβ: $(4.9\times0.9)\times10^6$ cells/mouse; rMuIFNβ and folinic acid: $(7.2\pm1.3)\times10^6$ cells/mouse]. In the peritoneal cavity cells, all forms of CD4+ and a few asialoGM1+CD8+ cells were present.

AsialoGM1+CD4+ and asialoGM1+CD8+ T cells increased on treatment with rMuIFNβ rMuIFNβ with folinic acid considerably induced asialoGM1+CD4+ and asialoGM1+CD8+ T cell formation (Table 2). No marked increase in asialoGM1+CD4+ or asialoGM1+CD8+ T cell production could be detected in the thymus or spleen following treatment witn rMuIFNβ and folinic acid (data not shown).

Table 4 Growth of Co 26Lu cells attached to peritoneal cells treated with anti-CD8 or anti-asialoGM1 antibody and complement. rMuIFNβ and folinic acid were administered to Co 26Lu-bearing BALB/c mice on days 7–13. The mice were killed 12 h after last treatment to obtain peritoneal cells (*PC*). Tumor cells were taken from untreated control mice. The numbers of peritoneal cells and tumor cells were 10×10^5 or 30×10^5 and 1×10^5 cells respectively. Tumor cells after combining with lymphocytes were implanted s.c. in new mice and the tumors were excised on day 14 and weighed. *rC* rabbit complement, *gC* guinea-pig complement

Expt.	Group	Treatment	Treatment weight (g) means ± SE (n = 4)
I	1	Co 26Lu cells (1×10^5 cells/mouse)	0.42 ± 0.07
	2	Co 26Lu cells + PC (1 : 10)	0.18 ± 0.03*1
	3	Co 26Lu cells + PC (1 : 10) + rC	0.15 ± 0.05*2
	4	Co 26Lu cells + PC (1 : 10) + anti-CD8 antibody + rC	0.31 ± 0.02*3
	5	Co 26Lu cells + PC (1 : 30)	0.00 ± 0.00*2
	6	Co 26Lu cells + PC (1 : 30) + anti-CD8 antibody + rC	0.39 ± 0.04*4
II	7	Co 26Lu cells (10^5 cells/mouse)	0.52 ± 0.09
	8	Co 26Lu cells + PC (1 : 10)	0.24 ± 0.09
	9	Co 26Lu cells + PC (1 : 10) + gC	0.31 ± 0.11
	10	Co 26Lu cells + PC (1 : 10) + anti-asialoGM1 antibody + gC	0.51 ± 0.11

*1 $P < 0.05$ compared with group 1
*2 $P < 0.01$ compared with group 1
*3 $P < 0.01$ compared with group 3
*4 $P < 0.01$ compared with group 5

Table 5 Growth of Co 26Lu cells attached to nonadhering peritoneal cells treated with anti-CD8 or anti-asialoGM1 antibody and complement. rMuIFNβ and folinic acid were administered to Co 26Lu-bearing BALB/c mice on days 7–13. The mice were killed 12 h after the last treatment to obtain peritoneal cells (*PC*). The numbers of peritoneal cells and tumor cells were 10×10^5 or 30×10^5 and 1×10^5 cells respectively. Tumor cells combined with lymphocytes were implanted s.c. in new mice and the tumors were excised on day 14 and weighed. *rC* rabbit complement, *gC* guinea-pig complement

Expt.	Group	Treatment	Treatment weight (g) means ± SE (n = 4)
I	1	Co 26Lu cells (1×10^5 cells/mouse)	0.42 ± 0.07
	2	Co 26Lu cells + PC (1 : 10)	0.04 ± 0.02*1
	3	Co 26Lu cells + PC + rC	0.10 ± 0.05*1
	4	Co 26Lu cells + PC (1 : 10) + anti-CD8 antibody + rC	0.24 ± 0.11
	5	Co 26Lu cells + PC (1 : 30)	0.00[a]
	6	Co 26Lu cells + PC (1 : 30) + anti-CD8 antibody + rC	0.40[a]
II	7	Co 26Lu cells (1×10^5 cells/mouse)	0.51 ± 0.02
	8	Co 26Lu cells + PC (1 : 30)	0.09 ± 0.05*2
	9	Co 26Lu cells + PC (1 : 30) + gC	0.13 ± 0.08*2
	10	Co 26Lu cells + PC (1 : 30) + anti-asialoGM1 antibody + gC	0.52 ± 0.04*3

*1 $P < 0.05$ compared with group 1
*2 $P < 0.01$ compared with group 1
*3 $P < 0.01$ compared with group 8 or group 9
[a] Results are means of two mice

Effects of anti-asialoGM1 and anti-CD8 antibodies on the extent of lung metastases following rMuIFNβ and folinic acid treatment

rMuIFNβ with folinic acid in combination markedly increased antimetastatic activity. This activity was significantly reduced by treatments with anti-asialoGM1 and/or anti-CD8 antibodies (Table 3). AsialoGM1-positive and/or CD8-positive cells would thus appear essential and effective for inhibition of lung metastasis.

Winn's assay

Subcutaneously implanted Co 26Lu cells (1×10^5 cells) grew well in syngeneic BALB/c mice. An admixture of Co 26Lu cells and total peritoneal cells from tumor-bearing BALB/c mice treated with rMuIFNβ and folinic acid at 10:1 or 30:1 (peritoneal cells:tumor cells) significantly prevented tumor growth, as did nonadhering peritoneal cells. The results of adding unfractionated peritoneal cells (10×10^5 or 30×10^5 cells), treated with anti-CD8 and anti-asialoGM1 antibodies and complement prior to being mixed with the tumor cells (1×10^5 cells), are shown in Table 4. When $CD8^+$ and asialoGM1$^+$ populations were eliminated from unfractionated peritoneal cells, there ceased to be any activity toward tumor cells, as also noted when $CD8^+$ and asialoGM1$^+$ populations were eliminated from nonadhering peritoneal cells (Table 5).

Discussion

The production and circulation of lymphocytes, both related to homeostasis of the host, are regulated by interferons and other cytokines. The thymus is the primary site for lymphocyte maturation. With aging, the thymus decreases in size and cells become less functional. Other sites for lymphocyte maturation may thus possibly develop as the host defense system. Extrathymic $CD4^+$ or $CD8^+$ T cells increase gradually in the spleen of healthy nude mice with aging [6, 7]. AsialoGM1$^+$$CD8^+$ and asialoGM1$^+$$CD4^+$ T cell formation was markedly induced by rMuIFNβ with folinic acid or cytokines and a cytotoxic drug co-administered to Co-26Lu-tumor-bearing BALB/c mice [3, 4]; marked antimetastatic activity was noted in all cases. Extrathymic T cells in most cases are TcRγδ$^+$ [9] or $CD8^+$ T cells as lymphocyte-activated killer cell subsets [1]. AsialoGM1$^+$$CD4^+$ T cells were markedly increased by rMuIFNβ in athymic nude mice, but asialoGM1$^+$$CD8^+$ cells were not [8]. rMuIFNβ with folic or folinic acid caused no significant inhibition of metastatic colony formation in Co-26Lu-bearing athymic nude mice lacking $CD8^+$ T cells. AsialoGM1$^+$$CD4^+$ T cells would thus appear to have no or only very weak killing activity toward these tumor cells, though the cells produced interleukin-2 (IL-2) and IFNγ as reported previously [8]. Antimetastatic activity of rMuIFNβ and folinic acid in Co-26Lu-bearing BALB/c mice was prevented by anti-asialoGM1 and/or anti-CD8 antibodies, but

not completely. In in vitro culture, folinic acid did not enhance the cytotoxicity of rMuIFNβ. One of the mechanisms of increased antimetastatic activity may possibly be the induction of asialoGM1$^+$ and CD8$^+$ effector-type cells, the activity of which was significantly reduced by anti-asialoGM1 and anti-CD8 antibodies, as shown by Winn's assay. Most peritoneal CD8$^+$ cells were asialoGM1$^+$ marker cells. AsialoGM1$^+$CD8$^+$ cells need to kill tumor cells. The cells could not be found in the thymus (less than 1%) following rMuIFNβ administration. The precursors of asialoGM1$^+$CD8$^+$ cells, possibly originating from thymocytes, must undergo differentiation in the peritoneal cavity under the influence of interferon, increased by the co-administration of folic or folinic acid. Such proliferation would augment host defense against tumor development and metastasis. After increasing, T cells disappeared from the peritoneal cavity within 2 days, possibly through their slow release into the bloodstream and disruption in most cases. These T cells could not be detected in the blood after rMuIFNβ treatment, and asialoGM1$^+$CD8$^+$ and asialoGM1$^+$CD4$^+$ T cells in the spleen were present at levels of 10% and 5% respectively. Though the mechanisms of induction of asialoGM1$^+$CD8$^+$ and asialoGM1$^+$CD4$^+$ T cell are not clear, clarification of the interactions between the T cells and metastatic solid tumor cells may provide the basis for developing therapeutic strategies. Interferon and folinic acid are used as biochemical modulators of 5-fluorouracil for treating colon carcinoma and this same combination may serve as a very effective means for preventing the metastasis of colon carcinoma.

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