Jpn. J. Cancer Res. 86, 761–769, August 1995

Dipyridamole Combined with Tumor Necrosis Factor-α Enhances Inhibition of Proliferation in Human Tumor Cell Lines

Nobuo Suzuki,[1,7] Souei Sekiya,[2] Isamu Sugano,[3] Takayuki Kojima,[4] Hideo Yamamori[5] and Yoshiaki Takakubo[6]

Second Department of [1]Biochemistry, [2]Obstetrics and Gynecology, [4]Dermatology and [5]Surgery, Chiba University School of Medicine, 1-8-1, Inohana, Chiba 260, [3]Department of Pathology, Teikyo University School of Medicine, 3426-3, Anegasaki, Ichihara 299-01 and [6]Department of Neurosurgery, National Tosei Hospital, 762-1, Nagasawa, Shimizu-cho, Sunto-gun, Shizuoka 411

In the search for cytokines whose antiproliferative action could be enhanced by combination with dipyridamole, 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine, the combination of tumor necrosis factor-α (TNF-α) with this agent was evaluated in various human tumor cell lines. Inhibition of the proliferation of human melanoma cell lines MM-1CB and HMV-1 by TNF-α (1–10^2 U/ml) was enhanced in culture dishes by combination treatment with dipyridamole (0.1–10 μM). The enhancement effect was also detected in other tumor cell lines: T98 (glioma), SCC-1CB (squamous cell carcinoma), HAC-2 (ovarian clear-cell carcinoma), HLE (hepatoma), HEC-1 (endometrial adenocarcinoma) and HOC-21 (ovarian serous cystadenocarcinoma). The incorporation of [^{14}C]amino acids and [^{3}H]uridine into acid-insoluble cell materials in the combination-treated cells was not significantly different from that in cells treated with TNF-α or dipyridamole. However, the incorporation of [^{3}H]thymidine was specifically inhibited in all cell lines examined after more than 12 h of the TNF-α and dipyridamole combination treatment, although neither agent alone inhibited this incorporation. On the other hand, the growth of tumors induced by the injection of MM-1CB and HMV-1 cells into nude mice was more markedly inhibited by the subcutaneous administration of TNF-α in combination with orally administered dipyridamole than by either agent alone. The results presented suggested that dipyridamole is beneficial in assuring the effectiveness of anti-cancer cytokine therapy.

Key words: Dipyridamole — TNF-α — Human tumor cells

The clinical use of cytokines with the capacity to inhibit cell proliferation, such as tumor necrosis factor (TNF)-α and human interferon (HuIFN), is limited because of their relatively low therapeutic effectiveness in various kinds of tumor tissues and because of their side effects, particularly when they are used at high doses.[1] We therefore consider it important to develop a method in which low doses of cytokines can be administered in combination with drugs that enhance their antiproliferative activity, but, themselves, have few side effects. In our previous studies,[2,3] we have used dipyridamole, 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido[5,4-*d*]-pyrimidine, as such a drug.

Dipyridamole is a well known vasodilator which also inhibits platelet aggregation. It is therefore widely used in the treatment of chronic coronary insufficiency and myocardial infarction, and in the prophylaxis of arterial thrombosis.[4–6] We found that dipyridamole enhanced the antiproliferative activity of HuIFN-α, -β, and -γ in melanoma-derived cell lines,[2] and in cell lines derived from various other kinds of tumor tissues.[3]

However, this dipyridamole combined treatment was not effective in cell lines resistant to the antiproliferative effect of HuIFN.[3] Some cell lines appeared to be non-susceptible to the enhancement effect of dipyridamole, even though they were susceptible to the antiproliferative effect of HuIFN alone and that of dipyridamole alone.[3] To extend the number of tumor cell lines in which the dipyridamole plus cytokine combination is effective, other cytokines whose antiproliferative activity can be enhanced by the combination must be identified. In this study, we examined the effectiveness of TNF-α in this connection.

TNF-α was originally described as a serum mediator of tumor necrosis in animals given injections of endotoxin.[7] TNF-α is now regarded as a cytotoxic cytokine that causes tumor regression, and it has been used clinically as an anti-cancer agent.[8–12] Many side effects have been reported with this cytokine, however: fever, chills, anorexia and nausea.[13] In particular, the toxic effects of TNF-α, including its induction of liver function abnormalities, hypotension, transient neurological changes, and hematological abnormalities, place serious limitations on its clinical use.[14,15]

Combination therapy consisting of low doses of TNF-α with other biological response modifiers has been attempted, such as therapy with HuIFN-α,[1,16] HuIFN-γ[17–20] and interleukin-2.[21] The antiproliferative activity of TNF-α combinations with various chemother-

[7] To whom correspondence should be sent.

apeutic drugs has also been investigated *in vitro*[22-30]; however, the drugs themselves caused some serious side effects.[31,32] Other drugs that can enhance the antiproliferative effect of TNF-α but that have few side effects are required.[1,33]

In this study we first evaluated the effectiveness of the TNF-α and dipyridamole combination in human melanoma cell lines, and subsequently in cell lines derived from various other kinds of tumor tissues. Cell lines refractory to HuIFN alone and to the HuIFN and dipyridamole combination were also tested.

MATERIALS AND METHODS

Agents Recombinant human TNF-α preparations (3×10^6 U/mg protein) of more than 99% purity and less than 1 ng/mg protein endotoxin contents were provided by Dainippon Pharmaceutical Co., Ltd. (Osaka)[34] and Suntory Co., Ltd. (Osaka).[35] The activity of these preparations was monitored by measuring the inhibition of L-M cell proliferation according to the method described by Bachwich *et al.*[36]

Dipyridamole was provided by Boehringer Ingelheim GmbH (Germany). The chemical was dissolved in HCl solution and used after dilution with phosphate-buffered saline (PBS). When administered orally to nude mice, the agent was diluted with 0.25% carboxylmethylcellulose (CMC), as described elsewhere.[3] The HuIFN-α (10^8 IU/mg protein) product used has been described elsewhere.[3] Eagle's minimal essential medium (EMEM) (Gibco, Grand Island, NY), calf serum (Gibco), streptomycin (Meiji Seika, Tokyo) and penicillin G (Meiji Seika) were purchased from Sanko Junyaku Co., Ltd. (Tokyo).

Other chemical agents were purchased from Nacalai Tesque Co., Ltd. (Kyoto).

Cells and culture conditions Murine L-M cells were provided by Dainippon Pharmaceutical Co., Ltd. The human tumor cell lines examined in antiproliferative tests were as follows; MM-1CB (melanoma),[2] HMV-1 (melanoma),[2] T98 (glioma),[3] SCC-1CB (squamous cell carcinoma),[37] HAC-2 (ovarian clear-cell carcinoma),[3] HLE (hepatoma),[38] HEC-1 (endometrial adenocarcinoma),[3] and HOC-21 (ovarian serous cystadenocarcinoma).[3] Cells were cultured with EMEM containing 10% calf serum and antibiotics (10^2 μg streptomycin/ml and 10^2 U penicillin G/ml), at 37°C in a humidified atmosphere containing 5% CO_2.

Cell survival test The cell proliferation assay was carried out as described before.[3] Briefly, logarithmically growing cells of each cell line were seeded, at 7×10^4 cells per 60-mm dish (Iwaki Co., Ltd., Tokyo), and 20 h later replenished with medium either containing or not containing various agents. Changes of medium were carried out every other day. After 8 days of this continuous treatment, viable cells were determined by the trypan-blue dye exclusion test and counted with a hemocytometer. The percent survival was calculated: number of viable cells in test dishes/number of viable cells in control dishes $\times$ 100%. The concentration of the agent that produced 50% survival (ID_{50}) was calculated. Evaluation of the effectiveness of TNF-α plus dipyridamole combination was also performed with isobolograms according to the method described by Tsai *et al.*[39]

For cloning efficiency assays, $1.5\text{–}1.0\times10^3$ cells were seeded in 100-mm dishes with culture medium containing an agent. At various times after seeding, the cells were washed and cultured according to procedures described previously.[3]

Detection of HuIFN antigens HuIFN levels in the culture medium were determined by monoclonal antibody-based immunoassays, as described previously.[3] The minimum detectable doses of HuIFN-α, β and γ were 2, 0.1 and 0.2 IU/ml, respectively.

Measurement of [^{14}C]amino acid, [^{3}H]uridine, and [^{3}H]deoxythymidine incorporation The incorporation of L-[^{14}C (U)]amino acid mixture (55 mCi/m atom carbon, New England Nuclear, Boston, MA) into acid-insoluble cellular materials was determined in cells treated with dipyridamole and/or TNF-α, essentially according to a method described elsewhere.[40] Briefly, after the cells had been treated with the agents for the appropriate time, they were pulse-labeled with 1 μCi/ml [^{14}C]amino acid mixture for 20 min. Cell numbers were then counted and the radioactivity of trichloroacetic acid-insoluble cell materials was determined. Values were expressed as percentages, dividing the radioactivity in TNF-α- and/or dipyridamole-treated cells by that in non-treated (control) cells.

The incorporation of [methyl-^{3}H]deoxythymidine ([^{3}H]dThd) (62 Ci/mmol, New England Nuclear) and [5,6-^{3}H]uridine (30 Ci/mmol, New England Nuclear) into acid-insoluble cell materials was measured by a pulse-labeling method, as previously described.[41] Briefly, 20 h after the seeding of cells that had been pre-cultured with 0.02 μCi/ml [2-^{14}C]dThd (63 mCi/mmol) for 24 h, the cells were treated with the cytokine and/or dipyridamole. After appropriate times, the cells were pulse-labeled with 5 μCi/ml [^{3}H]dThd or [^{3}H]uridine for 10 min, followed by determination of the radioactivity ratio (^{3}H/^{14}C) in the trichloroacetic acid-insoluble cell materials. Values were expressed as percentages by dividing the radioactivity ratio in cytokine and/or dipyridamole-treated cells by that in non-treated (control) cells.

Tumor growth inhibition assay in nude mice Anti-tumor activity in nude mice was determined essentially according to a method described previously.[2] Briefly, 1×10^7 cells were injected into the upper right thigh of each

mouse (BALB/cA JcL-nu) and from 3 days after the injection the test agents were administered (once a day) every second day. TNF-α (5×10^3 U/mouse/day) was injected subcutaneously around the tumor site. Dipyridamole, in 0.25% CMC, was given at a dose of 0.2 μmol/mouse/day by the oral route. Control subcutaneous and oral administration was done with PBS and 0.25% CMC solution, respectively. An approximation of tumor weight was obtained, as described elsewhere, by employing the formula: $(a\times b^2)/2$, where a and b are the length and width of the tumor, respectively.[2]

Other conditions Values are expressed as means of values obtained from two independent experiments, unless specifically stated otherwise. The significance of differences between groups treated with TNF-α or HuIFN-α alone and the combination treatment groups was evaluated by using Student's t test.

RESULTS

Effects of TNF-α and dipyridamole combination in tumor cell lines *in vitro* We first examined the effectiveness of the TNF-α and dipyridamole combination in the melanoma cell line MM-1CB. Treatment with TNF-α (10 U/ml) or dipyridamole (1 μM) alone had a slight inhibitory effect on MM-1CB cell proliferation, while more marked inhibition was achieved by treatment with the combination of the two agents in equal concentrations for more than 6 days (Fig. 1A). The percent survival after 8 days of continuous exposure to the two-agent combination was enhanced at various concentrations of TNF-α (10–10^2 U/ml) and at all concentrations (0.1–10 μM) of dipyridamole tested (Table I). The dose of TNF-α required to obtain 10% survival on day 8 was more than 10^2 U/ml for TNF-α alone, whereas the dose of each agent in the combination was only 1–20 U/ml (Table I). The dipyridamole-enhanced antiproliferative activity of TNF-α was confirmed by determination of cloning efficiency after 24–48 h of the combination treatment (Fig. 1B).

In contrast to MM-1CB, the melanoma cell line HMV-1 was relatively resistant to TNF-α. The ID_{50} value in MM-1CB cells was only 2 U/ml or less after 8 days of TNF-α treatment, whereas that in HMV-1 cells was about 15-fold higher (Table I). However, in combination with dipyridamole (1–10 μM) the inhibition of HMV-1 cell proliferation was substantially enhanced (Table I), and the ID_{50} value dropped to less than 10 U/ml TNF-α. There was no great difference between HMV-1 and MM-1CB cells in sensitivity to dipyridamole alone, and both showed more than 70% survival after 8 days of treatment with 1–10 μM dipyridamole (Table I).

In addition to melanoma cell lines, various other tumor cell lines were also examined with respect to their susceptibility to the combination of TNF-α and dipyridamole by cell proliferation assays *in vitro*.

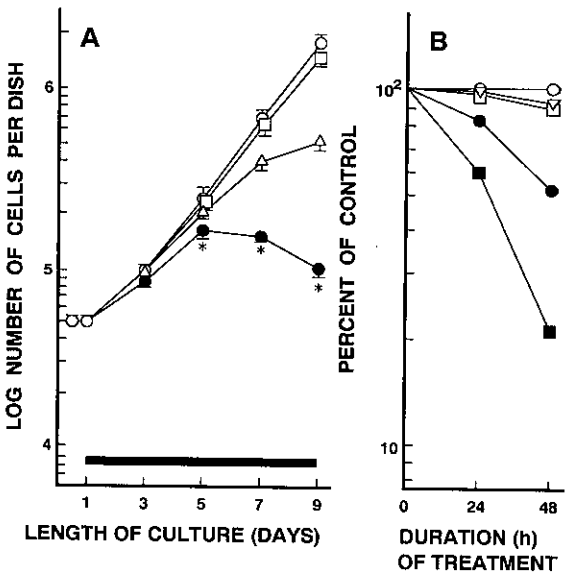

Fig. 1. Effects of TNF-α with and without dipyridamole on cell proliferation curves (A) and colony formation (B) in MM-1CB cells. (A) ○, control; △, 10 U/ml TNF-α; □, 1 μM dipyridamole; ●, 10 U/ml TNF-α plus 1 μM dipyridamole; ▬, duration of treatment with TNF-α and/or dipyridamole. (B) ○, 1 μM dipyridamole alone; ▽, 10 U/ml TNF-α alone; □, 10^2 U/ml TNF-α alone; ●, 10 U/ml TNF-α combined with 1 μM dipyridamole; ■, 10^2 U/ml TNF-α combined with 1 μM dipyridamole. Points in (A) show the mean of 4 determinations, and points in (B) show the mean of 2 determinations; bars, SD; difference between treatment with TNF-α alone and combination: *, $P<0.01$.

T98 glioma cells had almost the same sensitivity to dipyridamole alone (1–10 μM) as did MM-1CB and HMV-1 cells, but much more resistance to TNF-α, with ID_{50} of more than 100 U/ml (Table I). However, cell proliferation inhibition of T98 cells by TNF-α was also enhanced by the combination with dipyridamole (1–10 μM), and ID_{50} of TNF-α fell to less than 20 U/ml (Table I).

Of the other three cell lines listed in Table I, SCC-1CB and HAC-2 cells were more sensitive to TNF-α alone than HLE cells. SCC-1CB and HAC-2 cells had an ID_{50} of 20–30 U/ml, while HLE cells had an ID_{50} of more than 10^2 U/ml. SSC-1CB and HAC-2 cells were all more sensitive to dipyridamole alone than HLE cells (Table I). Despite these individual differences in susceptibility to the agents, in all three cell lines, the inhibition of cell proliferation by the two agents in combination (TNF-α, 10–10^2 U/ml, plus dipyridamole, 1–10 μM) was greater than that evoked by either agent alone (Table I).

Table I. Antiproliferative Activity of TNF-α and Dipyridamole in Combination against Various Tumor Cell Lines[a)]

Cell line	Dose of dipyridamole combined with TNF-α (μM)	Percent survival after combined treatment with TNF-α		
		0 U/ml	10 U/ml	100 U/ml
MM-1CB	0	100±4	24±3	13±2
	0.1	94±6	12±3	6.0±1.5
	1	80±2	5.2±2.5	2.0±2
	10	68±5	2.0±1	0.9±1.2
HMV-1	0	100±5	62±5	41±4
	1	89±2	30±4	14±3
	10	75±3	14±6	5.5±4.2
T98	0	100±4	92±3	84±4
	1	88±3	69±2	30±5
	10	74±5	48±4	10±3
SSC-1CB	0	100±2	59±2	36±3
	1	89±3	30±3	20±2
	10	52±3	19±2	9.3±0.5
HAC-2	0	100±3	72±3	29±2
	1	74±3	34±4	19±3
	10	55±4	19±1	3.8±1.3
HLE	0	100±3	82±2	52±3
	1	92±2	71±3	29±2
	10	88±3	48±3	11±3

a) Cell survival was determined after 8-day treatment with the agents, as described in "Materials and Methods." Each value (mean±SD, $P<0.01$) was obtained from 4 determinations.

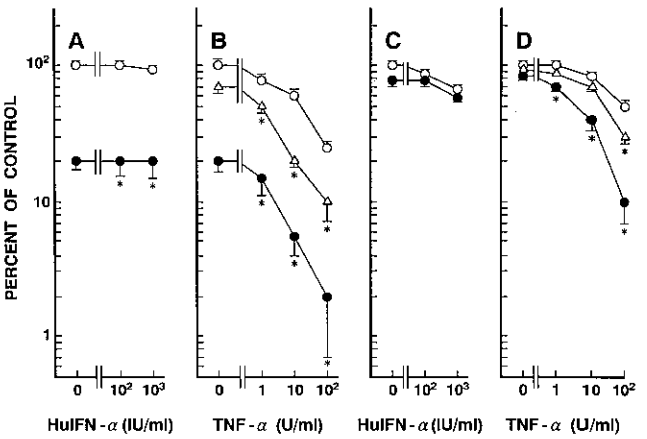

Fig. 2. Percent survival of HEC-1 cells (A and B) and HOC-21 cells (C and D) after 8-day treatment with HuIFN-α (A and C) or TNF-α (B and D) with or without dipyridamole. ○, HuIFN alone or TNF-α alone; △, combination with 1 μM dipyridamole; ●, combination with 10 μM dipyridamole. Points show mean of 4 determinations; bars, SD; *, $P<0.01$.

Effectiveness of TNF-α plus dipyridamole combination in cells resistant to HuIFN plus dipyridamole HEC-1 cells are resistant to HuIFN-β-induced inhibition of cell proliferation and this resistance is not overcome by the combination with dipyridamole.[3)] As shown in Fig. 2A, the percent survival after treatment with 10^2–10^3 IU/ml HuIFN-α alone was much the same as that in the control (without HuIFN-α treatment) and was not modulated by combination with 10 μM dipyridamole, although HEC-1 cells were highly sensitive to dipyridamole alone. HEC-1 cells were, however, susceptible to TNF-α, with an ID_{50} of about 20 U/ml (Fig. 2B). Moreover, the inhibition of cell proliferation evoked by TNF-α (1–10^2 U/ml) was greatly enhanced by combination with dipyridamole (1–10 μM): less than 10% survival was seen after 8 days of treatment with more than 10 U/ml TNF-α plus 10 μM dipyridamole (Fig. 2B).

HOC-21 cells were also refractory to the combination of HuIFN-α (10^2–10^3 IU/ml) plus dipyridamole (10 μM), although they showed slight susceptibility to each agent alone (Fig. 2C). These cells were, however, susceptible to TNF-α, with an ID_{50} of about 10^2 U/ml, and this susceptibility was enhanced by combination with dipyridamole (1–10 μM) (Fig. 2D).

Furthermore the combination effect of TNF-α plus dipyridamole was evaluated at the point of ID_{50}, using an isobologram analysis. As shown in Fig. 3, the combined data points fell in regions far from or near to the left isoeffect curves (meaning supra-additive regions) in all eight cell lines tested. Thus, the combination effect was indicated as synergistic.

Determination of HuIFN antigens in culture medium To examine whether the enhanced inhibition of cell proliferation evoked by treatment of cells with dipyridamole and TNF-α was due to extracellular HuIFN molecules produced and released into the culture medium, HuIFN antigen concentrations were estimated in the medium from all the cell lines tested here. However, no detectable amount of HuIFN-α, -β or -γ antigens was found 2 and 4 days after 1 μM dipyridamole and/or 10 U/ml TNF-α treatment (data not shown). HuIFNs added exogenously to the culture medium were detected (data not shown).

Incorporation of [^{14}C]amino acid mixture, [^{3}H]uridine, and [^{3}H]dThd into acid-insoluble cellular materials To investigate the effects of TNF-α plus dipyridamole on the synthesis of macromolecules (protein, RNA, and DNA), we determined the incorporation of radio-labeled precursors into acid-insoluble cellular materials in combination-treated cells and cells treated with TNF-α and/or dipyridamole.

The incorporation of [^{14}C]amino acids (Fig. 4, A-1, B-1 and C-1) and [^{3}H]uridine (Fig. 4, A-2, B-2 and C-2) in MM-1CB, HEC-1, and HOC-21 cells treated with 10 U/ml TNF-α plus 1 μM dipyridamole for up to 48 h was not significantly different from that in cells treated with 1 μM dipyridamole or that in control cells not treated with the agents, although a slight and transiently-enhanced

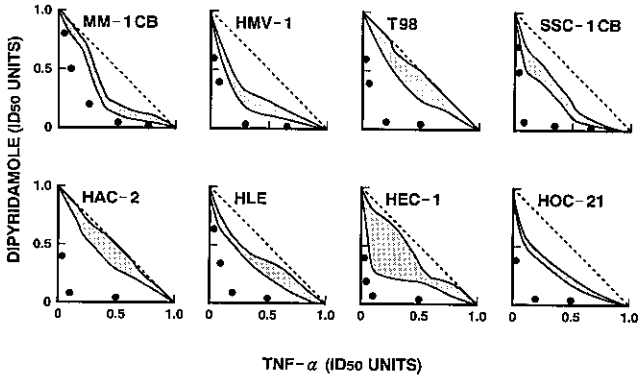

Fig. 3. Isobologram analysis for combination of TNF-α and dipyridamole producing ID_{50}. The fractional inhibitory concentrations of 1.0 for TNF-α and dipyridamole are equal to 2 U/ml and 20 μM in MM-1CB, 30 U/ml and 25 μM in HMV-1, 200 U/ml and 25 μM in T98, 30 U/ml and 10 μM in SSC-1CB, 20 U/ml and 10 μM in HAC-2, 100 U/ml and 30 μM in HLE, 20 U/ml and 5 μM in HEC-1, 100 U/ml and 30 μM in HOC-21, respectively. ●, points of the combination of TNF-α and dipyridamole which produced ID_{50}; diagonal dashed lines, idealized straight lines of additivity; shaded areas, envelopes surrounded by isoeffect curves, derived by plotting the relative doses of each agent which yield 50% survival when added together.

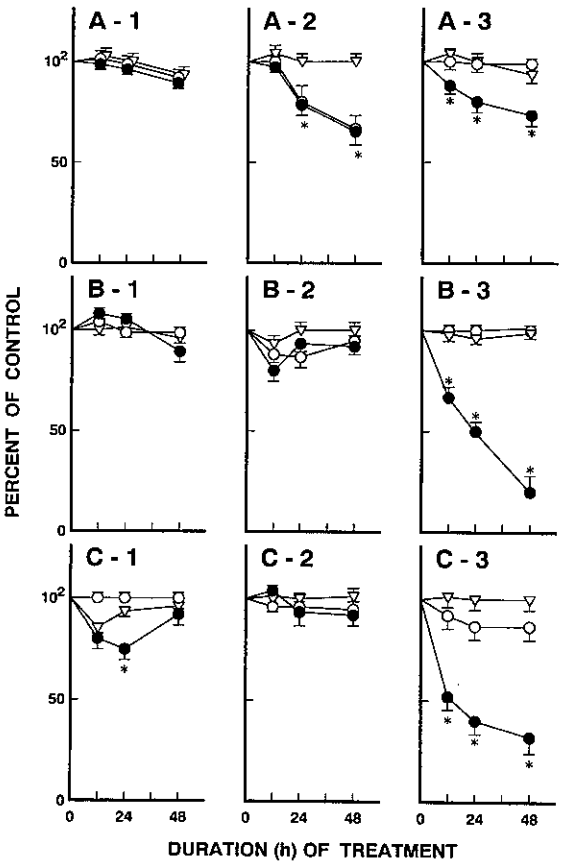

Fig. 4. Effects of treatment with 10 U/ml TNF-α with and without 1 μM dipyridamole for various times on incorporation of protein, RNA and DNA synthesis precursors into acid-insoluble matarials of MM-1CB cells (A-1, A-2, and A-3), HEC-1 cells (B-1, B-2, and B-3) and HOC-21 cells (C-1, C-2, and C-3). [^{14}C]Amino acids are shown in A-1, B-1, and C-1; [^{3}H]uridine in A-2, B-2, and C-2; and [^{3}H]dThd in A-3, B-3, and C-3. ○, TNF-α alone; ▽, dipyridamole alone; ●, combination of TFN-α and dipyridamole. Points show the mean of 4 determinations; bars, SD; difference between treatment with TNF-α alone and combination: ∗, $P<0.01$.

decrease of [^{14}C]amino acids incorporation was observed in HOC-21 cells 24 h after the combination treatment (Fig. 4C-1).

However, [^{3}H]dThd incorporation decreased in a time (0–48 h)-dependent manner in all of the cells, MM-1CB, HEC-1, and HOC-21, treated with 10 U/ml TNF-α plus 1 μM dipyridamole (Fig. 4, A-3, B-3 and C-3). At only 12 h after the combination treatment, a significant decrease was detectable (Fig. 4, A-3, B-3 and C-3) and this occurred in a TNF-α dose ($1–10^2$ U/ml)-dependent manner (Table II). This early response, i.e., a marked decrease in [^{3}H]dThd incorporation, was not observed after treatment with dipyridamole alone or with TNF-α alone (Fig. 4, A-3, B-3 and C-3). Treatment with HuIFN-α ($1–10^2$ IU/ml) in combination with 1 μM dipyridamole for 12 h did not decrease [^{3}H]dThd incorporation in any of these three cell lines (data not shown).

Other cell lines, HMV-1, T98, SCC-1CB, HAC-2, and HLE, also showed a marked decrease of [^{3}H]dThd incorporation 12 h after treatment with the combination of TNF-α ($10–10^2$ U/ml) plus 1 μM dipyridamole (Table II).

Effects of TNF-α and dipyridamole on tumor growth in nude mice The antitumor effect of TNF-α (5×10^3 U/mouse/day) plus dipyridamole (0.2 μmol/mouse/day) was tested following administration of the agents every second day after the injection of MM-1CB cells into nude mice. Subcutaneous administration of TNF-α alone had little effect on tumor growth, whereas oral administration of dipyridamole suppressed growth to about one half that of the control level (in mice not treated with TNF-α or dipyridamole) (Fig. 5A). The combination treatment with the two agents suppressed tumor growth much more markedly than when dipyridamole was given alone (Fig. 5A).

In another melanoma HMV-1 tumor, growth was not or was only slightly suppressed by subcutaneous administration of TNF-α alone (Fig. 5B), as was the case with MM-1CB tumor (Fig. 5A). Treatment with dipyridamole alone was moderately effective in suppressing

Table II. Effects of 12-h Treatment with TNF-α, Dipyridamole and a Combination of the Two Agents on [^{3}H]dThd Incorporation into Acid-insoluble Materials in Various Tumor Cell Lines[a)]

Cell line	TNF-α (U/ml)	Dipyridamole (μM)	[^{3}H]dThd incorporation (percent of control)
MM-1CB	0	1	95±5
	1	0	98±4
	1	1	90±6
	10	0	96±4
	10	1	80±7
	100	0	100±4
	100	1	52±8
HEC-1	0	1	98±6
	1	0	98±4
	1	1	80±6
	10	0	95±5
	10	1	70±8
	100	0	90±6
	100	1	51±7
HOC-21	0	1	90±8
	1	0	104±6
	1	1	76±8
	10	0	103±7
	10	1	52±6
	100	0	100±5
	100	1	48±8
HMV-1	0	1	100±8
	10	0	100±6
	10	1	50±7
	100	0	60±5
	100	1	20±6
T98	0	1	100±6
	10	0	100±7
	10	1	40±5
	100	0	90±8
	100	1	30±6
SCC-1CB	0	1	110±10
	10	0	70±6
	10	1	52±5
	100	0	61±4
	100	1	21±7
HAC-2	0	1	80±6
	10	0	100±3
	10	1	50±4
	100	0	61±7
	100	1	21±5
HLE	0	1	100±7
	10	0	80±4
	10	1	52±6
	100	0	69±5
	100	1	37±6

a) Treatment of cells with TNF-α, dipyridamole, and the combination of the two agents and measurement of [^{3}H]dThd incorporation are described in "Materials and Methods." Each value (mean±SD, $P<0.01$) was obtained from 4 determinations.

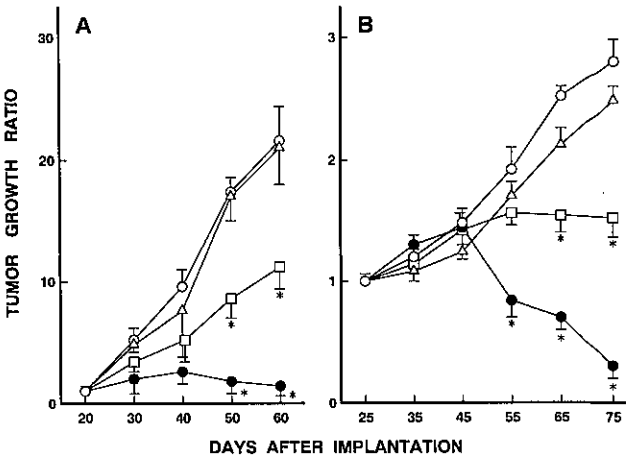

Fig. 5. Effects of TNF-α in combination with and without dipyridamole on growth of tumors in nude mice after the implantation of MM-1CB cells (A) and HMV-1 cells (B). Administration of agents (29 times/mouse from day 3 through day 60 for MM-1CB-implanted mice, and 36 times/mouse from day 3 through day 75 for HMV-1-implanted mice), and approximation of tumor weight were done as described in "Materials and Methods." ○, control; △, TNF-α alone; □, dipyridamole alone; ●, combination of TNF-α and dipyridamole. Points show the mean of 4 determinations; bars, SD; difference between treatment with TNF-α alone and the combination therapy: *, $P<0.01$.

HMV-1 tumor growth 55 days after cell implantation (Fig. 5B). In this case too, greater suppression of tumor growth was observed when TNF-α and dipyridamole were administered in combination than when either was administered alone (Fig. 5B).

DISCUSSION

This study demonstrated the enhanced antiproliferative activity exerted by the TNF-α and dipyridamole combination compared to the antiproliferative activity of either agent alone. The human tumor cell lines used here were divided into three groups on the basis of their *in vitro* sensitivity to TNF-α alone: the MM-1CB cell line, with an ID_{50} of less than 2 U/ml; a number of relatively susceptible cell lines, with ID_{50} of 10–10^2 U/ml (HMV-1, SSC-1CB, HAC-2, HLE, HEC-1, and HOC-21); and the T98 cell line, with an ID_{50} of more than 10^2 U/ml. With respect to dipyridamole sensitivity, the cell lines were divided into two groups: the HEC-1 cell line, with a survival of about 20% 8 days after continuous treatment with dipyridamole alone, and the other cell lines, in which there was more than 50% survival. Irrespective of the cell line's difference in intrinsic sensitivity to TNF-α and dipyridamole, the combination of these agents enhanced the antiproliferative effect of each one alone.

HEC-1 cells were resistant to HuIFN-α and to the combination of this agent with dipyridamole, as shown in Fig. 2A. In contrast, the proliferation of HEC-1 cells was markedly inhibited by the combination of TNF-α and dipyridamole compared to the inhibition evoked by TNF-α alone (Figs. 2B and 3). In HOC-21 cells, the antiproliferative activity of HuIFN-β was not enhanced when the agent was combined with dipyridamole,[3] nor was the antiproliferative activity of HuIFN-α enhanced by dipyridamole (Fig. 2C). HOC-21 cells were, however, susceptible to TNF-α, and combination treatment with dipyridamole resulted in more marked inhibition of cell proliferation than treatment with TNF-α alone (Figs. 2D and 3). Interestingly, HOC-21 cells were found to be insusceptible to the dipyridamole-enhanced activity of various chemotherapeutic agents.[3] Thus, if dipyridamole is used in combination with TNF-α or HuIFN as an anti-cancer treatment, the effectiveness of both the cytokines should be evaluated.

There was no detectable HuIFN antigen in any of the culture media of cells treated with dipyridamole alone. TNF-α has been reported to enhance the antiproliferative effect or production of HuIFN.[42,43] However, here we found no HuIFN antigens in any of the culture media of cells treated with TNF-α or with TNF-α plus dipyridamole. The enhancement of TNF-α antiproliferative activity by dipyridamole in HuIFN-resistant HEC-1 cells (Figs. 2A, 2B and 3) suggests that the enhancement effect does not require a contribution from HuIFN.

There have been reports on the mechanisms whereby TNF-α exerts its antiproliferative effects on target cells, including DNA fragmentation, alterations in arachidonic acid metabolism, lipid peroxidation, and the involvement of lysosomal enzymes such as protease.[1] We showed here that the incorporation of a DNA synthesis precursor, [^{3}H]dThd, was specifically inhibited by the TNF-α plus dipyridamole combination, while the incorporation of RNA and protein synthesis precursors, [^{3}H]uridine and [^{14}C]amino acids, respectively, was not affected by this treatment (Fig. 4 and Table II). Pusztai *et al.*[44] demonstrated inhibition of [^{3}H]dThd incorporation into cellular DNA after 24-h TNF-α exposure in a breast cancer cell line. In all the cell lines examined here, only slight inhibition of [^{3}H]dThd incorporation was observed after treatment with TNF-α alone, whereas inhibition was marked after treatment with the combination of TNF-α plus dipyridamole (Fig. 4 and Table II). After only 12 h of the combination there was greatly enhanced inhibition (Fig. 4 and Table II). We have already reported that the HuIFN-α and dipyridamole combination inhibited [^{3}H]dThd incorporation.[2,3] However, in this study, we observed no marked inhibition of [^{3}H]dThd incorporation 12 h after treatment with HuIFN-α plus dipyridamole. Modulation of the DNA precursor metabolism and/or DNA synthesis may play a role in the antiproliferative effects exerted by the TNF-α plus dipyridamole combination, at least *in vitro*.

We have already reported that induction of antipain-sensitive protease activity is involved in cellular functions responding to HuIFN-α actions.[45] We are also focusing our attention on the possible role of protease activity in the TNF-α plus dipyridamole combination effects. Addition of serine-type protease inhibitors during the combination treatment has been found to result in suppression of the enhanced antiproliferative activity (unpublished results), although how the protease activity is interrelated with the modulation of DNA precursor metabolism and/or DNA synthesis remains to be established. Interestingly, Camussi *et al.*[46] suggested a critical role of protease activation for TNF-α actions. We will deal with protease-related mechanisms for the TNF-α plus dipyridamole combination effects in a later publication.

Although most phase I studies and early phase II work have found that the systemic administration of recombinant TNF-α had no significant therapeutic effect, administration at the tumor site has proven more promising.[1] In the present study, administration of TNF-α at the tumor sites of melanoma cells transplanted into nude mice did not suppress or only weakly suppressed tumor growth, whereas the oral administration of dipyridamole (0.2 μmol/mouse/day) combined with TNF-α led to remarkable suppression of tumor growth (Fig. 5). Unexpectedly, the oral administration of dipyridamole alone effectively suppressed the growth of MM-1CB tumors (Fig. 5), in contrast to our findings for the subcutaneous injection of dipyridamole at the same dose,[2] which treatment was not effective in suppressing MM-1CB tumor growth. The reason for the efficacy via the oral route is not clear.

When dipyridamole was given orally to cancer patients, the agent was detected in plasma at levels of a few micromolar.[47] Importantly, as was evident in a previous study of ours,[3] and in the present study, the antiproliferative activity of HuIFN and TNF-α *in vitro* was enhanced at doses of 1–10 μM dipyridamole; in some cell lines such as MM-1CB, the enhancement was observed at concentrations of less than 0.1 μM. Thus, it would appear that dipyridamole may be beneficial in increasing the effectiveness of anti-cancer cytokine therapy.

ACKNOWLEDGMENTS

We thank Drs. M. Tatibana and H. Kohei for valuable support. We also thank Miss Y. Oiwa, Miss H. Kobayashi and Dr. Y. Tsunenari for their technical support. *In vivo* studies were done at the Laboratory Animal Center of Chiba University.

(Received January 17, 1995/Accepted April 27, 1995)

REFERENCES

1) Rege, A. A., Huang, K. and Aggarwal, B. B. Tumor necrosis factor. *In* "Cytokine Therapy," ed. D. W. Galvani and J. C. Cawley, pp. 152–176 (1992). Cambridge University Press, New York.
2) Kojima, T., Suzuki, N., Sugano, I. and Hayata, I. Enhancement of an anti-tumor effect of interferon by dipyridamole in established human malignant melanoma cell strains. *Int. J. Cancer,* **46,** 853–857 (1990).
3) Suzuki, N., Oiwa, Y., Sugano, I., Inaba, N., Sekiya, S., Fukazawa, I., Yoshida, J., Takakubo, Y., Isogai, E. and Saito-Ebihara, M. Dipyridamole enhances an antiproliferative effect of interferon in various types of human tumor cells. *Int. J. Cancer,* **51,** 627–633 (1992).
4) Caine, I. and Eldor, A. Dipyridamole. *Harefuah,* **111,** 240–244 (1986) (in Hebrew).
5) Batlouni, M. Dipyridamole and ischemic heart disease. *Arq. Bras. Cardiol.,* **50,** 69–74 (1988).
6) Shalaev, S. V. Platelet aggregation inhibition in the prevention of myocardial infarction: the results of prospective studies. *Kardiologiya,* **29,** 116–120 (1989).
7) Carswell, E. A., Old, L. J, Kassel, R. L., Green, S., Fiore, N. and Williamson, B. An endotoxin-induced serum factor that causes necrosis in tumors. *Proc. Natl. Acad. Sci. USA,* **72,** 3666–3670 (1975).
8) Blick, M., Sherwin, S. A., Rosenblum, M. and Gutterman, J. Phase I study of recombinant tumor necrosis factor in cancer patients. *Cancer Res.,* **47,** 2986–2989 (1987).
9) Wiedenmann, B., Reichardt, P., Rath, U., Theilmann, L., Schule, B., Ho, A. D., Schlick, E., Kempeni, J., Hunstein, W. and Kommerell, B. Phase I trial of intravenous continuous infusion of tumor necrosis factor in advanced metastatic carcinomas. *J. Cancer Res. Clin. Oncol.,* **115,** 189–192 (1989).
10) Aulitzky, W. E., Tilg, H., Gunther, G., Mull, R., Flener, R., Vogel, W., Herold, M., Berger, M., Judmaier, G. and Huber, C. Recombinant tumor necrosis factor α administered subcutaneously or intramuscularly for treatment of advanced malignant disease: a phase I trial. *Eur. J. Cancer,* **27,** 462–467 (1991).
11) Mittelman, A., Puccio, C., Gafney, E., Coombe, N., Singh, B., Wood, D., Nadler, P., Ahmed, T. and Arlin, Z. A phase I pharmacokinetic study of recombinant human tumor necrosis factor administered by a 5-day continuous infusion. *Invest. New Drugs,* **10,** 183–190 (1992).
12) Yoshida, J., Wakabayashi, T., Mizuno, M., Sugita, K., Yoshida, T., Hori, S., Mori, T., Sato, T., Karashima, A., Kurisu, K., Kiya, K. and Uozumi, T. Clinical effect of intra-arterial tumor necrosis factor-α for malignant glioma. *J. Neurosurg.,* **77,** 78–83 (1992).
13) Schiller, J. H., Storer, B. E., Witt, P. L., Alberti, D., Tombes, M. B., Arzoomanian, R., Proctor R. A., McCarthy, D., Brown, R. R., Voss, S. D., Remick, S. C., Grem, J. L., Borden, E. C. and Trump, D. L. Biological and clinical effects of intravenous tumor necrosis factor-α administered three times weekly. *Cancer Res.,* **51,** 1651–1658 (1991).
14) Bocci, V. Central nervous system toxicity of interferons and other cytokines. *J. Biol. Regul. Homeost. Agents,* **2,** 107–118 (1988).
15) Piper, B. F., Rieger, P. T., Brophy, L., Haeuber, D., Hood, L. E., Lyver, A. and Sharp, E. Recent advances in the management of biotherapy-related side effects: fatigue. *Oncol. Nurs. Forum,* **16,** 27–34 (1989).
16) Orita, K., Fuchimoto, S., Kurimoto, M., Ando, S. and Minowada, J. Early phase II study of interferon-α and tumor necrosis factor-α combination in patients with advanced cancer. *Acta Med. Okayama,* **46,** 103–112 (1992).
17) Demetri, G. D., Spriggs, D. R., Sherman, M. L., Arthur, K. A., Imamura, K. and Kufe, D. W. A phase I trial of recombinant human tumor necrosis factor and interferon-γ: effects of combination cytokine administration *in vivo. J. Clin. Oncol.,* **7,** 1545–1553 (1989).
18) Kurzrock, R., Fienberg, B., Talpaz, M., Saks, S. and Gutterman, J. U. Phase I study of a combination of recombinant tumor necrosis factor-α and recombinant interferon-γ in cancer patients. *J. Interferon Res.,* **9,** 435–444 (1989).
19) Fiedler, W., Zeller, W., Peimann, C. J., Weh, H. J. and Hossfeld, D. K. A phase II combination trial with recombinant human tumor necrosis factor and gamma interferon in patients with colorectal cancer. *Klin. Wochenschr.,* **69,** 261–268 (1991).
20) Smith, J. W., Urba, W. J., Clark, J. W., Longo, D. L., Farrell, M., Creekmore, S. P., Conlon, K. C., Jaffe, H. and Steis, R. G. Phase I evaluation of recombinant tumor mecrosis factor given in combination with recombinant interferon-gamma. *J. Immunother.,* **10,** 355–362 (1991).
21) Yang, S. C., Grim, E. A., Parkinson, D. R., Carinhas, J., Fry, K. D., Mendiguren-Rodriguez, A., Licciardello, J., Owen-Schaub, L. B., Hong, W. K. and Roth, J. A. Clinical and immunomodulatory effects of combination immunotherapy with low-dose interleukin 2 and tumor necrosis factor-α in patients with advanced non-small cell lung cancer: a phase I trial. *Cancer Res.,* **51,** 3669–3676 (1991).
22) Alexander, R. B., Nelson, W. G. and Coffey, D. S. Synergistic enhancement by tumor necrosis factor of *in vitro* cytotoxicity from chemotherapeutic drugs targeted at DNA topoisomerase II. *Cancer Res.,* **47,** 2403–2406 (1987).
23) Itano, S., Fuchimoto, S. and Orita, K. The synergistic antitumor effect of natural-human TNF and anticancer drugs. *Hiroshima J. Med. Sci.,* **36,** 157–161 (1987).
24) Regenass, U., Muller, M., Curschellas, E. and Matter, A. Antitumor effect of tumor necrosis factor in combination with chemotherapeutic agents. *Int. J. Cancer,* **39,** 266–273 (1987).
25) Watanabe, N., Niitsu, Y., Yamauchi, N., Ohtsuka, Y.,

Sone, H., Neda, H., Maeda, M. and Urushizaki, I. Synergistic cytotoxicity of recombinant human TNF and various anti-cancer drugs. *Immunopharmacol. Immunotoxicol.*, **10**, 117–127 (1988).
26) Mutch, D. G., Powell, C. B., Kano, M.-S. and Collins, J. L. *In vitro* analysis of the anti-cancer potential of tumor necrosis factor in combination with cis-platinum. *Gynecol. Oncol.*, **34**, 328–333 (1989).
27) Krosnick, J. A., Mule, J. J., McIntosh, J. K. and Rosenberg, S. A. Augmentation of antitumor efficacy by the combination of recombinant tumor necrosis factor and chemotherapeutic agents *in vitro*. *Cancer Res.*, **49**, 3729–3733 (1989).
28) Bonavida, B., Tsuchitani, T., Zighelboim, J. and Berek, J. Synergy is documented *in vitro* with low-dose recombinant tumor necrosis factor, cisplatin, and doxorubicin in ovarian cancer cells. *Gynecol. Oncol.*, **38**, 333–339 (1990).
29) Cimoli, G., Valenti, M., Venturini, M., Conte, P. and Russo, P. Augmentation of antineoplastic effects by the combination of recombinant human tumor necrosis factor and mitoxantrone on primary culture of human ovarian cancer cells. *Anticancer Res.*, **12**, 1411–1414 (1992).
30) Matsuo, S., Takano, S., Yamashita, J. and Ogawa, M. Synergistic cytotoxic effects of tumor necrosis factor, interferon-γ and tamoxifen on breast cancer cell lines. *Anticancer Res.*, **12**, 1575–1580 (1992).
31) Schuchter, L. M., Luginbuhl, W. E. and Meropol, N. J. The current status of toxicity protectants in cancer therapy. *Semin. Oncol.*, **19**, 742–751 (1992).
32) Hill, A. D. K., Redmond, H. P., Croke, D. T., Grace, P. A. and Bouchier-Hayes, D. Cytokines in tumor therapy. *Br. J. Surg.*, **79**, 990–997 (1992).
33) Frei, E., III. and Spriggs, D. Tumor necrosis factor: still a promising agent. *J. Clin. Oncol.*, **7**, 291–294 (1989).
34) Taguchi, T. and Sohmura, Y. Clinical studies with TNF. *Biotherapy*, **3**, 177–186 (1991).
35) Tsujimoto, M., Tanaka, S., Sakuragawa, Y., Tsuruoka, N., Funakoshi, K., Butsugan, T., Nakazato, H., Nishihara, T., Noguchi, T. and Vilcek, J. Comparative studies of the biological activities of human tumor necrosis factor and its derivatives. *J. Biochem.*, **101**, 919–925 (1987).
36) Bachwich, P. R., Lynch, J. P., Larrick, J., Spengler, M. and Kunkel, S. L. Tumor necrosis factor production by human sarcoid alveolar macrophage. *Am. J. Pathol.*, **125**, 421–426 (1986).
37) Watanabe, F., Kojima, T., Morita, E., Kobayashi, M. and Fujita, M. Establishment of a human skin squamous cell carcinoma cell line (SCC-1CB) *in vitro* and its characteristics. *Jpn. J. Dermatol.*, **99**, 793–800 (1989) (in Japanese).
38) Doi, I., Namba, M. and Sato, J. Establishment and some biological characteristics of human hepatoma cell lines. *Gann*, **66**, 385–392 (1975).
39) Tsai, C.-M., Gazdar, A. F., Venzon, D. J., Steinderg, S. M., Dedrick, R. L., Mulshine, J. L. and Kramer, B. S. Lack of *in vitro* synergy between etoposide and *cis*-diammine-dichloroplatinum(II). *Cancer Res.*, **49**, 2390–2397 (1989).
40) Fuse, A. and Kuwata, T. Effects of interferon on the human clonal cell line, RSa: inhibition of macromolecular synthesis. *J. Gen. Virol.*, **33**, 17–24 (1976).
41) Sugita, K., Suzuki, N., Kojima, T., Tanabe, Y., Nakajima, H., Hayashi, A. and Arima, M. Cockayne syndrome with delayed recovery of RNA synthesis after ultraviolet irradiation but normal ultraviolet survival. *Pediatr. Res.*, **21**, 34–37 (1987).
42) Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino, M. A., Jr. and Shepard, H. M. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells *in vitro*. *Science*, **230**, 943–945 (1985).
43) Reis, L. F. L., Lee, T. H. and Vilcek, J. Tumor necrosis factor acts synergistically with autocrine interferon-β and increases interferon-β mRNA levels in human fibroblasts. *J. Biol. Chem.*, **264**, 16351–16354 (1989).
44) Pusztai, L., Lewis, E. C. and McGee, J. O. Growth arrest of the breast cancer cell line, T47D, by TNFα; cell cycle specificity and signal transduction. *Br. J. Cancer*, **67**, 290–296 (1993).
45) Isogai, E. and Suzuki, N. Involvement of antipain-sensitive protease activity in suppression of UV-mutagenicity by human interferon-α. *Mutat. Res.*, **325**, 81–85 (1994).
46) Camussi, G., Albano, E., Tetta, C. and Bussolino, F. The molecular action of tumor necrosis factor-α. *Eur. J. Biochem.*, **202**, 3–14 (1991).
47) Casper, E. S., Baselga, J., Smart, T. B., Magill, G. B., Markman, M. and Ranhosky, A. A phase II trial of PALA+dipyridamole in patients with advanced soft-tissue sarcoma. *Cancer Chemother. Pharmacol.*, **28**, 51–54 (1991).