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Induction of Differentiation of Human Myeloid Leukemia Cells by 2′-Deoxycoformycin in Combination with 2′-Deoxyadenosine

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2′-Deoxycoformycin (dCF), a specific and potent inhibitor of adenosine deaminase, has demonstrated significant antitumor effect on lymphoid malignancies. The drug induced functional and morphologic differentiation of myeloid leukemia cells in combination with 2′-deoxyadenosine (dAd), but not dCF alone. NB4, a cell line derived from a patient with t(15;17) acute promyelocytic leukemia (APL) underwent granulocytic differentia tion when treated with all-*trans* retinoic acid (ATRA) or dCF plus dAd, but not with cytosine arabinoside. Pre-exposure of NB4 cells to ATRA greatly potentiated differentiation induced by dCF plus dAd, but pretreatment with dCF plus dAd before exposure to ATRA was less effective. Differentiation of NB4 cells was effectively induced by clinically applicable concentrations of dCF in combination with dAd. These findings may provide useful information about induction of differentiation *in vivo*.

Differentiation therapy with ATRA leads to a predictable clinical remission in APL patients (1,2). However, hyper-leukocytosis is observed during the induction of remission by ATRA in a high frequency of APL patients, leading to severe adverse effects in some patients. A more effective therapeutic strategy is needed for differentiation therapy. Although ATRA can induce differentiation of many myelomonocytic leukemia cell lines *in vitro* (3,4), ATRA is less effective in clinical trials against other hematologic malignancies. Further development of differentiation therapy for leukemia requires new and potent inducers of differentiation. Alternatively, a suitable combination of known differentiation inducers might be effective therapy for AML or MDS, since combined treatment with some differentiation inducers is synergistic for the induction of differentiation, terminal cell division and apoptotic cell death (5-8). The application of synergy between combinations of differentiation inducers may lead to a potent differentiation therapy for some types of leukemia.

Some nucleoside analogs can induce differentiation of leukemia cells (9-12). AraC, an analog of cytosine that is effective in chemotherapy of AML, is effective as a differentia tion therapy for patients with AML or MDS, although most patients with complete remission underwent a hypoplastic or aplastic phase (13,14). The limited success of differentiation therapy with low-dose AraC led us to find potent analogs for use in differen tiation therapy for AML and MDS. Although all the nucleoside analogs have been used as cytotoxic drugs in treatment of leukemia and lymphoma, some nucleoside analogs might be effective as differentiation inducers for control of leukemia cells. Therefore, we examined the differentiation-inducing activity of nucleoside analogs which are clinically used in treatment of leukemia and lymphoma, and combined effect of the analogs and ATRA or other non-toxic differentiation inducers.

MATERIALS AND METHODS

Chemicals and differentiation inducers. dCF was obtained from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). dAd, ATRA and NBT were purchased from Sigma Chemical Co.(St Louis, MO), and VD3 was from Wako Pure Chemicals (Osaka, Japan). dCF was dissolved in phosphate-buffered saline, and the stock solution was prepared in 0.1 M and kept at 4°C.

Cells and cell culture. Human myeloid leukemia K562, HL-60 and NB4 cells (15) were cultured in suspension in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air.

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Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; MDS, myelodysplastic syndrome; ATRA, all-*trans* retinoic acid; AraC, cytosine arabinoside; VD3, 1α,25-dihydroxyvitamin D_3, dAd, 2′-deoxyadenosine; dCF, 2′-deoxycoformycin; NBT, nitroblue tetrazolium.

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Assay of cell growth and differentiation. Cell numbers were counted with a Model ZM counter (Coulter Electronics, Luton, UK) after culture for the indicated times. Erythroid differentiation was assayed by benzidine staining (16). Myelomonocytic differentiation was measured by reduction of NBT, non-specific α-naphthyl acetate esterase, lysozyme and morphology using light microscopy of cytospin preparations stained with May-Grünwald-Giemsa solution (Merck, Darmstadt, Germany) (5,6,9).

Flow cytometry. Expression of myelomonocytic antigen CD11b and CD14, and erythroid marker glycophorin A on the surface was determined by indirect immunofluorescent staining and flow cytometry. Cells were incubated for 30 min at 4°C in the presence of an appropriate monoclonal antibody. After three washes, cells were incubated for 30 min at 4°C with goat anti-mouse Ig-labeled with fluorescein, then analyzed in an Epics EX flow cytometer (Coulter Electronics, Hialeah, FL)(17).

Cell cycle analysis. Cells were washed, fixed by 100% ethanol, and left for 30 min on ice. After RNase treatment, cells were stained with 50 μg/ml of propidium iodide in 1.12% sodium citrate and analyzed in a cytometer (18).

Determination of mRNA level by reverse transcription-PCR. RNA was extracted by modification of the method of Chomczynski and Sacchi (19). Total RNA (0.2 μg) from NB4 cells was converted to first strand cDNA primed with random hexamer using a GeneAmp RNA PCR kit (Takara Shuzo Co, Tokyo, Japan). The primers and profiles of the amplification reactions were as follows: bcl-2-F (forward); GGTGCCACCTGTGGTCCACCTG, bcl-2-R (reverse); CTTCACTTGTGGCCCAGATAGG, bax-F; TGCTTCAGGGTTTCATCCAGG, bax-R; TGGCAAAGTAGAAAAGGGCGA, bcl-XL-F; CCAGCCGCCGTTCTCCTGGAT, bcl-XL-R; CCGGGATGGGGTAAACTGGGG, 95°C for 45 sec-60°C for 45 sec-72°C for 2 min for 35 cycles. The primers for c-myc and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared as described (20). Amplification cycles were preceded by a denaturation step (95°C for 2 min) and followed by an elongation step (72°C for 10 min). After amplification, PCR products were analyzed on 1.5% agarose gels with ethidium bromide staining.

RESULTS

We examined ability of several anticancer nucleosides to induce differentiation of human erythroleukemia K562 and promyelocytic leukemia HL-60 cells, and found that dCF effectively induced differentiation of K562 and HL-60 cells in the presence of dAd. Neither dCF nor dAd alone induce erythroid differentiation of K562 cells even in a high concentration of the drugs, but combined treatment with dCF and dAd effectively induced the hemoglobin-producing cells in a dose-dependent manner (Table 1 and data not shown). Expression of glycophorin A, another typical erythroid differentiation marker, was expressed in untreated K562 cells, and the expression was greatly enhanced by dCF plus dAd, but not by dCF or dAd alone. With respect to growth-inhibitory effect, the combined treatment of dCF and dAd was prominent but the single treatment was modest (Table 1). We next examined the effect on differentiation of HL-60 cells. NBT reduction, a typical marker of myelomonocytic differentia tion of the cells, was significantly induced by dCF alone and the inducing effect was augmented in the presence of dAd (Fig. 1). Morphologic examination reveals that dCF induces

TABLE 1

Effect of dCF and dAd on Growth Inhibition and Erythroid Differentiation of K562 Cells

Treatment	Growth (% of control)	Benzidine-positive (%)	Glycophorine A expression (mean intensity)
None	100	4.3 ± 0.3	2.0 ± 0.1
dCF	103 ± 2	5.2 ± 0.3	2.1 ± 0.1
dAd	79 ± 2	10.8 ± 1.6	2.9 ± 0.3
dCF + dAd	47 ± 1	74.3 ± 6.3	12.9 ± 1.8

Cells were cultured with or without 1 μM dCF and 50 μM dAd for 5 days. Values represent the means±SD of three separate experiments.

granulocytic differentiation of HL-60 cells in the presence of dAd, mainly into myelocytes and metamyelocytes (Table 2). This was confirmed by the results that α-naphthyl acetate esterase activity was not detected in the treated cells (data not shown). On incubation with dCF plus dAd for 4 days, lysozyme activity was increased about 2-fold (Table 2) and the growth was dose-dependently inhibited, $5.1 \pm 0.5 \times 10^{-5}$ M dCF in the presence of 50 μM dAd causing 50 % inhibition (IC_{50}). This combined treatment also induced differentiation of other human myeloid leukemia cells, such as promyelocytic leukemia NB4 cells (Fig. 1) and monocytic leukemia U937 and THP-1 cells, although dCF alone did not induce any differentiation-associated markers of the cells.

Combined treatment of dCF plus dAd significantly induced differentiation of several myeloid leukemia cells, but the drug in the clinically applicable concentrations (less than 5 μM dCF) seemed to be insufficient to use as an agent of differentiation therapy of AML, since the concentrations were similar to IC_{50} concentration of HL-60 cells. Therefore, we next examined the differentiation-inducing effect of dCF plus dAd in the presence of clinically applicable concentrations of ATRA or VD3 (Table 2). The NBT reducing activities of cells treated with 3 nM VD3 and 10 μM dCF plus 50 μM dAd were 1.4 and 1.8 $A_{560}/10^7$ cells, respectively. The combined effect of VD3 and dCF+dAd was more than additive to induction of NBT reduction of HL-60 cells (6.4 $A_{560}/10^7$ cells). Similar results were obtained in induction of the other differentiation-associated phenotypes, such as lysozyme production, expression of CD11b (β-integrin subunit, expressed by both granulocytes and monocytes), morphologic changes (Table 2) and expression of CD14, a late monocytic cell surface marker (data not shown). ATRA at clinically applicable concentrations and the drugs also cooperated in induction of differentiation of HL-60 cells (Table 2).

AraC effectively induced differentiation of K562 and HL-60 cells, but not differentiation of promyelocytic

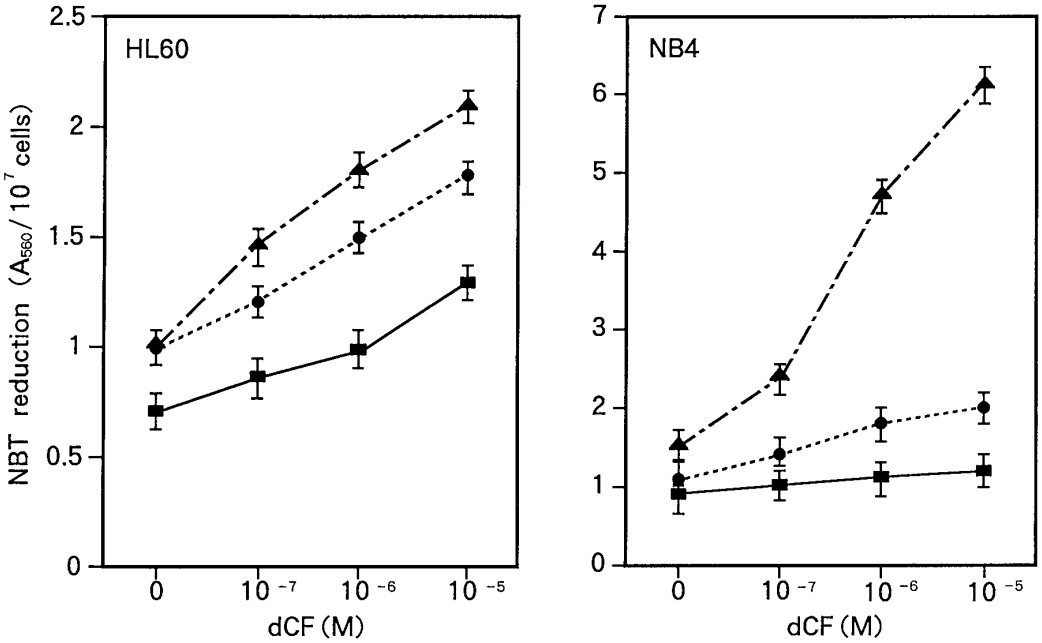

FIG. 1. Induction of NBT reduction of HL-60 and NB4 leukemia cells by dCF and dAd. Cells were cultured with various concentrations of dCF in combination with 0 (■), 30 (●), or 50 (▲) μM dAd for 5 days.

NB4 cells. On the other hand, differentiation of NB4 cells was significantly induced by dCF plus dAd in a dose-dependent manner (Fig. 1 and Table 3). ATRA also induced differentiation of the cells, and the combined effect of ATRA and dCF plus dAd was additive when the cells were simultaneously treated for 5 days (Fig. 2A). To determine effect of pretreatment with dCF plus dAd on ATRA-induced NBT reduction, NB4 cells were treated with various concentrations of dCF in the presence of 40 μM dAd for 2 days, washed in fresh medium, and then incubated with ATRA for 5 days. The results were almost same to those of the simultaneous treatment (Figs. 2A and 2B), indicating that effect of the pretreatment was just additive. For the post-treatment with dCF plus dAd, cells were treated with ATRA for 2 days and then with various concentrations of dCF in the presence of 40 μM dAd (Fig. 2C). The treatment was more effective than that of the simultaneous treatment in induction of NBT reduction of NB4 cells, suggesting that the priming effect of ATRA was synergistic to treat-

TABLE 2

Effect of dCF and dAd on Differentiation of HL-60 Cells Treated with VD3 or ATRA

Treatment	Growth (% of control)	NBT reduction ($A_{560}/10^7$ cells)	Lysozyme ($U/10^6$ cells)	CD11b expression (%)	Morphologic change (%)			
					Bla	Mye	Met	Mono
					+Pro		+Gr	
None	100	0.6 ± 0.2	0.16 ± 0.02	4 ± 1	99	1	0	0
dCF	99.4 ± 0.6	0.9 ± 0.2	0.17 ± 0.01	3 ± 1	99	1	0	0
dAd	96.1 ± 1.4	1.0 ± 0.1	0.17 ± 0.02	4 ± 1	98	2	0	0
dCF+dAd	69.4 ± 4.4	1.8 ± 0.2*	0.23 ± 0.03	6 ± 1	78	19	3	0
VD3	96.4 ± 1.8	1.4 ± 0.1*	0.23 ± 0.04	10 ± 2	68	8	2	22
VD3+dCF+dAd	47.4 ± 4.5	6.4 ± 1.4**	0.61 ± 0.08**	34 ± 3	34	14	1	51
ATRA	95.1 ± 2.6	1.2 ± 0.4*	0.18 ± 0.03	38 ± 4**	62	18	20	0
ATRA+dCF+dAd	48.4 ± 3.1	8.5 ± 1.5***	0.26 ± 0.04	48 ± 2**	24	27	49	0

HL-60 cells were cultured with 1 μM dCF or/and 50 μM dAd in the presence or absence of 3 nM VD3 or 4 nM ATRA for 4 days. Values are the means ± S.D. of three separate experiments.

Statistical analyses; *$P<0.01$, **$P<0.001$, ***$P<0.0001$ compared with the data of untreated cultures. Bla+Pro; blasts and promyelocytes, Mye; myelocytes, Met+Gr; metamyelocytes and mature granulocytes, Mono; monocytes.

TABLE 3

Expression of CD11b on NB4 Cells Treated with dCF+dAd and ATRA

Regimen	Days 1–2	Days 3–7	CD11b expression (%)
1-1	None	None	1.9 ± 0.4
1-2		ATRA	4.9 ± 0.9
1-3		dCF+dAd	8.4 ± 1.4
1-4		ATRA+dCF+dAd	52.8 ± 3.8
2-1	dCF+dAd	None	7.3 ± 1.2
2-2		ATRA	50.6 ± 4.1
3-1	ATRA	None	3.8 ± 1.1
3-2		dCF+dAd	71.9 ± 4.4

NB4 cells were cultured for 2 days in the presence or absence of 10 μM dCF plus 40μM dAd or 40 nM ATRA. On day 2, the cultures were washed with fresh medium, and the cultures reincubated in the presence or absence of the same concentration of dCF plus dAd or ATRA. Means ± SD of three determination.

ment with dCF plus dAd. Similar results were observed when examined the other differentiation-associated phenotypes, such as expression of CD11b (Table 3) and morphologic changes (data not shown). NB4 cells were incubated with ATRA various times and then the priming effect was examined in the dCF-induced NBT reduction. The enhancing effect of ATRA was significantly obtained after 1 day of incubation, and reached a maximum after 2 days (data not shown).

Exposure of NB4 cells to dCF plus dAd induced G_1 accumula tion: 32.4 ± 3.2 % of untreated cells and 52.1 ± 4.4 % of the treated cells in G_1 phase, when cultured with 10 μM dCF plus 40 μM dAd for 2 days. ATRA significantly enhanced the G_1 accumulation of the dCF+dAd-treated cells, although ATRA at 40 nM alone did not affect the pattern of cell cycle distribution (data not shown).

Continuous treatment with dCF and dAd caused significant growth inhibition for 4 days, but thereafter the growth-inhibitory effect was minimum. ATRA at 40 nM hardly affected the proliferation of NB4 cells, but combined treatment with 40 nM ATRA and 10 μM dCF plus 40 μM dAd induced complete growth arrest of the cells: the cell number increased until day 4 but did not change thereafter (Fig. 3). The treated cells were mainly metamyelocytes.

To understand a relationship between the complete growth arrest induced by combination of dCF+dAd with ATRA and expression of apoptosis-associated genes. NB4 cells express significant amounts of c-myc, bcl-2, bcl-XL, and bax mRNA when determined by reverse transcription-PCR (Fig. 4). ATRA or dCF plus dAd significantly reduced the expression of c-myc and bcl-2 mRNA, while the bcl-XL expression was inhibited

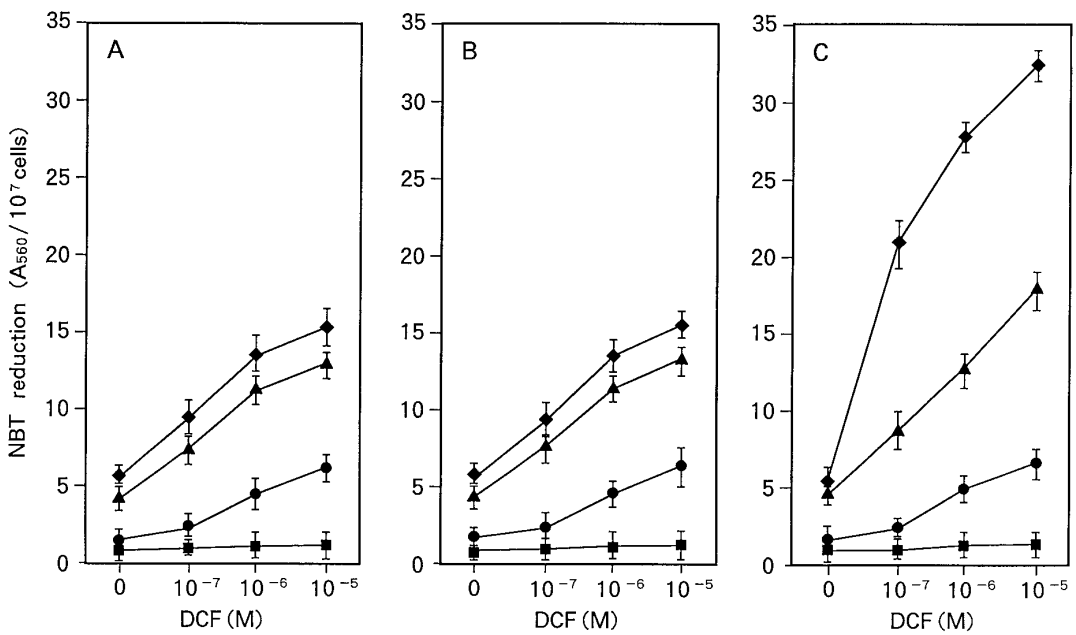

FIG. 2. Enhancement by dCF plus dAd of ATRA-induced NBT reduction of NB4 cells. A; Cells were incubated with various concentrations of dCF in the absence (■) or presence of 40 μM dAd (●), 40 μM dAd plus 4 nM ATRA (▲), or 40 μM dAd plus 40 nM ATRA (◆) for 5 days. B; Cells were incubated with various concentrations of dCF plus 40 μM dAd for 2 days. The cells were washed with fresh medium and reincubated without (■) or with 40 μM dAd (●), 40 μM dAd plus 4 nM ATRA (▲), or 40 μM dAd plus 40 nM ATRA (◆) for 5 days. C; Cells were cultured without (■) or with 40 μM dAd (●), 40 μM dAd plus 4 nM ATRA (▲), or 40 μM dAd plus 40 nM ATRA (◆) for 2 days, and then reincubated with various concentrations of dCF plus 40 μM dAd for 5 days. Means ± SD of three determinations.

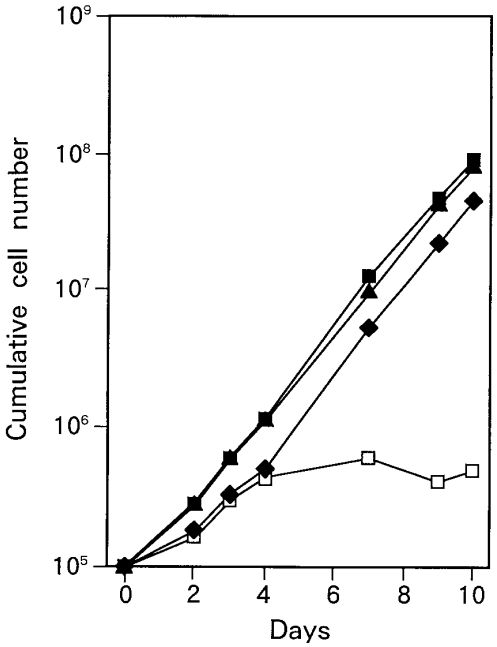

FIG. 3. Proliferation of NB4 cells in culture with ATRA and dCF plus dAd. Cells were cultured without (■) or with 40 μM dAd plus 1 μM dCF (▲), 40 nM ATRA (◆), or 40 nM ATRA and 40 μM dAd plus 1 μM dCF (□). The culture medium was replaced on days 4 and 7. The cumulative cell number was calculated from the counts and the dilution used when feeding the culture.

by combined treatment with ATRA and dCF+dAd, but not with ATRA or dCF+dAd. The decreased expression of these mRNAs was prominent 24 hrs after the treatment (data not shown). On the other hand, expression of bax mRNA was hardly affected by the treatments.

DISCUSSION

dCF, a potent inhibitor for adenosine deaminase, has found clinical applications for the treatment of several types of lymphocytic leukemia, such as hairy cell leukemia (21-23). On the other hand, the drug has been reported to be less effective in clinical trials against myelogenous malignancies. By preventing the catabolism of dAd to deoxyinosine, adenosine deaminase inhibitors result in a high level of intracellular dATP, which in turn inhibit ribonucleotide reductase (24). This may lead to inhibition of DNA synthesis and cell proliferation of the malignant cells. High levels of adenosine deaminase were found in T-lymphoblast cells (25), and the selective action of dCF in this cell type has been related to their peculiar purine enzyme pattern which, by virtue of high deoxynucleoside-kinase and reduced cytosolic 5′-deoxynucleotidase activity, seems to preserve the dATP pool (26).

There are some reports concerning effect of dCF on differentiation of leukemia cells. Two cases of leukemia treated with dCF experienced conversion from lymphoid to myeloid morphology (27,28). Exposure *in vitro* of lymphoblastic cells to dCF induced expression of myeloid surface antigens (29). Triozzi and Vicstein had examined the effect of dCF on differentiation of HL-60 cells (30). Their results indicate that dCF alone does not induce differentiation of HL-60 cells, nor does it interact with ATRA, VD3 or interferon-γ in inducing differentiation of the cells. Our present results also show that dCF alone does not affect the differentiation of HL-60 cells, but we now demonstrate that the drug in combination with dAd can effectively induce morphologic and functional differentiation of the cells. The mechanism of action of dCF plus dAd in inducing differentiation of leukemia cells is unclear, but may be related to the accumula tion of dATP following adenosine deaminase inhibition. Early studies demonstrated that dCF in combination with dAd greatly increased intracellular dATP levels (24-26). Starvation or addition for a natural or synthetic nucleoside leads to intracellular nucleotide pool imbalance, and induces differentiation of some myeloid leukemia cells. An extremely high concentration of dAd alone did slightly induce differentiation of K562 and HL-60 cells. By preventing catabolism of dAd, dCF greatly enhanced the differentiation induced by dAd. However, it is not clear by what mechanism(s) the changes

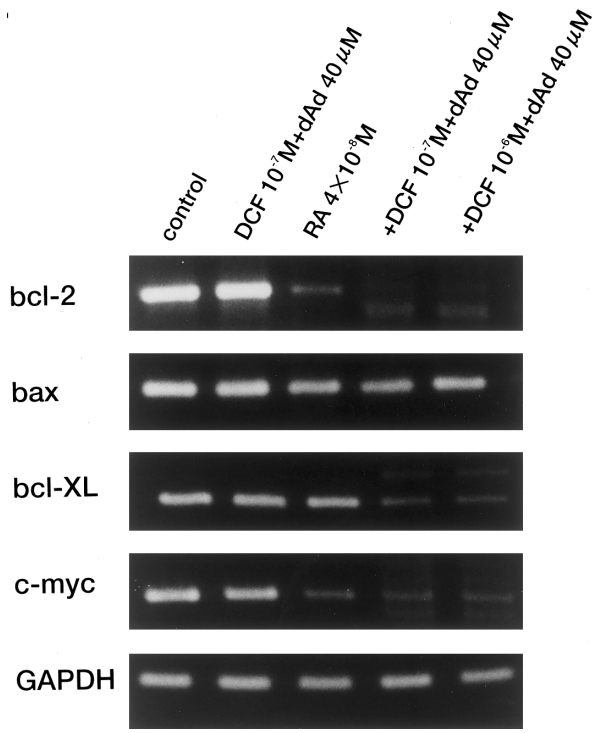

FIG. 4. Down-regulation of bcl-2, bcl-XL and c-myc RNA in NB4 cells treated with ATRA and dCF plus dAd. Cells were treated with the indicated compounds for 48 hrs.

in nucleotide pools influence differentiation of leukemia cells (31).

ATRA at 40 nM alone did not induce complete growth arrest, whereas the combined treatment with dCF+dAd induced NB4 cells to terminal differentiation, and then to apoptosis. Exposure of NB4 cells to ATRA down-regulated expression of bcl-2 and c-myc mRNA, but not of bcl-XL mRNA. However, the combined treatment with ATRA and dCF+dAd significantly down-regulated expression of bcl-XL mRNA. Down-regulation of both bcl-2 and bcl-XL might be required to the complete growth arrest. The present finding is consistent with the recent report that expression of blc-XL is down-regulated during differentiation of hematopoietic progenitor cells along granulocytic lineage (32).

Among antimetabolic anticancer agents, AraC is a potent inducer of differentiation of myeloid leukemia HL-60 and K562 cells, but not effective in inducing differentiation of NB4 cells (33). However, dCF in combination with dAd significantly induces differentiation of the APL cell line, suggesting that dCF is more suitable than AraC in differentiation therapy of APL. Since the APL patients treated with ATRA alone frequently relapse, ATRA should be used with anticancer agents or other differentiation inducers which followed by anticancer chemothera py in order to prevent the recurrence. dCF in combination with dAd can synergistically induce differentiation of NB4 cells with ATRA, especially in the case of ATRA priming. The present results are consistent to the previous findings that pre-exposure of NB4 cells to ATRA abolishes resistance to non-retinoid inducers such as butyrate or hexamethylene bisacetamide (HMBA), and potentiates differentiation (33). However, pretreatment with ATRA before exposure to AraC failed to stimulate differenti ation (33). Neither hexamethylene bisacetamide nor butyrate can be used clinically because of the impertinent duration of action and adverse effects, whereas dCF has been used clinically on therapy of lymphoid malignancies. Moreover, clinically applicable concentrations of dCF (less than 5 μM) effectively induced differentiation of human myeloid leukemia cells in combination with dAd, and synergized with ATRA. Therefore, the combination of dCF with ATRA might reduce the dose of ATRA in treatment of APL patients. The cooperation of dCF and ATRA or VD3 was also observed in inducing differentiation and growth inhibition of other myelomonocytic leukemia cells. The combined treatment might be useful for the other myeloid malignancies. Further studies are required to explore the possibilities.

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