RARα is a regulatory factor for Am-80-induced cell growth inhibition of hematologic malignant cells

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Abstract. Retinoids are used for treatment of acute promyelocytic leukemia (APL). Am-80, Tamibarotene, binds to retinoic acid receptor alpha (RARα) more specifically than all-*trans* retinoic acid. We studied the tumor cell suppressive effects of Am-80, with respect to cytotoxicity and growth inhibition using eight myeloid and lymphoid malignant cells in culture (HL-60, HL-60R, K-562, Kasumi-1, MEG01, Raji, U266B1, and U937). The effects of Am-80 were examined during 9 days of incubation with 10^{-7}-10^{-5} M of Am-80 in culture medium, which was changed every 3 days. HL-60 were the only cells sensitive to Am-80-induced cytotoxicity; the latter reached more than 95% after 9 days of incubation, and death was primarily through apoptosis. The total mass of RARα in HL-60 was significantly greater ($p<0.006$) than in ATRA-resistant HL-60 (HL-60R) as well as all of other cells tested. However, in all cells excluding HL-60, Am-80 induced time- and dose-dependent cell growth inhibition without noticeable cytotoxicity. TGF-ß2 was released into the media containing cells incubated with Am-80 for 3 days. A dose-dependent increment of phosphorylation of Smad-2 was also detected. The relative amount of secreted TGF-ß2 correlated with the growth inhibition rates in all cells tested excluding HL-60, and with the total mass of RARα in the cells ($p=0.0137$). Our results indicate that Am-80-induced cell-type non-specific growth inhibition is mediated by TGF-ß2, where the total mass of RARα could be an important regulatory factor in hematologic malignant cells.

Introduction

Vitamin A acts like a hormone, and is involved in ontogeny, morphogenesis and cell differentiation (1). The growth and differentiation of various tissues are regulated by vitamin A metabolite retinoic acid (RA) (2). Three types of RA receptors (RARs) and retinoid X receptors (RXRs) localized in the nuclei have been described so far; α, ß, and γ, and RAR and RXR form a dimeric construction (3). RA promotes differentiation of myeloid precursor cells in the hematopoietic system (4), and all-*trans* retinoic acid (ATRA) has been used for treatment of acute promyelocytic leukemia (APL) with chromosomal translocation t(15;17) (5-7) or t(11;17) (8). Such translocation induces the formation of chimeric genes of RARα and promyelocytic leukemia (PML) or promyelocytic leukemia zinc finger (PLZF), by which transcription is constitutively prohibited, results in cessation of post-promyelocytic differentiation and uncontrolled growth. Treatment with retinoids (also known as differentiation-inducing therapy) leads to apoptosis of APL cells through differentiation. However, in the past, HL-60 were used as APL cells, but these cells neither possess chromosomal translocation (9) nor PML/RARα (10). However, it is also well known that ATRA induces differentiation and apoptosis of the cells.

It has been reported that treatment with ATRA results in a high complete remission rate of relapsed or unresponsive APL patients after chemotherapy (11-13). However, one problem arising from ATRA treatment is the appearance of tolerance to the drug and relapse of the disease (14). One reason for the development of tolerance is thought to be the formation of cellular retinoic acid-binding protein (CRABP) (15,16), which eventually reduces the effect of ATRA.

Kagechika *et al* (17) developed a new synthetic retinoid, Am-80, which was subsequently approved as a drug for relapsing or refractory APL in 2005 in Japan. The specific binding activity of Am-80 to RARα is greater than ATRA (17,18), and results in 10-fold increase in tumor cell differentiation. Moreover, Am-80 has a low affinity to CRABP (19). On the other hand, several recent studies indicated that retinoids can be used also against malignant tumors other than APL (20). However, the mechanisms of the selective action of Am-80 on tumor cells remain obscure. In the present study, we investigated the effect of Am-80 on cell growth using eight types of cultured myeloid and lymphoid malignant cells, and determined the growth suppression mechanisms of Am-80.

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Materials and methods

Cells and reagents. HL-60 and HL-60R were obtained from RIKEN Inc. (Tokyo, Japan), and Kasumi-1, K562, Meg01, MOLT3, Raji, U266B1, and U937 were from American Type Culture Collection (VA, USA). Am-80 was kindly provided by TMRC Inc. (Tokyo). RPMI medium 1640, Iscove's-modified Dulbecco's medium (IMDM), and PSN antibiotic mixture were from Invitrogen Japan Inc. (Tokyo). We also purchased the following items from the indicated suppliers. Fetal bovine serum (JRH Biosciences, Lenexa, KS), culture flasks (BD Bioscience Inc., Tokyo), FACS Flow™ (BD Bioscience Co., Tokyo), rabbit anti-human RARα and ß antibodies and mouse anti-human RARγ antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human Smad2 antibody (Zymed Laboratories Inc., San Francisco, CA), anti-Smad2 [pT8] phosphospecific antibody (BioSource, Camarillo, CA), mouse anti-human p53 antibody (Santa Cruz Inc.), HRP-labeled donkey anti-rabbit IgG antibody (Abcam plc, Cambridge, UK), PE-labeled anti-mouse IgG antibody (Rockland, PA), PE-labeled anti-rabbit IgG antibody (Dako Japan, Kyoto, Japan), ECL Advance Western Blot Detection Kit (GE Healthcare Biosciences Japan, Tokyo), DAPI: 4',6-Diamidino-2-phenylindole (Sigma-Aldrich, Tokyo), 7-aminoactinomycin D (7-AAD, Invitrogen Japan), Guava ViaCount™ , Guava Nexin™ , Guava MutliCaspase™ , and Guava TUNEL™ (GE Healthcare Biosciences Japan), human TGF-ß2 Immunoassay (Quantikine, R&D Systems, Minneapolis, MN), polyvinylidene fluoride (PVDF) (Bio-Rad, Hercules, CA), and chemiluminescent reagent (ECL Advance, GE Healthcare Biosciences Japan).

Cell culture. Cells were cultured in T-75 culture flasks with 10 ml of media, i.e., RPMI-1640 or IMDM, containing 10% FBS under an atmosphere of 5% CO_2 95% air in a humid atmosphere. Cells were subcultured every 3 days to prevent them reaching a confluence of cell density more than $1x10^7$ cells/flask. Cells were constantly maintained in the best condition, and cell viability was monitored by ViaCount reagent at each subculture. If cell viability decreased 15% from the common condition, cells were discarded, and culture was restarted. For analysis of cell growth, cells were initially cultured at $5x10^5$ viable cells/10 ml in each flask containing 50 μl of 200 time concentrated Am-80 in ethanol, $2x10^{-5}$ M for 10^{-7} M, $2x10^{-4}$ M for 10^{-6} M, $2x10^{-3}$ M for 10^{-5} M, and $8x10^{-3}$ M for $4x10^{-4}$ M, or ethanol vehicle alone. After incubation for 3 or 6 days, the cell number was counted and the viable cell density determined using ViaCount reagent. The total cell number was adjusted to $5x10^5$ cells/10 ml/flask. The cells were again treated with the same concentration of Am-80 and cultured for a total of 9 days.

Flow cytometric analysis of RAR expression. After incubation in the medium, cells were harvested and centrifuged at 1300 rpm for 5 min. They were then fixed with 1% paraformaldehyde/0.01 M PBS (pH 7.3) for 1 h in ice. After fixing, cells were washed twice with 0.01 M PBS (pH 7.4), and treated with -20˚C cooled 70% ethanol for membrane permeabilization, and thereafter stored under -20˚C at a density of $3x10^6$ cells/ml. Cells in ethanol at a density of $3x10^5$ cells/100 μl were transferred into three polystyrene tubes as a set of measurement for 1st and 2nd antibody combination of -/-, -/+ and +/+, and 1 ml of PBS was added to each tube. After centrifugation, cells were washed three times with 0.5% FBS containing FACS Flow. Cells were incubated with 10-times diluted rabbit antibody against human RARs α, ß or γ for 1 h in ice. After washing three times with 0.5% FBS containing FACS Flow, cells were then incubated with 200-times diluted PE-conjugated anti-rabbit or 165-times diluted PE-conjugated anti-mouse antibody for 15 min in ice. After washing three times with 0.5% FBS containing FACS Flow, total sample volume was adjusted to 500 μl. Next, 5 μl of 200 μg/ml 7-aminoactinomycin D (7-AAD) (Molecular Probes, Eugene, OR) was added, and the cells were incubated for 10 min at room temperature. After centrifugation, 500 μl of FACS Flow was added to the samples, and intracellular expression of retinoid receptors was measured by Guava PCA™ (GE Healthcare Biosciences Japan) based on a flow cytometry technique. The median value of fluorescence intensity was used, and relative expression value was calculated using following formula: relative expression value = {[(+/+) - (-/+)] / (-/-)}.

Analysis of apoptosis. Apoptotic cells were analyzed by three different assays, and analytical methods were followed as described by the manufacturers with minor modifications. Nexin assay is based on the detection of phosphatidylserine (PS) on the outer surface of the plasma membrane. Early in the apoptotic pathway, PS is translocated from the inner to the outer surface of the cell membrane, to which Annexin V can readily bind. MultiCaspase assay is based on the detection of activated caspase enzymes (caspase-2, -8, -9 and -10), which initiate the apoptotic cascade. The assay uses a fluorochrome-conjugated inhibitor of caspases (SR-VAD-FMK), which binds covalently to multiple caspases activated during the process of apoptosis. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) assay is based on the detection of degradation products of chromatin structures and nuclear DNAs by activated nucleases. TdT catalyzes the incorporation of Br-DU residues into the fragmented DNA at 3'-hydroxy ends by nicked end-labeling. Apoptotic cells were analyzed by computer-assisted specific software connected to the flow cytometer Guava PCA.

Quantification of TGF-ß2 secreted in media. A serum-free growth medium (AIM V medium, Invitogen Japan) was initially used, however, cell viability could not be maintained in this medium during 3-day incubation period, which resulted in low cell viability. Therefore, a minimum level of FBS (1%) was added to each medium, and the medium alone without cells was used as a background level of TGF-ß2 in FBS. Cells at $1x10^6$/5 ml medium were incubated with $1x10^{-5}$ M, $2x10^{-5}$ M or $4x10^{-5}$ M concentration of Am-80 or vehicle alone for 3 days. After incubation, the medium was collected and acidified with 1 N HCl to activate latent formed TGF-ß2. The concentration of TGF-ß2 in the medium was measured by an enzyme-linked immunosorbent assay (ELISA) kit. Secreted TGF-ß2 in the medium was expressed as ng/10^6 viable cells.

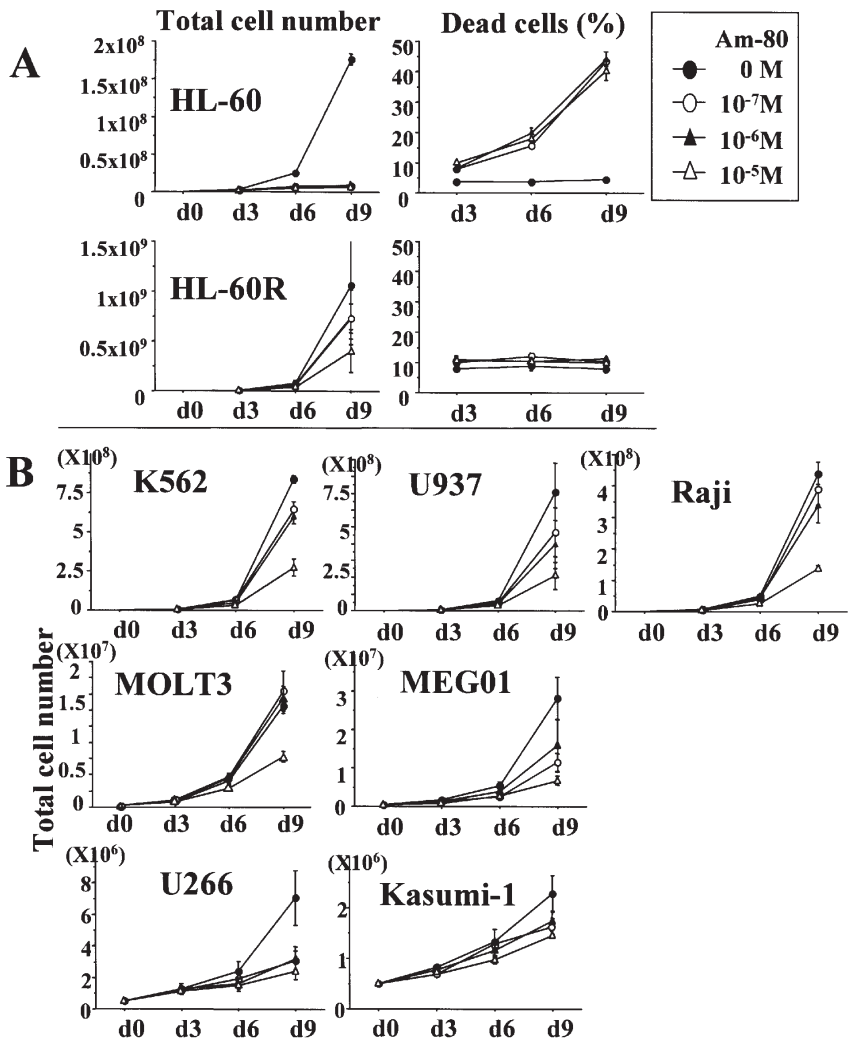

Figure 1. Effects of Am-80 on cell growth and cytotoxicity in different cells. Eight different cells derived from myeloid and lymphoid malignant cells were cultured for 9 days in growth media containing 0, 10^{-7}, 10^{-6} and 10^{-5} M of Am-80. Cell number was always adjusted to $5x10^5$ cells/10 ml/flask every 3 days by changing the culture medium. A, Total cell number (left) and dead cell number (right) of HL-60 and HL-60R during 9 days of incubation with Am-80. B, Total cell number of Raji, K-562, MEG01, U937, U266, and Kasumi-1 during 9 days of incubation with Am-80. Data are mean ± SEM of three experiments.

Western blotting. After incubation with/without Am-80 for 3 days, cells were washed three times with PBS, and viable cells at density of $1x10^6$ were dissolved in 50-μl sample buffer and sonicated. ß-mercaptoethanol (2 μl) added to 20 μl of cell lysate ($2x10^4$ cells), was incubated for 10 min at 90˚C. The cell lysate was applied to a lane of 8.2% polyacrylamide gel, and electrophoresed at 200 V/15 mA for 1 h. The gel was attached to PVDF and blotted at 100 V/350 mA for 1 h. After blotting, PVDF was soaked in 2% blocking reagent containing PBS with Tween-20 for 1 h, then incubated with rabbit anti-human Smad 2 antibody (x333 dilution) or rabbit anti-human pSmad 2 (phospho T8) antibody (x1000 dilution) at 20˚C for 1 h. After extensive washing with PBST, PVDF was incubated with HRP-labeled anti-rabbit antibody (x7500 dilution) at room temperature for 1 h. After washing, chemiluminescent reagent was spread on the PVDF. PVDF was exposed to X-ray film and developed.

Statistical analysis. All data were expressed as mean ± SEM unless otherwise indicated. Identical experiments were performed at least twice, and similar results were obtained. Differences between groups were examined for statistical significance using the Student's t-test and one-way analysis of variance (ANOVA). A $p<0.05$ denoted the presence of a statistically significant difference.

Results

Effects of Am-80 on cell death and growth. Am-80 at 10^{-7}-10^{-5} M significantly reduced the total cell number of HL-60 incubated for 9 days (Fig. 1A), and increased the number of dead cells in a time-dependent manner (Fig. 1A). In contrast, Am-80 did not increase the number of dead cells when incubated with HL-60R (Fig. 1A), although it significantly reduced the total cell number (Fig. 1A). Am-80 also inhibited the cell growth of other cells (Raji, U937, K562, U266, MEG01, and Kasumi-1) but had no cytotoxic effect on these cells (Fig. 1B).

With regard to the effect of Am-80 on apoptosis, it induced apoptosis of HL-60 cells as evident by condensation of nuclear

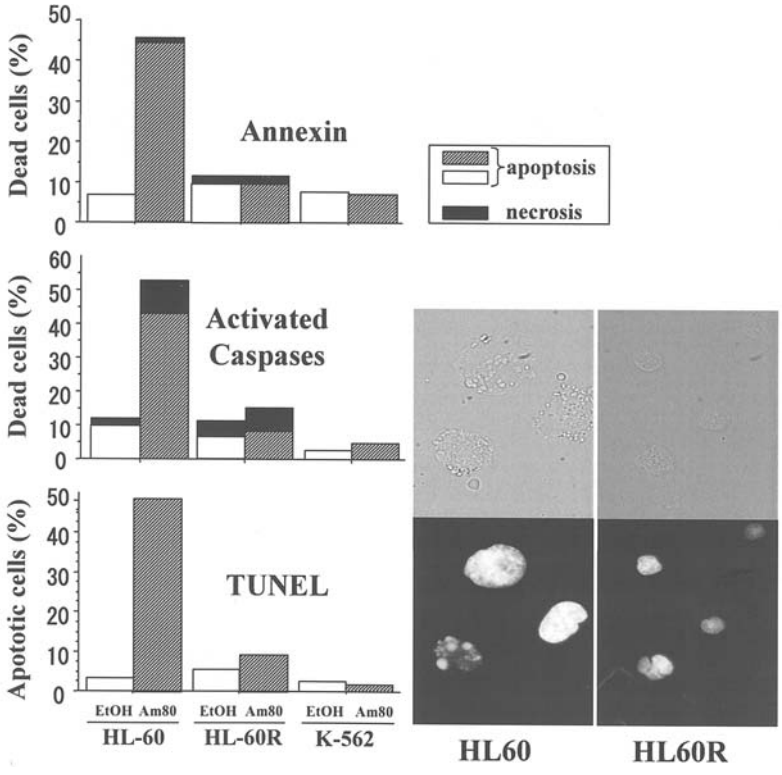

Figure 2. Cell death induced by Am-80. HL-60 incubated with 10^{-5} M of Am-80 for 9 days showed condensation of nuclear chromatin and small nuclear fragments stained by DAPI, i.e., apoptotic bodies (top, upper: phase-contrast microscopy). However, no such changes were found in HL-60R. HL-60, HL-60R and K562 incubated with Am-80 were assessed for appearance of cells positive for annexin V, activated caspases and TUNEL. Am-80 induced apoptosis of HL-60, and the extent of apoptosis was about 5 times that of other cells. In comparison, Am-80 only had a weak apoptotic activity (<10%) against HL-60R and K562 cells.

chromatin stained by DAPI, and formation of small nuclear fragments, i.e., apoptotic bodies (Fig. 2). However, no such changes were noted when Am-80 was added to cultures of HL-60R (Fig. 2). Incubation of HL-60 with 10^{-5} M for 9 days resulted in the appearance of cells positive for annexin V, activated caspases and TUNEL. These results indicated that HL-60, but not other tested cells (HL-60R and K562), undergo apoptosis when treated with Am-80 (Fig. 2).

RAR protein expression. Next, we used immunohistochemistry-based flow cytometry to examine protein expression of the total mass of RARs α, ß and γ in cells incubated under normal growth conditions. The total mass of RARs α and ß was greater (40-60 times) than that of RARγ (Fig. 3). The expression levels of RARs α and ß were similar in various cells. However, the expression levels of RARα ($p<0.05$) and RARγ ($p<0.05$) were significant higher in HL-60 possessed than in HL-60R and other cells depicted in Fig. 3.

Growth inhibition by Am-80. Growth inhibition of cells incubated with different concentrations of Am-80 was calculated: total cell density in cells incubated with Am-80 relative to that of cells incubated with ethanol vehicle alone. With the exception of HL-60, Am-80 inhibited the growth of all cells in a time-dependent manner (Fig. 1). Table I shows the percentage of growth inhibition induced by Am-80 after 9 days of incubation. Table I shows that Am-80 induced a dose-dependent increase of growth inhibition in all tested cells, with a mean value at 10^{-7}, 10^{-6} and 10^{-5} M Am-80 of about 35, 40 and 60%, respectively. There was no significant relationship between RARα mass and growth inhibition or dead cell number in the cells incubated with Am-80 for 9 days (data not shown).

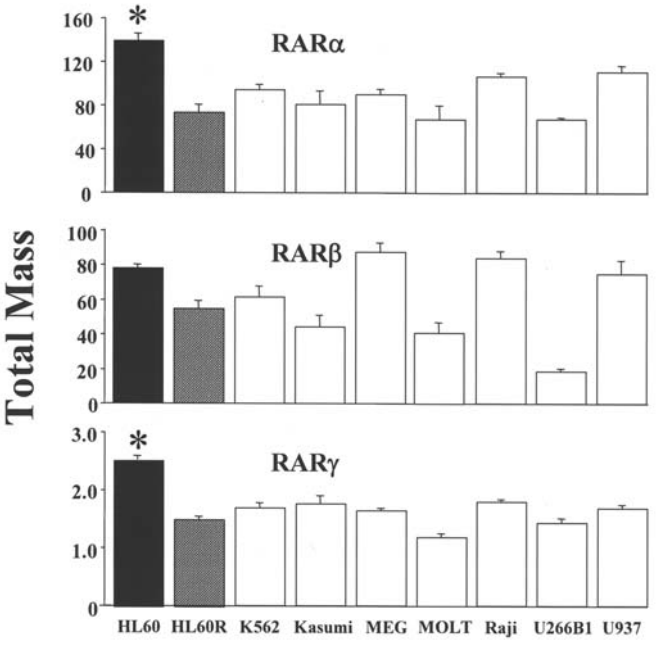

Figure 3. Protein expression of RARs α, ß and γ. Cellular protein expression of total mass of RARs was examined under normal growth conditions by flow cytometry. HL-60 possessed significantly greater amounts of RARs α and γ ($p<0.05$) than HL-60R and other cells. Data are mean ± SEM of three experiments. *$p<0.05$.

Table I. Growth inhibition by Am-80.

Day-9	Am-80 (M) 10^{-7} (%)	10^{-6} (%)	10^{-5} (%)	RARα expression
HL60R	32.0±19.8	33.3±6.2	62.8±4.9	72.3±7.3
K562	23.7±5.3	28.8±4.2	67.5±5.4	93.0±5.0
Kasumi-1	27.9±5.1	23.2±3.2	33.4±10.3	80.0±12.3
Meg01	59.2±3.5	47.8±12.0	75.7±1.1	89.0±5.2
MOLT3	3.7±11.7	2.7±0.43	50.0±5.4	82.3±11.9
Raji	20.6±7.4	37.2±8.1	67.9±3.9	99.0±3.5
U266	54.5±4.3	54.7±2.2	65.4±1.6	66.3±2.2
U937	36.7±7.8	46.9±10.6	72.8±7.0	110.3±5.9
				mean ± SE (n=3)

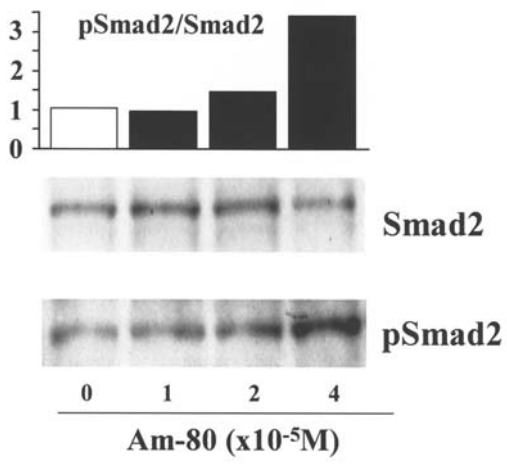

Figure 5. Intracellular signal transduction of TGF-ß2. Since Raji cells are sensitive to growth inhibition when incubated with Am-80, they were used in this experiment. Raji cells incubated with different concentrations of Am-80 for 3 days were subjected to Western blot analysis for Smad-2 and phosphorylated-Smad-2 (pSmad2). The ratio of pSmad relative to Smad-2 increased dose-dependently.

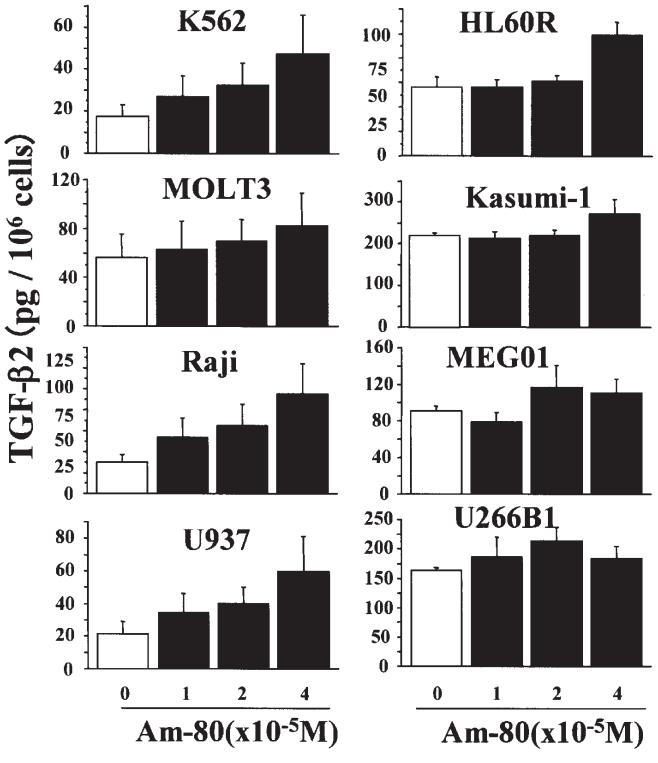

Figure 4. Am-80 stimulates TGF-ß2 secretion. To quantify TGF-ß2 secreted by cells treated with Am-80, we modified the experimental protocol by limiting the incubation period to 3 days and the concentration of Am-80 used to 1, 2, and $4x10^{-5}$ M. TGF-ß2 concentration in the medium (pg/10^6 cells) increased in all cells, and an apparent dose-dependent increment was found in K562, MOLT3, Raji, and U937. Data are mean ± SEM of three experiments.

Am-80-induced TGF-ß2 secretion. To clarify the role of TGF-ß2 in the inhibitory effects of Am-80, we used cells incubated in serum-free media. However, such cells could not be maintained. Therefore, we changed the experimental protocol to shorten the incubation period to 3 days and increased the concentration of added Am-80 to 1, 2, and $4x10^{-5}$ M. In all cells exposed to Am-80, TGF-ß2, including both active and latent forms, was secreted by the cells, and such secretion was AM-80-dose-dependent in K562, MOLT3, Raji, and U937, and weakly in other cell types (Fig. 4).

Intracellular signal transduction of TGF-ß2. Among the cells sensitive to Am-80 with regard to growth inhibition and TGF-ß2 secretion, we selected Raji cells to examine the signal transduction for TGF-ß2. Raji cells were incubated with 1, 2, or $4x10^{-5}$ M concentrations of Am-80 for 3 days and then subjected to Western blotting to analyze Smad-2 and phosphorylated Smad-2. Fig. 5 shows the bands of Smad-2 and phosphorylated Smad-2 (pSmad-2). The density of pSmad-2 relative to Smad-2 increased in Am-80 dose-dependent manner, and was 3-times greater than the control culture (no Am-80). These results indicate that phosphorylation of Smad activated the intracellular signal of TGF-ß2.

Relationship between increased TGF-ß2 secretion and cell growth inhibition. To correlate the increased TGF-ß2 secretion with Am-80-induced growth inhibition, we expressed cell growth inhibition in the presence of different concentrations of Am-80 as percentage value relative to the vehicle alone (Table II). With the exception of U266 and TGF-ß2, Am-80 induced a dose-dependent inhibition of cell growth, with Raji, U937, and K562 being the most sensitive cells (Table II). Regression analysis showed that the amount of secreted TGF-ß2 correlated significantly with the Am-80-induced cell growth inhibition (p=0.0272) (Fig. 6, bottom panel).

Relationship between TGF-ß2 secretion and total mass of RARα. The amount of secreted TGF-ß2 by all cells except HL-60, correlated significantly with the total mass of RARα (p=0.0137) (Fig. 6, top panel).

Table II. Secretion of TGF-ß2 and growth inhibition by Am-80.

Day-3	vs. vehicle alone (%)	Am-80 (x10^{-5} M) 1	2	4
HL60R	TGF-ß2	100.9	109.1	177.5
	Growth inhibition	19.4	14.2	62.0
K562	TGF-ß2	156.7	188.9	275.8
	Growth inhibition	6.2	4.5	36.0
Kasumi-1	TGF-ß2	96.8	100.1	123.3
	Growth inhibition	1.3	3.4	20.5
Meg01	TGF-ß2	85.8	128.5	121.5
	Growth inhibition	9.8	33.3	42.3
MOLT	TGF-ß2	112.8	124.5	147.0
	Growth inhibition	2.5	2.7	29.9
Raji	TGF-ß2	180.9	218.4	321.4
	Growth inhibition	44.4	47.2	69.4
U266	TGF-ß2	113.5	130.5	112.0
	Growth inhibition	22.3	25.0	18.8
U937	TGF-ß2	160.3	187.4	279.3
	Growth inhibition	24.5	21.0	52.4

mean of triplicated data

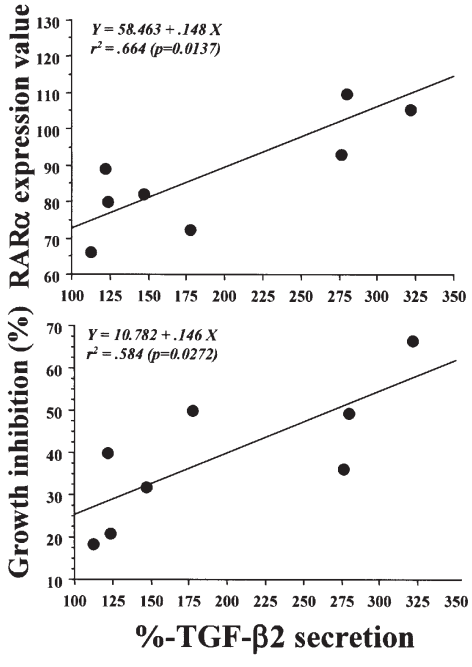

Figure 6. Relationship between TGF-ß2 secretion and total mass of RARα and growth inhibition. Top: the total mass of RARα correlated significantly (p=0.0137) with the relative amount of TGF-ß2 in the media. Bottom: the relative amount of TGF-ß2 secreted (in reference to that by the vehicle control) correlated significantly with Am-80-induced growth inhibition (p=0.0272).

Discussion

The present study used eight different hemotologic malignant cultured cells, which originated from myeloid and lymphoid tissues, and showed that Am-80 is cytotoxic to human leukemia HL-60 cells. However, no such effect was noted in cells resistant to ATRA, namely HL-60R, which harbor a mutated RARα gene (21), suggesting that Am-80 and ATRA act through a similar cytotoxic mechanism. HL-60 is widely used as an APL cell in many studies. However, recent criteria for APL is restricted to cells that possess chromosomal translocations, especially t(15:17) (9), which forms PML/RARα (10). In cells harboring t(15;17), it is shown that ATRA restores PU.1 expression, a transcription factor for normal hematopoiesis (22). However, no such translocation has been found in HL-60. Alternatively, Altucci *et al* (23) indicated that the mechanism of ATRA-induced cell death in APL is related to a paracrine production of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). However, the precise mechanism of retinoid-induced cytotoxicity in HL-60 is still uncertain.

It is well known that HL-60 have similar features to APL cells, especially in terms of differentiation induced by ATRA (24). The present study showed that HL-60 contained the largest protein mass of RARα among eight different types of cells, which may explain the selective induction of cytotoxicity and differentiation in HL-60 by Am-80 and ATRA (25).

Growth inhibition is a well-known effect of retinoids in cultured cells, and previous studies suggested the involvement of intranuclear retinoid receptor in this effect. Am-80 suppressed the cell growth of all cells used in our study, although the extent of growth inhibition varied among the cells. Am-80 was developed as a RARs α- and ß-specific synthetic retinoid (17,18), and thus its binding activity to other retinoid receptors might be negligible. Interestingly, the growth inhibition rate induced by 10^{-5} M Am-80 after a 3-day incubation correlated with RARα expression rate, but no such relation was found with RARs ß and γ. A previous gene transfer study (21) to convey normal RARα gene to HL-60R indicated that ATRA can induce differentiation of HL-60R with normal RARα, thus suggesting that ligand-induced activation of RARα is sufficient to induce differentiation in HL-60. Moreover, it has been shown that inhibition of anchorage-dependent growth in an estrogen-receptor positive carcinoma cell line treated with retinoid correlated with binding to RARα, but not to its antagonist (26). RARα may therefore act as a cell-growth regulating transcription factor through differentiation in tumor cells.

TGF-ß is one of the most important cytokines involved in cell growth regulation, and therefore controls biological homeostasis in different tissues. In oncogenesis, failure of response to TGF-ß due to abnormality of the receptor or signal transduction is thought to be the underlying cause of abnormal proliferation acquired by malignant cells (27). Previous studies reported that retinoids stimulate the production of TGF-ß in variety of cells including HL-60 (28) and U937 (29). As expected, production of TGF-ß2 increased in the present study in all cells incubated with the RARα-specific retinoid Am-80.

Han and coworkers indicated previously that changes induced in RARα by ATRA in human bronchial epithelial cells correlate directly with TGF-ß2 (30). In the present study, we measured the amount of TGF-ß2 secreted in the media, which included both active and latent forms. The amount correlated significantly with the extent of Am-80-induced growth inhibition among the cells. On the other hand, the amount of TGF-ß2 secreted varied widely among cells. Therefore, we normalized the amount of secreted TGF-ß2 relative to that induced by ethanol vehicle alone. The results showed that Am-80-induced growth inhibition correlated with TGF-ß2 (Fig. 6), suggesting that Am-80-induced growth inhibition is mediated through secreted TGF-ß2. Furthermore, we also demonstrated a direct correlation between RARα expression level and TGF-ß2 secreted by the cells.

Activation of latent-form TGF-ß, latency-associated peptide (LAP) and latent-TGF-ß binding protein (LTBP) requires various catabolic enzymes including plasmin (31) and thrombospondin-1 (32). In the present study, we could not detect the active form of TGF-ß2, probably due to the presence of serum in the culture media, which was added to maintain cell viability in a prolonged incubation study; serum may hinder the measurement of a trace amount of the active form of TGF-ß2. Nevertheless, it is likely that cells incubated with Am-80 may have a better chance to respond to TGF-ß secreted and activated by them.

After binding of TGF-ß to its receptor, Smad bound to the intracellular domain of receptor protein is phosphorylated and liberated, and then acts as a transcriptional signal onto the target gene in the cell nucleus, which leads to cell differentiation and growth suppression (33). Our results showed increases in phosphorylated Smad in cells incubated with Am-80, indicating that intracellular signals initiated by TGF-ß2 is activated in the cells when treated with Am-80. On the other hand, Cordenonsi *et al* (34) reported that Smad2 incorporates with p53, a tumor suppressive gene, to synergistically activate TGF-ß-induced transcription. However, we could not find any change in p53 in cells treated with Am-80 (data not shown).

In conclusion, we examined the effects of Am-80 using cultures of malignant cells that originated from myeloid and lymphoid tissues. Am-80 exhibited a cytotoxic effect on HL-60 cells but not other cell types. On the other hand, Am-80 inhibited the cells growth of all cells tested, and this effect was mediated through TGF-ß2 secretion in an autocrine/paracrine manner. These results indicate that Am-80 induces growth inhibition in a cell-type non-specific manner, and such effect seems to be mediated through TGF-ß2. Our results suggest that Am-80 could be potentially useful therapeutically to induce cell growth inhibition as an antitumor agent.

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