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Differential modulation of PI3-kinase/Akt pathway during all-*trans* retinoic acid- and Am80-induced HL-60 cell differentiation revealed by DNA microarray analysis

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Abstract

All-*trans* retinoic acid (ATRA) and Am80 are natural and synthetic derivatives of Vitamin A and have been used in the fields of oncology and dermatology for years. Their action was considered to be achieved mainly through binding to nuclear hormone receptors, retinoic acid receptors (RARs), although they have been observed to have different biological effects. For example, the two compounds have similar effects on differentiation but different effects on proliferation in human promyelocytic leukemia cell line HL-60 cells. To elucidate the genes responsible for this and other differences, we attempted for the first time to determine the genes whose expressions were differentially modulated during the time course of HL-60 cell differentiation by ATRA and Am80 treatment up to 72 h utilizing DNA microarray and clustering analyses. As a result, the expressions of 204 genes were found to be modulated differentially by ATRA and Am80. Among them, we focused on two components of the PI3-kinase/Akt signal transduction pathway, *phosphoinositide-3-kinase*, *β-catalytic subunit* and *ribosomal protein S6 kinase polypeptide 1*, which are related to the regulation of cell proliferation and apoptosis. Their expressions were specifically suppressed by ATRA, which coincided with the suppressive effects of ATRA on the HL-60 cell proliferation. Moreover, PI3-kinase inhibitors suppressed the proliferation of Am80-treated cells to the same extent as ATRA did. These results indicated that these gene products play a role in HL-60 cell growth suppression during the late stage of differentiation. The complete data and a list of the genes are available at http://www.nihs.go.jp/mpj/index-e.htm.

Keywords: All-*trans* retinoic acid; Am80; HL-60; Cell proliferation; Global gene expression profiling analysis; PI3-kinase/Akt pathway

1. Introduction

Retinoids are natural or synthetic derivatives of Vitamin A and have potential chemopreventive and therapeutic applications in the fields of oncology and dermatology. One of the successful applications of retinoids is for differentiation therapy in acute promyelocytic leukemia (APL) using all-*trans* retinoic acid (ATRA) (Fig. 1). In most cases, high complete remission rates were achieved in APL with ATRA treatment, a result much better than that provided by conventional chemotherapy [1]. Now, ATRA is the first-choice drug in APL treatment. As the therapeutic applications of retinoids have become wider, a number of synthetic retinoids have been developed. Among them, Am80 (Fig. 1) has been used already in the treatment of APL in a clinical trial and showed better potency [2,3]. Am80 was able to introduce a second complete remission in 58% of the patients who relapsed after the first ATRA treatment and with fewer adverse effects. Am80, as well as many other synthetic retinoids, has been developed by an in vitro differentiation assay

Abbreviations: APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; 9-*cis* RA, 9-*cis* retinoic acid

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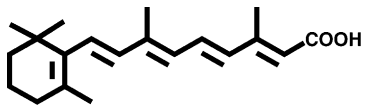
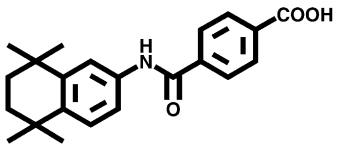


ATRA



Am80

Fig. 1. Structures of retinoids used in this study.

using HL-60 human promyelocytic leukemia cell line [4] and it was approximately 10 times more potent than ATRA in differentiation induction activity. This and other unique features of Am80 were suggested to explain its higher potency in APL treatments [2,3]. In addition to these features of Am80, Am80 showed different effects on HL-60 cell growth during the differentiation assay compared to ATRA. The cells treated with Am80 for 4 days (the normal period of the assay) were growing with the slower growth rate, while ATRA-treated cells almost ceased growing [5][1], indicating that ATRA suppressed the cell growth much more strongly than Am80.

Both ATRA and Am80 are thought to exert their biological effects through binding to retinoic acid receptors (RARs), members of the nuclear steroid hormone receptors. Biochemical analysis of their binding proteins in HL-60 cell extracts clearly showed that the major binding proteins were RARs [6,7]. This suggested that they should have common biological activities. However, ATRA showed binding to several other proteins in the same assay. This implies that ATRA and Am80 should have different biological or clinical activities, such as the differences in the effects of growth suppression observed in HL-60 cell cultures or the side-effects experienced in clinical applications which are usually severer in ATRA treatment. These facts prompted us to clarify the differences and the similarities of ATRA and Am80, because it should provide important information for the development of retinoids with more potency and/or fewer side-effects. For this purpose, we conducted a large-scale analysis of the gene expression using DNA microarray and clustering analysis to elucidate the genes whose expressions were differentially modulated during the time course of HL-60 cell differentiation by ATRA and Am80.

2. Materials and methods

2.1. Chemicals

ATRA and LY294002 were purchased from Sigma Chemical Co. Wortmannin was purchased from Wako Pure Chemicals. Am80 [8] and PA024 [9] were synthesized at The University of Tokyo. All chemicals were dissolved in ethanol.

2.2. Cells, cell culture, and cell treatments

The human promyelocytic leukemia cells, HL-60, were provided by Dr. F. Takaku (Faculty of Medicine, The University of Tokyo). The cells were cultured in suspension in RPMI-1640 (Biomedicals Inc.) supplemented with 5% fetal bovine serum (BioWhittaker or Wako Pure Chemicals) and penicillin–streptomycin (Invitrogen) under a humidified atmosphere of 5% CO_2 at 37 °C. Fetal bovine serum from either provider gave identical results in terms of HL-60 cell differentiation. The following numbers of cells were seeded at the beginning of culture according to the culture period, i.e. 1×10^6 cells/ml for 1 and 9 h culture, 8×10^5 cells/ml for 24 h culture, 3×10^5 cells/ml for 72 h culture, and 1×10^5 cells/ml for 96 h culture. ATRA, Am80, wortmannin, or LY294002 was added to the cells at the indicated time of the culture, and the cells were harvested and processed further at the end of the indicated culture period. The same volume of ethanol was added to the control culture (0.5% (v/v)), which did not affect the HL-60 cell growth and differentiation.

2.3. Total RNA preparation

After washing the cells twice with PBS, total RNA was prepared with RNeasy Mini total RNA Preparation Kit (Qiagen) according to the manufacturer's instructions.

2.4. DNA microarray analysis

Converting total RNA to the targets for Affymetrix GeneChip DNA microarray hybridization was done according to the manufacturer's instructions. The targets were hybridized to a human genome U95A GeneChip DNA microarray (Affymetrix) for 16–24 h at 45 °C. After the hybridization, the DNA microarrays were washed and stained on Fluidics Station (Affymetrix) according to the protocol provided by Affymetrix. Then, the DNA microarrays were scanned, and the images obtained were analyzed by Microarray Suite Expression Analysis Software (version 5.0; Affymetrix). The DNA microarray analysis of each sample was done in duplicate. The results of the DNA microarray analyses are available at our web site, http://www.nihs.go.jp/mpj/index-e.htm.

2.5. Cluster analysis of gene expression patterns induced by ATRA and Am80

The first step was selecting genes whose expressions were changed by either ATRA or Am80 with statistical significance at least at one time point and whose expressions were reproducible through the time course. The data

[1] Ishida et al. unpublished results.

acquired through the absolute analysis by Microarray Suite Expression Analysis Software were imported to the GENFO program [10] due to the limited replicates of the DNA microarray data. GENFO is a suitable program in this case because it selects the genes whose expressions changed by a given treatment with statistical significance based upon the ''a priori S.D.'' ''A priori S.D.'' was obtained from independent experiment beforehand, in which the variation of given signal intensity was determined from six replicate measurements of human genome U95A DNA microarrays [10]. Using this ''a priori S.D.'' avoids the need for many replicates in the actual experiment, which are usually required for conducting *t*-test etc. Genes that showed $p < 0.01$ were selected. Genes whose duplicate measurements by GeneChip differed more than those expected by the a priori S.D. were also eliminated during this step. Then the fold change of each gene by either ATRA or Am80 treatment at each time point was calculated. Genes whose expressions changed more than or equal to 2.5-fold were selected. In addition to this, the average expression level (''Signal'' value of the Microarray Suite Expression Analysis Software) of a given gene through all the time points of three samples, control, ATRA, and Am80, was calculated and genes which had an average more than or equal to 1000 were selected. The genes which passed all three criteria above were assumed to be the genes whose expressions were changed by either ATRA or Am80 with statistical significance at least at one time point and whose expressions were reproducible through the time course. The number of genes left by this selection was 610. Next, the relative expression level (expression level of ATRA- or Am80-treated sample/expression level of the control sample, the average of duplicate measurements) of each gene during the time course was plotted and successively subjected to hierarchical clustering and *k*-mean clustering (GeneSpring, Silicon Genetics).

3. Results

3.1. Delineation of distinct patterns of gene expressions induced by ATRA and Am80

To elucidate the different effects of ATRA and Am80 on the gene expressions, the expression levels of 12,559 genes in HL-60 cells treated with 0.1 μM ATRA or Am80 for 1, 9, 24, and 72 h were analyzed by Affymetrix human genome U95A GeneChip and genes whose expressions were reproducible and changed more than 2.5-fold by either ATRA or Am80 treatment were selected according to the procedure described in Section 2. Next, to select the genes differentially modulated by ATRA and Am80, the relative expression level (expression level of ATRA- or Am80-treated sample/expression level of control sample) of each gene during the time course was plotted and successively subjected to the hierarchical clustering and *k*-mean clustering. Fifty seven patterns (set 1–set 57) were obtained and are shown in Fig. 2A. The number, 57, was obtained as the result of serial trials to identify the tight clustering by comparing the ''percent explained variability'' calculated by GeneSpring. To select the patterns which showed different gene expressions by ATRA and Am80, the average relative expression levels of the genes included in each set were calculated, then the similarity of the calculated average relative gene expression levels of ATRA and Am80 were compared by evaluating the standard correlation between them. The sets that showed a standard correlation of less than 0.965, which was set arbitrarily, were judged as ''differentially controlled'' sets. These sets are highlighted in Fig. 2A and the number of genes involved in these sets was 204. The genes involved in the sets whose standard correlations were greater than or equal to 0.965 (406 genes) were modulated in their expression almost identically by ATRA and Am80. These genes were interesting from a different point of view in so far as they were regulated by both retinoids through RARs in a similar manner, and are thus considered candidate retinoid target genes. The complete list of 610 genes available at our website includes a comparison with the list of retinoic acid target genes reviewed by Balmer and Blomhoff [11]. The fact that two-thirds of the genes clustered showed identical expression patterns by ATRA and Am80 treatment also indicated that the reproducibility of the time course analysis conducted in this study was fairly high (see discussion).

The expression patterns of some of the ''differentially controlled'' sets appeared visually similar to each other. Thus, to group these sets, hierarchical clustering was applied which compared the similarity of each set by calculating the Pearson correlation coefficients. The result is shown in Fig. 2B. Each node contained the sets whose expression patterns were similar to each other. As a result, there were several groups of genes whose expressions were differentially modulated by ATRA and Am80. The results showed that about one-third of the genes (204 genes out of 610 genes) were controlled differentially by ATRA and Am80, and indicated that ATRA and Am80 actually had different effects upon the gene expressions.

3.2. Identification of genes responsible for the growth suppression by ATRA

One node, which consisted of sets 36, 39, 40, and 47, was interesting for two reasons: The first is that the expressions of the genes involved in this node were suppressed by ATRA but not by Am80. The second is that many of the genes involved in this node were related to cell proliferation and anti-apoptosis (Table 1). Considering that the growth of HL-60 cells was suppressed by ATRA more efficiently than by Am80 during the course of differentiation, the difference in the expression pattern of this node

that contained many cell growth-related genes should explain the different effects of ATRA and Am80 on the cell growth. Thus, we looked closer into the gene list and found two interesting genes, (*phosphoinositide-3-kinase*, *β-catalytic subunit* and *ribosomal protein S6 kinase polypeptide1*) because they were involved in the same signal transduction pathway, the PI3-kinase/Akt pathway. Keeping this pathway active is important for the cell growth and prevents the cells from undergoing apoptosis [13,18]. The expressions of these two genes were suppressed by ATRA but not by Am80 (see Fig. 4 for the individual expression pattern). Thus, the PI3-kinase/Akt pathway might be suppressed in the ATRA-treated HL-60 cells, while it might be still active in the Am80-treated HL-60 cells. This hypo-

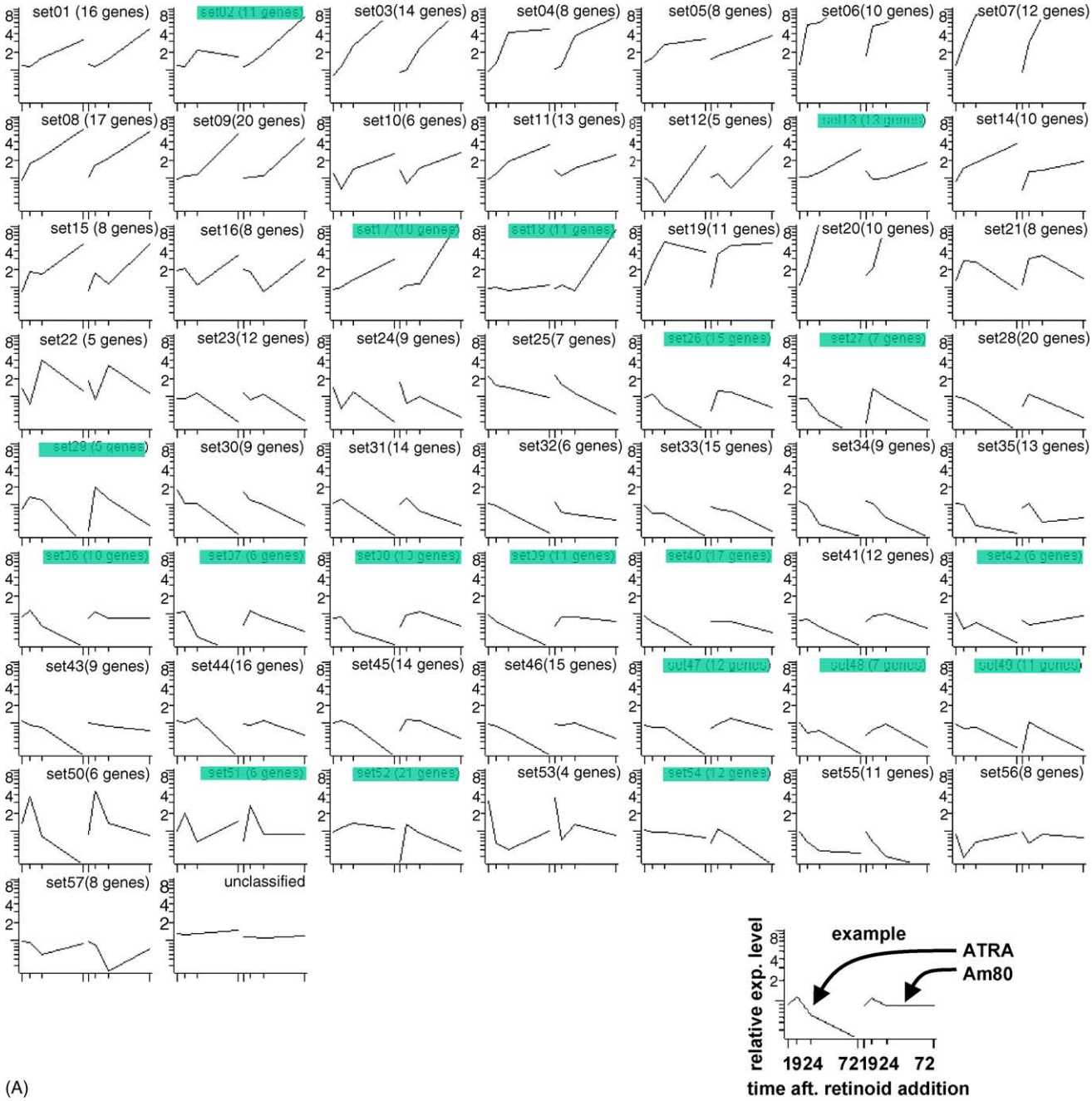


Fig. 2. Selection of gene sets whose expressions were differentially controlled by ATRA and Am80. (A) HL-60 cells were incubated with ATRA, Am80, or ethanol (vehicle) for 1, 9, 24, and 72 h, and the expressions of 12,559 genes on Affymetrix human genome U95A DNA microarray were assayed in duplicate. Genes whose expressions were changed by either ATRA or Am80 with statistical significance were selected and clustered by *k*-mean clustering. Averaged relative expression level of genes included in each set is plotted. Vertical axis is relative expression level and horizontal axis is the time after retinoid addition. Left part of each graph is the expression pattern of the ATRA-treated sample and the right part is that of the Am80-treated one. The sets in which genes were differentially regulated by ATRA and Am80 are highlighted. The set "unclassified" included genes that were eliminated during the selection step. (B) The sets in which genes were differentially regulated by ATRA and Am80 were grouped by hierarchical clustering. Relative expression level of each set is depicted in pseudocolor scale.

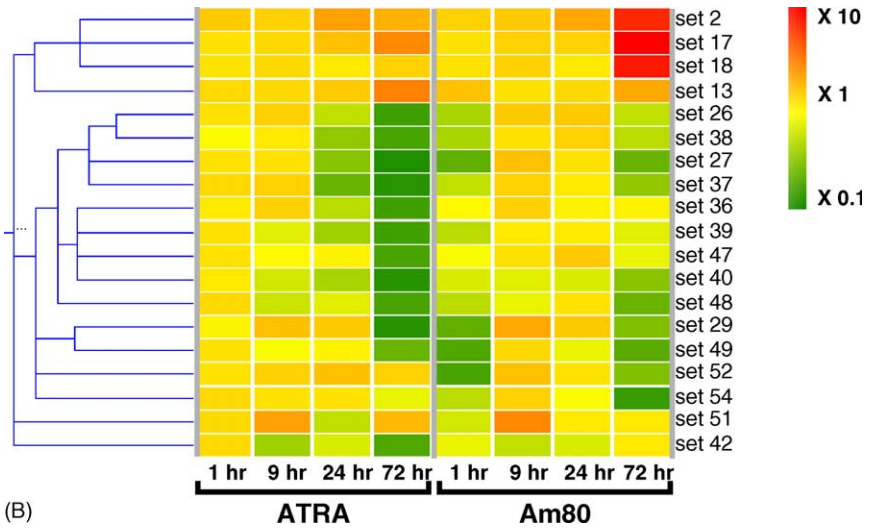


Fig. 2. (*Continued*).

thesis led the idea that if the activity of this pathway were inhibited in the Am80-treated cells, the growth of those cells should be suppressed. We employed two inhibitors, wortmannin and LY294002, for this purpose. HL-60 cells were cultured with or without Am80 (0.1 μM) for 3 days, and then wortmannin (150 nM) or LY294002 (4 μM) was added to the culture medium. To the control culture the same amount of ethanol was added. After 1 day culture with or without inhibitors, the cell number was counted. As shown in Fig. 3A, neither wortmannin nor LY294002 affected the growth of HL-60 cells cultured without Am80. In contrast, when wortmannin or LY294002 was added to the Am80-treated HL-60 cells, both inhibitors suppressed the growth of the cells. The effect of wortmannin was more significant than that of LY294002 since the growth of the cells was suppressed to the same level as ATRA-treated HL-60 cells. The fact that wortmannin is an irreversible inhibitor and LY294002 is a reversible inhibitor might explain this difference. We also checked the dose dependency of the effects of wortmannin and LY294002 in HL-60 cell growth inhibition same as Fig. 3A. The results (Fig. 3B and data not shown) showed that their effects were dose-dependent. These indicated that the suppression of growth at the late stage of differentiation induced by ATRA was caused by the reduced expression of PI3-kinase/Akt pathway component genes, and suppression of this pathway by inhibitors in Am80-treated HL-60 cell mimicked the effect of ATRA on cell growth suppression.

The effects of these inhibitors on HL-60 cell differentiation were also examined using an NBT reduction assay. Am80 alone induced the differentiation of almost 90% of cells at the end of the 4 days of treatment. HL-60 cells treated with Am80 and either inhibitor differentiated almost the same (around 90%; data not shown), indicating that these inhibitors did not affect the HL-60 cell differentiation at this stage.

3.3. Specificity of effects of ATRA on phosphoinositide-3-kinase, β-catalytic subunit and ribosomal protein S6 kinase polypeptide 1 expressions

Both ATRA and Am80 bind RARs selectively. However, ATRA easily transforms to the isomer, 9-*cis* retinoic acid (9-*cis* RA), photochemically. There is a possibility that 9-*cis* RA existing in the culture medium of ATRA-treated cells caused the different effect on HL-60 cell growth, since 9-*cis* RA binds and activates RXR in addition to RAR. To exclude this possibility, RXR ligand PA024 (10 nM) was added with Am80 to the culture medium and the same GeneChip analysis was conducted. PA024 is an RXR-specific ligand [9] and both Am80 and PA024 are stable photochemically or in normal assay conditions. The expression patterns of *phosphoinositide-3-kinase, β-catalytic subunit*, *ribosomal protein S6 kinase polypeptide 1*, and *c-myc* in ATRA-treated cells, Am80-treated cells, and Am80 with PA024-treated cells were compared (Fig. 4). Treatment of HL-60 cells with Am80 and PA024 suppressed cell growth [19] and, in accordance with this, also suppressed the expression of *c-myc*. In contrast, the expressions of *phosphoinositide-3-kinase*, *β-catalytic subunit* and *ribosomal protein S6 kinase polypeptide 1* were not suppressed by simultaneous stimulation of RAR by Am80 and RXR by PA024. The addition of 9-*cis* RA alone into the culture medium also showed the same gene expression patterns (data not shown). These results indicated that the difference observed in HL-60 cells treated with ATRA and Am80 was not caused by contamination of 9-*cis* RA.

4. Discussion

In this report, we firstly tried to identify the genes whose expressions were differentially modulated by ATRA and Am80 in HL-60 cells during a culture period of 72 h by a

Table 1
List of genes differentially modulated by ATRA and Am80

Set	Identifier	Title	Cell proliferation	Anti-apoptotis
36	S67334	**Phosphoinositide-3-kinase, catalytic, beta polypeptide**	GO[a]: 74; regulation of cell cycle	
	X85753	Cyclin-dependent kinase 8	GO: 74; regulation of cell cycle	
	L07540	Replication factor C (activator 1) 5, 36.5 kDa	GO: 6260; DNA replication	
	AB000450	Vaccinia related kinase 2	[12]	
	AL079273	Dead box protein 73D-like		
	M83822	LPS-responsive vesicle trafficking, beach and anchor containing		
	U33429	Potassium voltage-gated channel, shaker-related subfamily, beta member 2		
	U82328	E3-binding protein		
	AC004472	Hypothetical protein FLJ11560		
	HG1139–HT4910	N/A		
39	M60725	**Ribosomal protein S6 kinase, 70 kDa, polypeptide 1**	[13]	
	L19161	Eukaryotic translation initiation factor 2, subunit 3 gamma, 52 kDa	GO: 6414; translational elongation	
	X98743	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 18 (Myc-regulated)		
	D25547	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase		
	D30037	Phosphotidylinositol transfer protein, beta		
	M28209	RAB1A, member RAS oncogene family		
	U40462	Zinc finger protein, subfamily 1A, 1 (Ikaros)		
	W27675	Eukaryotic translation initiation factor 2A eIF2a		
	X17576	NCK adaptor protein 1		
	Z85986	Hypothetical protein MGC14254		
	AA013087	Homo sapiens, clone MGC: 17296 IMAGE: 3460701, mRNA, complete cds		
47	J04088	Topoisomerase (DNA) II alpha 170 kDa	GO: 6259; DNA metabolism	
	X76770	Poly(A) polymerase alpha	GO: 6350; transcription, [14]	
	X15331	Phosphoribosyl pyrophosphate synthetase 1	GO: 9165; nucleotide biosynthesis	
	AL080127	Tumor necrosis factor receptor superfamily, member 6b, decoy		GO: 6916; anti-apoptosis
	W28869	Testis enhanced gene transcript (BAX inhibitor 1)		[15]
	X63753	SON DNA binding protein		GO: 6916; anti-apoptosis
	AL049758	Protein kinase C and casein kinase substrate in neurons 2		
	X97544	Translocase of inner mitochondrial membrane 17 homolog A (yeast)		
	AB029032	Hypothetical protein KIAA1109		
	H15872	Hypothetical protein H41		
	AA189161	CGI-150 protein		
	U08997	Homo sapiens, clone MGC: 13241 IMAGE: 4026312, mRNA, complete cds		
40	U22376	v-myb myeloblastosis viral oncogene homolog (avian)	[16]	
	V00568	v-myc myelocytomatosis viral oncogene homolog (avian)	GO: 8283; cell proliferation;	
	U10564	WEE1 homolog (S. pombe)	GO: 74; regulation of cell cycle	
	U52960	SRB7 suppressor of RNA polymerase B homolog (yeast)	[17]	
	Z46376	Hexokinase 2	GO: 74; regulation of cell cycle	
	U29185	Prion protein (p27-30)		

Table 1 (*Continued*)

Set	Identifier	Title	Cell proliferation	Anti-apoptotis
	X98296	Ubiquitin specific protease 9, X chromosome (fat facets-like Drosophila)		
	AJ132440	Putative DNA/chromatin binding motif		
	Z24724	Hypothetical protein FLJ20986		
	M14219	N/A		
	HG3523-HT4899	N/A		

[a] GO: onthology defined by gene onthology consortium (http://www.godatabase.org/htdocs/docs.html).

large-scale analysis of the gene expression using a DNA microarray. By selecting genes whose expressions were changed by either ATRA or Am80 with statistical significance at least at one time point and whose expressions were reproducible through the time course, 610 genes out of 12,559 genes were left as the candidates. Next, we applied hierarchical and *k*-mean clustering algorithms to the time course expression data of these 610 genes. As the result, one-third of these genes (204 genes) were selected as the differentially controlled genes, while two-thirds of genes behaved similarly by both retinoid treatments. This fact suggested that the existence of (at least) two kinds of pathways which regulate HL-60 cell growth and differentiation, one is controlled specifically by ATRA (see below), and the other is by both retinoids.

Time course experiments involve multiple points and clustering is an algorithm that clarifies the patterns of gene expression. Since the pattern is dependent on not just one time point but on many, our analysis essentially represents the repetition of several assays and is more reproducible compared to a one time point type experiment, such as by treating HL-60 cells with either ATRA or Am80 for a certain period and then selecting differentially controlled genes at that point. Actually, the changes of the gene expression patterns induced by ATRA and Am80 were

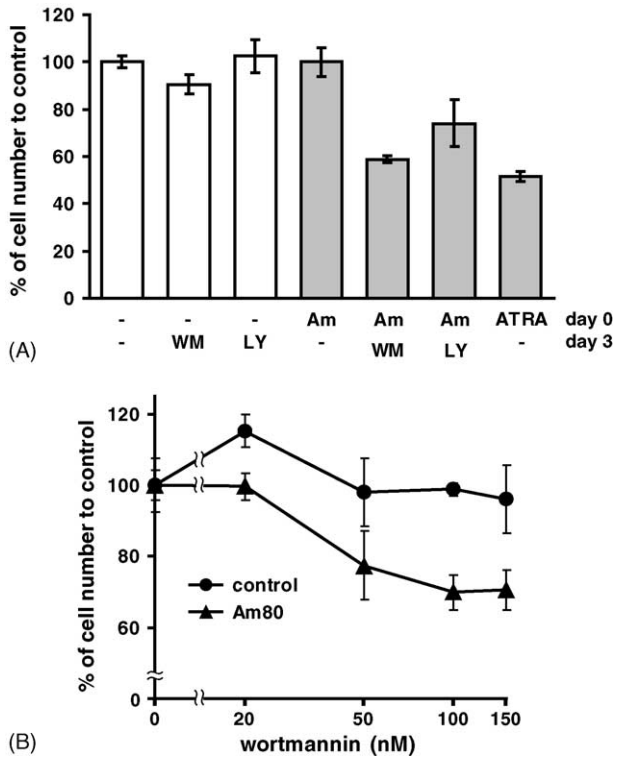


Fig. 3. Effect of PI3-kinase inhibitors on the growth of HL-60 cells treated with Am80. (A) Open bars: after 3 day culture of HL-60 cells without Am80, ethanol (−), wortmannin (WM), or LY294002 (LY) was added into the culture medium and the cells were cultured one more day before counting the number of the cells. Relative cell number is depicted as the number of cells in control (−, −) sets 100%. Each experiment was done in triplicate and error bar indicates standard error. Gray bars: same inhibitor treatments were done with HL-60 cells treated with Am80 for 3 days. The relative cell number is depicted as the number of cells in control (Am, −) sets 100%. Each experiment was done in triplicate. The relative number of HL-60 cells treated with ATRA for 4 days without inhibitors is also shown with that of the control experiments to (Am, −). (B) Different concentrations of wortmannin were added same as (A) and the number of the cells were counted. The relative cell number is depicted as the number of cells in control sets 100%. Each experiment was done in triplicate and error bar indicates standard error.

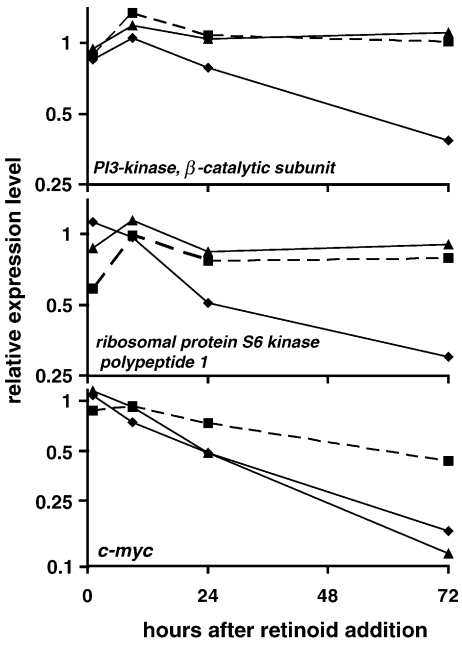


Fig. 4. Effects of RXR stimulation on the expressions of *phosphoinositide-3-kinase*, *β-catalytic subunit*, *ribosomal protein S6 kinase polypeptide 1*, and *c-myc*. RXR in HL-60 cells was stimulated by PA024 (retinoid synergist, [9]) with Am80 and the time courses of the expression levels of the three genes were measured by Affymetrix human genome U95A GeneChip. The relative expression levels were plotted: Am80 + PA024, (▲); ATRA (◆); and Am80 alone (■).

almost identical in two-thirds of the sets generated by *k*-mean clustering (Fig. 2A). This result depicted the high reproducibility of the clustering method clearly, which is why we chose the clustering of the time course data instead of analyzing the expression data of a given fixed time point.

4.1. Biological characteristics of genes differentially modulated by ATRA and Am80

The 204 genes, which were differentially modulated by ATRA and Am80, were important for the elucidation of the different effects of ATRA and Am80. Among them, the genes included in sets 36, 39, 40, and 47, which comprised a node in the hierarchical clustering, were interesting because their expressions were suppressed only by ATRA (Fig. 2) and many of them were related to cell proliferation or anti-apoptosis (Table 1). Cell proliferation is thought to be conducted by the cooperative regulation of signal transduction cascades. Taking a closer look into Table 1, three hierarchical relationships were found: (a) *DEAD/H box polypeptide 18* was regulated by *c-myc* [20]; (b) *topoisomerase (DNA) II alpha*170 kDa was a *c-myb* target gene [21]; (c) two genes, *phosphoinositide-3-kinase, β-catalytic subunit* and *ribosomal protein S6 kinase polypeptide 1*, were involved in the PI3-kinase/Akt signal transduction pathway [13,18]. The first two are relationships between transcription factors (*c-myc* and *c-myb*) and the regulated genes (*DEAD/H box polypeptide 18* and *topoisomerase (DNA) II alpha*170 kDa). Our previous study combining DNA microarray analyses with biomolecular-functional network analyses [22] indicated that the existence of such a kind of relationship implied that the behaviors of the genes were not caused by false positive signals. The third one is more interesting than the others in the context of cell growth, since the PI3-kinase/Akt pathway is positioned immediately downstream of the cell surface growth factor receptors [13,18]. Thus, modulation of this cascade triggers a change of the cell growth directly, while alterations in the expressions of the other two might be the downstream events of this cascade. The modulations of some other genes involved in this group, such as *replication factor C (activator 1) 5*, *eukaryotic translation initiation factor 2*, *subunit 3 gamma*, *poly(A) polymerase alpha*, etc., were also thought to be the downstream events of PI3-kinase/Akt pathway, however, this remains to be clarified.

The uniqueness of the modulation of the two genes involved in the PI3-kinase/Akt pathway by ATRA was also demonstrated by the experiment in which RXR–RAR heterodimer was stimulated simultaneously with PA024 and Am80 (Fig. 4). Activation of the genes downstream of the RAR–RXR heterodimer caused cell growth arrest and apoptosis [23]. The expression of *c-myc* was suppressed in this case, but on the other hand, the expressions of *phosphoinositide-3-kinase*, *β-catalytic subunit* and *ribosomal protein S6 kinase polypeptide 1* remained unchanged. These changes indicate that the expressions of *phosphoinositide-3-kinase*, *β-catalytic subunit* and *ribosomal protein S6 kinase polypeptide 1* were uniquely suppressed by ATRA treatment.

4.2. Role of PI3-kinase/Akt pathway in the late stage of HL-60 cell differentiation

Several studies have been already reported concerning the relationship between PI3-kinase and HL-60 cell differentiation [24–27]. However, there are few reports that discuss the PI3-kinase activity and HL-60 cell proliferation during differentiation [28,29]. According to a report by Liu et al. [28], when HL-60 cells cultured in serum free medium were treated with ATRA, they differentiated poorly and underwent apoptosis. However, the addition of IGF-I, which induced PI3-kinase activity in the cells, prevented the apoptosis and increased the differentiated cell population. These results indicated that keeping PI3-kinase active is a prerequisite for the cell proliferation during the HL-60 cell differentiation process. According to our gene expression analyses, the expressions of two components belonging to the PI3-kinase/Akt pathway, *phosphoinositide-3-kinase*, *β-catalytic subunit* and *ribosomal protein S6 kinase polypeptide 1*, were suppressed only in ATRA-treated HL-60 cells but not in Am80-treated cells. Taking the results by Liu et al. into consideration, the different expression patterns of these two genes should explain the different effects of ATRA and Am80 on the HL-60 cell proliferation during the differentiation process, that is, ATRA suppresses HL-60 cell proliferation more effectively than Am80. If the different modulation of the expression of the two components of the PI3-kinase/Akt pathway were the main cause of the difference in the HL-60 cell growth suppression by ATRA and Am80, inhibition of this pathway in an alternative way might be enough to induce the cell growth suppression in Am80-treated HL-60 cells. To examine this hypothesis, wortmannin and LY294002, well known PI3-kinase inhibitors, were added to the HL-60 cells treated with Am80 for 3 days and the change in the cell growth was assayed 1 day later. Both inhibitors inhibited the cell growth at a concentration that did not suppress the growth of cells cultured without Am80 (Fig. 3) in a dose-dependent manner. This result meant that the inhibition of PI3-kinase alone was able to suppress the cell growth of the Am80-treated HL-60 cells and indicated that modulation of the PI3-kinase/Akt pathway was important in the cell growth control during the HL-60 cell differentiation induced by retinoids. A recent report by Ma et al. [29] supported this idea. In that report, they suggested that the survival of differentiated HL-60 cells induced by ATRA depends on the ability of the PI3-kinase pathway. The mechanism that explains why only ATRA suppressed the expressions of *phosphoinositide-3-kinase*, *β-catalytic subunit* and *ribosomal protein S6 kinase polypeptide 1* remains to be clarified.

In our case, neither inhibitor affected the HL-60 cell differentiation (data not shown). In experiments dealing with the relationship between PI3-kinase and HL-60 cell differentiation, when PI3-kinase inhibitors were used to assess the involvement of PI3-kinase activity, they were added to the culture several hours before or at the same time as the retinoid treatment [24–27]. In contrast, both inhibitors were added to the culture medium 3 days after the Am80 treatment in our study. It is plausible that the commitment of HL-60 cell differentiation into granulocytes was already established during the 3 day treatment with Am80, thus the inhibition of PI3-kinase showed no effect on the HL-60 cell differentiation.

4.3. Clinical potential of concomitant usage of Am80 and PI3-kinase inhibitors

As shown in the result section, we were able to mimic the effects of ATRA on HL-60 cell proliferation by using PI3-kinase inhibitors in Am80-treated cells (Fig. 3). This would be clinically important because the concomitant use of synthetic retinoids and PI3-kinase inhibitors has the potential to widen their activities with fewer side-effects. For example, inhibitors of PI3-kinase might help to attain complete remission in patients who do not respond to Am80 well, because the inhibitors should inhibit the proliferation of Am80-treated cells. Another possibility is the case of solid tumor treatment. PI3-kinase activity has been linked to a variety of human tumors including breast cancer, lung cancer, melanomas and so on [18]. Thus, inhibitors of PI3-kinase activity are promising as novel chemotherapeutic agents. The application of synthetic retinoids to solid tumors has also been tried, particularly Am555S (TAC-101), another synthetic retinoid of the benzanilide series [30]. It is interesting that our results are applicable to such cases, which may thus expand the use of retinoids for cancer treatment.

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