All-trans retinoic acid induces proliferation of an irradiated stem cell supporting stromal cell line AFT024

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Objective. We have previously shown that *all-trans* retinoic acid (ATRA) enhanced the maintenance of early human hematopoietic progenitor cells (HPCs) in the presence of an irradiated stromal cell line AFT024. In this study, we examined the effects of ATRA on the stromal cell component with particular reference to cellular proliferation and gene expression.

Methods. Irradiated AFT024 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum and were incubated with ATRA at 1 μ mol/L up to 21 days. The cells were examined in terms of immunostaining for proliferative cell nuclear antigen (PCNA) and BrdU incorporation, apoptosis assay, cell cycle analysis, and gene expression using semiquantitative reverse-transcriptase polymerase chain reaction.

Results. In the control experiments, AFT024 cells lost their confluence in culture after 15-Gy irradiation and were arrested in G2/M phase on days 7 and 21. ATRA restored the cellular confluence with an increase in proliferation on day 21 (BrdU incorporation: 20.6-fold; PCNA staining: 51.7-fold) with reversal of cell cycle arrest (S phase: 2.7-fold increase; G2/M phase: 2.0-fold decrease). There was no effect on apoptosis as shown by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining. ATRA significantly upregulated the expression of cell cycle genes for checkpoint transition, including cyclin A2, B2, and aurora kinase B, as well as genes associated with a putative role in HPC maintenance, including osteopontin, HoxA5, enhancer of zeste homolog 2, and peroxisome proliferator-activated receptor gamma.

Conclusion. We concluded that ATRA induced cellular proliferation of irradiated AFT024 cells and expression of a number of genes whose relevance to HPC homeostasis would have to be further examined. © 2007 International Society for Experimental Hematology. Published by Elsevier Inc.

Hematopoietic stem cells (HSCs) are characterized by their ability to self-renew and differentiate into multiple blood lineages and to radioprotect lethally irradiated hosts [1]. A challenge in HSC research has been the development of a culture system whereby stem cells can be induced to undergo self-renewal without differentiation so that limiting number of HSCs, as derived from umbilical cord blood (UCB), can be expanded and used for clinical transplantation in adults [2]. When primitive hematopoietic progenitor cells (HPCs) from human UCB were cultured in the presence of a murine fetal liver-derived stromal cell line,

AFT024, long-term culture initiating cells (LTC-ICs) can be expanded and severe combined immunodeficient repopulating cells (SRCs) can be maintained in cytokine-supplemented cultures without direct contact with stromal cells (stroma noncontact), suggesting that diffusible factors from AFT024 cells support maintenance of primitive human HPC in vitro [3].

Retinoic acid plays an important role in the development and differentiation of cells at different levels in the hematopoietic hierarchy [4,5]. At late progenitor levels, *all-trans* retinoic acid (ATRA) induces differentiation of leukemia cells from patients with acute promyelocytic leukemia in which myelopoiesis is arrested at promyelocytic stage due to t(15:17) translocation, resulting in an aberrant fusion of Promyelocytic Leukemia-Retinoic Acid Receptor α . At

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early stem/progenitor cell levels, ATRA has been shown to enhance the maintenance of murine HSCs in culture [6]. More recently, we demonstrated in human hematopoietic cell cultures that exogenous ATRA was able to increase LTC-IC expansion and improved the maintenance of more primitive myeloid-lymphoid initiating cells and SRCs in the AFT024 stroma noncontact culture system [7]. In the same study, we observed that ATRA maintained the confluence of irradiated AFT024 during prolonged coculture with hematopoietic cells. Upon review of the literature, reports on the effects of retinoic acid on stem cell-supporting stroma are scarce and the mechanism underlying the observed phenomenon is unclear. This unexpected observation led us to further examine the effects of ATRA on irradiated stromal cells with special reference to cellular proliferation and gene expression, which might be of relevance in patients who received total body irradiation conditioning before HSC transplantation.

Materials and methods

Cell cultures

AFT024 were maintained and subcultured at 32°C in Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum (10%), 2-mercaptoethanol (50 µmol/L), penicillin/streptomycin (1%) (GIBCO-BRL, Grand Island, NY, USA). Cells were grown to confluence and irradiated at 15 Gy using a gamma irradiator (Gammacell 3000 Elan, MDS Nordion, Ottawa, ON, Canada). Immediately after irradiation, all cultures were replaced with fresh media with either ATRA (1 µmol/L) or ethanol at equal volume and maintained at 37°C. Culture media were changed every 2 days. Previous study showed that ATRA produced the most pronounced effect on HPC maintenance at 1 µmol/L [7].

Flow cytometry

Cells were trypsinized and fixed by resuspending in 70% (v/v) ethanol overnight. Prior to data acquisition, cells were resuspended in 0.5 mL freshly prepared propidium iodide (PI) staining solution (0.2 mg/mL RNase A, 20 µg/mL PI and 0.1% (v/v) Triton X-100 in phosphate-buffered saline [PBS]) and incubated for 30 minutes at room temperature in the dark. They were thereafter analyzed by flow cytometry (Cytomics FC500 MPL flow cytometry system, Beckman Coulter, Fullerton, CA, USA).

Immunocytochemistry

To examine the proliferative state of irradiated stromal cells in cultures, we performed immunostaining for the proliferative cell nuclear antigen (PCNA) and BrdU incorporation. Briefly, the cells were fixed in paraformaldehyde (1%) and permeabilized with ethanol-acetic acid (v:v = 2:1). After blocking with 0.5% bovine serum albumin in PBS for 30 minutes at room temperature, cells were incubated with rabbit anti-PCNA primary antibody (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 hour at room temperature. The bound rabbit anti-PCNA antibody was visualized as brown color using the Dako Envision Plus System (Dako, Carpinteria, CA, USA). To examine for BrdU incorporation, the cells were incubated with BrdU (10 µmol/L) for 2 hours at 37°C, followed by immunostaining for BrdU according to the

manufacturer's instruction (Roche Applied Science, Penzberg, Germany). Positivity of BrdU and PCNA staining was scored quantitatively by visual inspection in three separate experiments.

Terminal deoxynucleotidyl

transferase-mediated dUTP nick end labeling

We also examine cell apoptosis by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Chemicon, Temecula, CA, USA). Briefly, cells were fixed in paraformaldehyde (1%) followed by ethanol-acetic acid (v:v = 2:1). Cells were then incubated with equilibration buffer for 10 seconds and subsequently incubated with terminal deoxynucleotidyl transferase for 1 hour at 37°C in humidified chamber. The reaction was then stopped by incubation in stop/wash buffer for 10 minutes at room temperature. The signal was viewed by fluorescence microscopy (Olympus IX70, Olympus Corporation, Tokyo, Japan).

RNA extraction and

reverse-transcriptase polymerase chain reaction

RNA from cells was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Genomic DNA was removed by RNase-free DNase I at 37°C for 30 minutes. cDNAs were synthesized from 1 µg of total RNA using SuperScript reverse-transcriptase polymerase chain reaction (RT-PCR)

Table 1. List of RT-PCR primers used for gene expression studies

Primers	Orientation	Sequence (5' to 3')
Cyclin A2	Sense	CCTTACCCAGTACTTCCTGCAC
	Antisense	AAGGTCCACAAGACAAGGCTTA
Cyclin B2	Sense	TCTGCAAGATCGAGGACATAGA
	Antisense	GAAACTTGGATGGACTTGGAC
Aurora kinase B	Sense	GCCAGAAGTTGGCTGAGAAC
	Antisense	GATCTTGAGTGCCACGATGA
Breast cancer 1	Sense	AACCTTGGAAATCGTGAGATCAG
	Antisense	AAAACCTCACAGCAGCCTCTTC
Survivin	Sense	CAACCCGATAGAGGAGCATA
	Antisense	ATTCACTGACGGTTAGTTCTTC
PCNA	Sense	AAGTTTCTGCAAGTGGAGAGC
	Antisense	CTTTAAGTGTCCCATGTCAGCA
HNF3α	Sense	CCCCTTTCTCCCTTTCACTC
	Antisense	TGGGCCTAACAAACAACAACA
Vimentin	Sense	ACGGTTGAGACCAGAGATGG
	Antisense	CGTCTTTTGGGGTGTGTCAGTT
ASMA	Sense	AGGACCTGTACGCCAACAAAC
	Antisense	ACATCTGCTGGAAGGTGGAC
GATA binding protein 6	Sense	TACACAAGCGACCACCTCAG
	Antisense	CCTCTTGGTAGCACCAGCTC
Thymopoietin	Sense	AGAGCCGCTTTTCTCTCTCC
	Antisense	GTGGGGTGGCTTCAGAAATA
Osteopontin	Sense	TCTGATGAGACCGTCACTGC
	Antisense	AGGTCTCATCTGTGGCATC
HoxA5	Sense	GTGAAGAAGCCCTGTTCTCG
	Antisense	GGGGGCTTTTGTGTGAGTA
Enhancer of zeste homolog 2	Sense	CTGTTACAGGGGAGCAAAGC
	Antisense	TCCCTCCAGATGCTGGTAAC
PPARγ	Sense	GCCCTTTACCACAGTTGATTTTC
	Antisense	GATGCTTTATCCCCACAGACTC
β-actin	Sense	CCAAGTGGGACGACATGGAGAA
	Antisense	CGACCAGAGGCATACAGGGACA

system (Invitrogen) with oligo(dT)₂₀ primer. cDNAs were then amplified using AmpliTaq Gold (Roche Applied Science). Primer sequences and polymerase chain reaction conditions were shown in Table 1. All poplymerase chain reaction results were verified by no-reverse-transcriptase-negative control and normalized to β-actin expression.

Statistics

Results were expressed as means ± standard error of the means. Comparison between groups of data was evaluated by Mann-Whitney *U*-test (SPSS, Chicago, IL, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

ATRA restored cell confluence after irradiation in AFT024

We first examined the effects of ATRA (1 μmol/L) on AFT024 cells that were irradiated when they reached confluence in cultures. In the control experiments, the cultures lost their confluence as the irradiated cells became nonadherent during continuous culture. In ATRA-treated cells, despite of a similar loss of confluence on day & postirradiation (Fig. 1A and B), there was a gradual increase in cell number as shown by the restoration of confluence on day 21

(Fig. 1C and D). This observation confirmed our previous observation in cytokine supplemented stroma noncontact system that ATRA restored the confluence of irradiated AFT024.

ATRA induced cell proliferation rather than reduced apoptosis of irradiated AFT024 feeders

The restoration of cellular confluence in ATRA-treated AFT024 cells 21 days postirradiation could be due to an increase in cellular proliferation and/or a decrease in apoptosis. To examine this, we performed BrdU incorporation assay, PCNA immunostaining, and TUNEL staining. ATRA-treated AFT024 cells exhibited a significant increase in BrdU incorporation (Fig. 1E and H) and PCNA staining (Fig. 1I–L) on day 7 and more markedly on day 21 postirradiation. However, apoptosis was negative at both time points in both ATRA-treated and control cultures (Fig. 1M–P). These observations support the thesis that the restoration of confluence in irradiated AFT024 cells resulted primarily from an increase in cellular proliferation rather than a decrease in apoptosis. The increase in BrdU and PCNA staining was further confirmed quantitatively by direct visual inspection in three separate experiments (Fig. 2A and B).

Figure 1. ATRA induced cellular proliferation of irradiated AFT024. AFT024 cells at confluence were irradiated at 15 Gy and exposed to either ATRA or equivolume amount of ethanol (control) for either 7 or 21 days. Although the cells died off in the control, the cultures maintained confluence in the presence of ATRA (A–D). The increase in cell number upon treatment with ATRA was due to enhanced proliferation and not due to decreased apoptosis. Cells were actively cycling with an increase in BrdU uptake (E–H) and PCNA staining (I–L). TUNEL assay showed that there was no significant decrease in apoptosis (M–P).

Figure 2. Quantitative analysis of ATRA induced cellular proliferation of irradiated AFT024. (A): Percentage of cells positive for BrdU incorporation in ATRA-treated and control samples on day 7 and day 21. (B): Percentage of cells positive for PCNA immunostaining. Results were based on direct scoring under visual inspection. Effects were more pronounced on day 21 than on day 7. Results represented average data from three separate experiments and were expressed in means $\pm$ standard error of the means.

ATRA relieved cell cycle arrest caused by irradiation in AFT024

The ATRA-induced proliferation of irradiated AFT024 cells led us to examine their cell cycle status. In the control experiments, irradiated AFT024 cells were arrested at both the G1/S and G2/M checkpoint as shown by the decrease in S phase and increase in G2/M phase (Fig. 3A and B). In the presence of ATRA, there was an apparent reversal of cell cycle arrest from these checkpoints at 7 and 21 days, characterized by a significant increase in S phase (fold-increase: day 7: 1.8; day 21: 2.7) and decrease in G2/M phase compared with the untreated cells (fold-decrease: day 7: 1.6; day 21: 2.0). These observations were consistent with the continuous proliferation of irradiated AFT024 cells when treated with ATRA. AFT024 is derived from immortalized murine fetal liver cells that resulted from transformation by temperature-sensitive simian virus 40 T antigen [8]. In a similarly transformed cell line derived from human fibroblast (HFL6-2), ATRA has been shown to relieve temperature-sensitive growth restriction through suppression of p21 expression [9]. To address this issue, we further examined the expression of p21 in irradiated AFT024 cultures treated with ATRA to ascertain if the observed proliferative effects might be related to downregulation of p21. However, there was no detectable change in p21 mRNA expression upon ATRA treatment (Fig. 3C).

Effects of ATRA on gene expression profile

To understand the mechanisms whereby ATRA induces proliferation in irradiated AFT024 and enhances HPC

maintenance via a stroma cell-dependent fashion, we examined the effects of ATRA on the gene expression of AFT024. We first examined the expression of retinoic acid receptors in AFT024 using RT-PCR. mRNA encoding for retinoic acid receptor α , β , and γ as well as those for retinoid x receptor α and β were readily detectable (Fig. 4A). In subsequent experiments, we studied the effects of ATRA on irradiated AFT024 by examining the expression of a panel of genes associated with cellular proliferation and cell cycle regulation, cellular differentiation, as well as hematopoiesis, based on a previous genomewide comparison between control and ATRA-treated AFT024 using microarray (Leung, Moore, and Verfaillie, unpublished). On day 7 postirradiation, there was no significant change in the expression of genes across the panel between ATRA-treated and control samples, with the exception of cyclin B2 and Aurora kinase B, which was upregulated in the presence of ATRA (Fig. 4B). However, on day 21, genes associated with cellular proliferation and cell cycle regulation, including cyclin A2, cyclin B2, aurora kinase B, breast cancer 1, survivin, and PCNA, were significantly upregulated by ATRA. As ATRA is known to be a differentiating agent in both cell cultures and acute promyelocytic leukemia, we next examined if it might induce differentiation of AFT024 cells, which are otherwise in an epithelial-to-mesenchymal transition state [10]. There was no significant change in gene expression associated with epithelial hepatocyte nuclear factor (HNF3 α) and mesenchymal (vimentin and α -smooth muscle actin [ASMA]) lineages as well as genes that are normally expressed in fetal liver (GATA binding protein 6 and thymopoietin) upon treatment with ATRA. Furthermore, we compared the expression of a panel of genes in AFT024 that have been implicated in the regulation of hematopoiesis. These include osteopontin, HoxA5, enhancer of zeste homolog 2 (ezh2), and peroxisome proliferation activated receptor gamma (PPAR γ). Their expression was significantly upregulated upon ATRA treatment.

Effects of ATRA on AFT024 depends on irradiation dose

In previous HSC expansion protocols, AFT024 used as feeder cells has been irradiated at 15 Gy [3,7] and the same dosage has been used throughout this study. However, much higher irradiation doses have been used in bone marrow stromal cells. Therefore, we examined if the proliferative effects of ATRA might depend on the irradiation dose used. AFT024 was irradiated at 2, 4, 10, and 20 Gy and was subsequently cultured with or without ATRA for 21 days. At 2, 4, and 10 Gy, ATRA restored the confluence of irradiated stromal cells similar to the effects seen at 15 Gy (Fig. 5). However, irradiation at 20 Gy produced significant toxicity in AFT024, and ATRA appeared to produce additional toxicity with the cells became nonadherent and many were removed during medium change.

Figure 3. ATRA relieved cell cycle arrest in irradiated AFT024 cells. On day 7 and 21 postirradiation, cells were harvested for cell cycle analysis. (A): Cell cycle profiles of a representative set of experiment. Although irradiated AFT024 cells were arrested at G1 and G2/M, treatment with ATRA relieved the cells from cell cycle arrest and the S-phase population was maintained. (B): Graph showing the average percentage of cells in each cell cycle phase of three sets of experiments. A significant reduction in S-phase population with a corresponding increase in G2/M population was noted in the control. Upon treatment with ATRA, the cell cycle profile was restored, similar to that of the nonirradiated cells. Blank column: G0/G1 phase; gray column: S phase; black column: G2/M phase. (C): RT-PCR of p21 in AFT024 showed that there was no change in p21 expression between ATRA-treated and control cells. Expression of β -actin was included as an internal control.

Discussion

The present study confirmed our previous observations that ATRA restored the confluence of irradiated AFT024 cells [7]. Although the previous stroma noncontact system contained thrombopoietin and Flt-3 ligand, which might have influenced AFT024 cells, the present study showed a direct effect of ATRA on AFT024 in the absence of hematopoietic cytokines. On days 7 and 21, there was increased cellular proliferation of irradiated AFT024. The effects were more

pronounced on day 21 at which there was increased expression of genes related to cellular proliferation and hematopoietic support in AFT024. Apoptosis, as shown by TUNEL staining, was not detectable. However, this does not preclude the occurrence of apoptosis at earlier time points when the dead cells became nonadherent and might have been removed during medium replacement.

We demonstrated that ATRA induced proliferation of irradiated AFT024 cells as shown by immunohistochemistry,

Figure 4. Gene expression in irradiated AFT024 cells. (A): Expression of the different retinoic acid receptors (except RxR γ) in AFT024 was confirmed by RT-PCR. (B): Using RT-PCR, upregulation of genes associated with cellular proliferation (cyclins A2, B2, Aurora kinase B, survivin, BRCA-1, and PCNA) were detected upon treatment with ATRA. The gene expression of ASMA and vimentin (mesenchymal markers) and HNF3 α (epithelial marker) were unchanged, indicating that cells have maintained their mesenchymal-epithelial phenotype. Genes with putative role in HPC maintenance and proliferation, including osteopontin, HoxA5, enhancer of Zeste homolog 2, and PPAR γ , were upregulated in the presence of ATRA. Non Irr., nonirradiated cells; Ctrl, ethanol-treated control cells; ATRA, ATRA-treated cell. All polymerase chain reaction results were verified by no-reverse-transcriptase-negative control and normalized to β -actin expression. The numbers under each band represented quantification of gene expression using TotalLab version 2005 (Nonlinear dynamics Ltd, Newcastle upon Tyne, UK) and the relative band intensity was calculated by normalizing to that of nonirradiated cells.

cell cycle analysis, and gene expression studies. Previous studies in fetal rat hepatocytes and HepG2 cell lines [11], as well as in irradiated acute promyelocytic leukemia cell-line HL-60 [12] and human lung fibroblast cells [13],

have demonstrated the antiproliferative and differentiating effects of ATRA. To our knowledge, this is the first report that ATRA may induce proliferation of irradiated stromal cells. Concomitant differentiation is unlikely, as expression of genes associated with epithelial and mesenchymal lineages, as well as those genes that are normally expressed in fetal liver, were unchanged upon ATRA treatment. Moreover, cell cycle analysis showed that irradiated AFT024 cells treated with ATRA were in active cycle, in contrast to differentiated cells, which would be in G0 state. The proliferative effect of ATRA was unlikely to be due to a relief of growth suppression at 37°C [9], as p21 expression was not affected upon ATRA treatment. Intriguingly, although increased cellular proliferation occurred as early as day 7, most of the gene expression changes did not manifest until day 21. Whether these changes, particularly of genes associated with cellular proliferation, might have occurred even earlier than day 7 remain to be determined.

In addition, ATRA upregulated expression of a number of genes in irradiated AFT024, including osteopontin, HoxA5, ezh2, and PPAR γ . Osteopontin is a matrix glycoprotein recently shown to regulate HSCs in bone marrow [14] and fetal liver [15]. HoxA5 is normally expressed in human HPCs and constitutive expression of HoxA5 has been shown to skew lineage differentiation toward myeloid lineage [16,17]. Expression of HoxA5 in the stroma was also important for the support of proliferation of adjacent epithelium [18]. The polycomb group gene enhancer of zeste homolog 2 (ezh2) is preferentially expressed in young as compared with senescent murine embryonic fibroblasts (MEFs) and overexpression of ezh2 in MEFs and HSCs endows the cells with the ability to escape senescence and grow continuously in several passages as well as the ability to maintain long-term repopulating potential during serial transplantations [19]. Human PPAR γ has also been shown to be expressed in bone marrow stromal cells [20], but the significance of its upregulation in murine fetal liver AFT024 by ATRA is presently unclear. In this respect, functional studies are needed to delineate how the reported changes in gene expression could be related to the improved HPC maintenance by ATRA as observed previously [7]. The effects of ATRA on the expression of these genes did not manifest until day 21. This is consistent with the observations that both ATRA-treated and untreated cells lost their confluence on day 7 but pronounced restoration of confluence was only seen in ATRA-treated cells for 21 days. It also corroborated with our previous observations that pronounced increased in LTC-IC expansion upon ATRA treatment occurred only after day 21 [7]. Intriguingly, genes encoding for secreted cytokines, which have been linked to HPC maintenance in vitro [21], were not significantly affected by ATRA treatment (Cheung et al., unpublished).

Results of the present study might be of relevance to clinical transplantation. At present, total body irradiation

Figure 5. Effects of ATRA on AFT024 depend on irradiation dose. AFT024 was irradiated at 2, 4, 10, and 20 Gy and was incubated with or without ATRA for 7 or 21 days. At 2 Gy (A–D), 4 Gy (E–H), and 10 Gy (I–L), ATRA restored the confluence of irradiated stromal cells similar to the effects seen at 15 Gy. However, irradiation at 20 Gy (M–P) produced significant toxicity in AFT024, and ATRA appeared to produce additional toxicity with the cells becoming nonadherent and many were removed during medium change.

is one of the standard regimens for bone marrow conditioning prior to HSC transplantation. Most patients suffer from its side effects including profound cytopenia, mucositis, and various organ toxicity. The observation that ATRA may restore the proliferation of irradiated stromal cells in vitro suggested that it may likewise hasten the recovery from organ and tissue damage after total body irradiation. This proposition would have to be carefully examined in vivo.

In conclusion, we demonstrated that ATRA relieves cell cycle arrest induced by irradiation in AFT024 and enhances cell proliferation through upregulation of various cell cycle genes. It also induces expression of a number of interesting genes whose relevance to HPC homeostasis would have to be further examined.

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