All-trans retinoic acid modulates radiation-induced proliferation of lung fibroblasts via IL-6/IL-6R system

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EXPOSURE OF CELLS TO IONIZING radiation can induce serious damage in multiple organs. Acute and subacute radiation pneumonitis and late fibrosis are well known to be complicated in the patients receiving radiotherapy to the lung field. Although the risk of radiation-induced pulmonary injury increases parallel to the radiation dose, the mechanism initiating the reaction is not fully understood. Several factors have been reported to be released after ionizing radiation, such as transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, interleukin (IL)-1, platelet-derived growth factor, vascular endothelial growth factor, or fibroblast growth factor (15, 34, 37, 45, 46). Ionizing radiation was also reported to be a potent activator of NF-κB in myeloid leukemia cells (5, 14), resulting in the induction of IL-6 (3, 4). According to clinical research investigating the circulating cytokines in relation to radiation-induced pulmonary injury, the levels of IL-6 or TGF-β may serve as a predictor of high incidence of radiation pneumonitis (1, 7, 8). Recently it has been shown that the levels of IL-6 and TGF-β in bronchoalveolar lavage fluid collected from irradiated areas increased progressively during lung irradiation, which suggests more direct effects of these cytokines than their plasma levels (2).

IL-6 displays a broad range of biological activities, including the stimulation of immunoglobulin production (17), acute phase protein induction (12), and hematopoiesis (11). The excessive production of IL-6 plays a major role in some disorders, such as Castleman’s disease (48), collagen-induced arthritis (42), and cancer cachexia (40). It has been reported that IL-6 is an autocrine growth factor for human myeloma cells (21, 23, 26) and Epstein-Barr virus-transformed B cells (47). There have been some reports demonstrating that all-trans retinoic acid (ATRA) induced growth inhibition of myeloma cell lines and freshly isolated myeloma cells (24, 32, 39). ATRA is a physiological metabolite of vitamin A, which affects cell differentiation, proliferation, and development and has been widely used in the differentiating therapy for acute promyelocytic leukemia (APL). In the majority of patients with APL, the chromosomal translocation t(15, 17) is formed, which generates a fusion gene composed of a putative transcription factor PML on chromosome 15q22 and the retinoic acid receptor (RAR)-α located on 17q12-21. The resultant PML-RARα fusion protein causes the arrest of cell differentiation and promyelocyte and leukemic cell proliferation to APL. Addition of pharmacological levels (10⁻⁷ to 10⁻⁶ mol/l) of ATRA can overcome this PML/RARα-induced leukemic cell proliferation by inducing the normal function of each gene.

The inhibitory mechanism of the proliferation of APL cells by ATRA is directly related to the fusion gene as mentioned above, whereas the effect of ATRA on other cells is not fully understood. Here we show the inhibitory effects of ATRA on a lung fibroblast cell proliferation induced by irradiation, through its suppressive effect on IL-6/IL-6 receptor (IL-6R) system in the cells, which can result in lung protection from fibrosis in radiation therapy.

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MATERIALS AND METHODS

**Cell culture.** WI38VA-13, a human embryonic lung fibroblastic cell line transformed by SV40, IMR-90, a cell line also derived from fetal lung fibroblasts, and BEAS-2B, a human epithelial cell line isolated from normal human lung bronchial epithelium and infected with SV40, were cultured in Dulbecco’s modified Eagle’s medium (Sigma Chemical, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum and antibiotics in a humidified incubator with 5% CO₂ at 37°C. Cells were grown in 10-cm plates, washed, exchanged to serum-free medium, and irradiated with γ-rays by a 137Cs source emitting at a fixed-dose rate of 1.2 Gy/min (Gammacell 40 Exactor, MDS Nordion International, Ontario, Canada, located in Radiation Biology Center, Kyoto University Graduate School of Medicine).

**Animals.** Eight-week-old C57Bl/6 female mice were purchased from Japan SLC (Shizuoka, Japan) and maintained in our specific pathogen-free animal facility. All animals were kept according to the Animal Protection Guidelines of Kyoto University. All protocols for animal use and euthanasia were reviewed by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto, Japan.

**Treatment of cells with ATRA.** ATRA (Sigma Chemical) was diluted in DMSO to a stock concentration of 2 × 10⁻⁶ M. For each experiment, ATRA was diluted from its stock solution and added to the growth medium so that the final DMSO solvent concentration was <0.2% (vol/vol). In preliminary experiments, this final concentration of DMSO had no gross effect on WI38VA-13 cells. In some experiments, the cells were preincubated with protein kinase C (PKC)β inhibitor rottlerin (5 μM) and PKCα inhibitor Gö-6976 (2.5 nM) (28) (Calbiochem, San Diego, CA) for 30 min before the addition of ATRA to the culture and then irradiated.

**Flow cytometry.** Cell surface expressions of IL-6R and gp130 were determined by flow cytometric analysis. Cells (5 × 10⁵) from single-cell suspensions were incubated with 2 μg of phycoerythrin (PE)-conjugated mouse anti-human IL-6R monoclonal antibody (Immuno-tech, Marseille, France) or 1 μg of FITC-conjugated mouse anti-human gp130 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min on ice and washed twice with PBS containing 1% BSA and 0.1% NaN₃. FITC- and PE-conjugated mouse IgG₁κ (BD Biosciences, San Jose, CA) were used as isotype controls. Flow cytometric analysis was performed on a FACS Calibur (Becton Dickinson, Mountain View, CA). Data were obtained with CellQuest soft-ware (Becton Dickinson).

**Immunofluorescence staining.** Cultured cells were fixed by 4% paraformaldehyde/PBS for 10 min on ice. After being washed with PBS, cells were incubated in 0.1% Triton X and blocked with 5% BSA/PBS for 30 min at room temperature. The fixed cells were stained with mouse anti-human IL-6R monoclonal antibody (1:100, Immuno-tech) followed by Alexa 488-conjugated donkey anti-mouse antibody (1:1,000, Molecular Probes). Hoechst 33258 fluorochrome (Sigma) was used for nuclear staining. The exposure time of fluorescence microscopy was fixed to 1.5 s. Fluorescence intensity was checked in at least 10 fields of vision in each of three independent experiments and was scored at five levels. The proportion of the positive cells was counted in the fields by three investigators blinded to the treatment groups.

**Cell proliferation assay.** Cells were cultured in 96-well flat-bottomed culture plates for 4 days with or without IL-6 (1–10,000 pg/ml), ATRA (10⁻⁶ M), and/or mouse anti-human IL-6R monoclonal antibody (20 ng/ml; Dako, Glostrup, Denmark) to inhibit the binding of IL-6 to its receptor. Cell Counting Kit-8 (Dojindo, Tokyo, Japan) was used to characterize the growth of cells. Water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenoxy)-3-(4-nitrophenoxy)-5-(2,4-di-sulfo phenyl)2H-tetrazolium, monosodium salt] was added to the culture for the final 1 h. The absorbance (450 nm) of Formosan, which is the product of the reduction of WST-8 by mitochondrial dehydrogenase, was measured with an enzyme-linked immunosorbant assay (ELISA) reader.

**Measurement of IL-6, soluble IL-6R, TNF-α, and IL-1β in culture supernatant.** Cells were cultured with or without ATRA (10⁻⁶ M), rottlerin (5 μM), and/or Gö-6976 (2.5 nM) for 24 and 48 h, and culture supernatants were collected. The concentrations of soluble IL-6R (sIL-6R), TNF-α, and IL-1β were measured by ELISA Kit (BioSource, Camarillo, CA). For IL-6, microtiter 96-well plates were coated with goat anti-human IL-6 antibody (R&D Systems, Minneapolis, MN) for overnight at 4°C, followed by washing three times with PBS containing 0.1% Tween 20. IL-6 standard (R&D Systems) and unknown samples were added and incubated at room temperature for 2 h, and then the plates were washed. To detect bound IL-6, rabbit anti-human IL-6 antibody (Pierce Biotechnology, Rockford, IL) was added and incubated for 2 h at room temperature, followed by washing three times. Peroxidase activity was determined using o-phenylenediamine (Zymed Laboratories, South San Francisco, CA) as a substrate. The absorbance was measured at 475 nm.

**Measurement of NF-κB p65.** After treatments, nuclear extracts and cytoplasmic extracts were prepared by Nuclear Extract Kit (Active Motif, Carlsbad, CA), and NF-κB p65 and p50 were detected by ELISA kit (BioSource and Active Motif, respectively).

**Measurement of PKC kinase activity.** After treatments, cellular extracts were prepared by Nuclear Extract Kit (Active Motif), and PKC kinase activity was measured by PKC kinase activity assay kit (Stressgen Bioreagents, Victoria, Canada).

**Quantitative real-time RT-PCR.** RNA was prepared by using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Single-stranded cDNA was synthesized from 1 μg of total RNA using SuperScript reverse transcriptase (Invitrogen), oligo (dT) primer (Invitrogen), and dNTPMix (Promega, Heidelberg, Germany). Quantitative real-time RT-PCR was performed with an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol, using TaqMan Gene expression products for IL-6, IL-6R, and gp130. 18sRNA served as an endogenous control (Applied Biosystems).

**Animal study.** After anesthesia with pentobarbital, γ-ray was administered as single dose (20 Gy) to the entire thorax. Other organs, above and beyond the thorax, were shielded. Mice were injected intraperitoneally with 0.5 mg of ATRA dissolved in 0.1 ml of cottonseed oil or 0.1 ml of cottonseed oil alone (controls). Injections were repeated three times weekly until the end of the observation period. The mice were killed at 2 mo after irradiation.

**Statistical analysis.** Results are given as means ± SD of values. Statistical analysis was performed using Tukey-Kramer multiple-comparison test, and P < 0.05 was considered the level of significance.

RESULTS

**Reduction of irradiation-induced production of IL-6 by ATRA.** We first studied the effect of irradiation on IL-6 release from lung fibroblastic cell lines. The concentration of IL-6 in the culture supernatant from WI38VA-13 cells increased in a dose-dependent manner up to 16 Gy (Fig. 1A). The viability of WI38VA-13 cells at 48 h of culture was not affected by the irradiation (see Fig. 5C), and in some experiments, the concentration of IL-6 was adjusted parallel to cell number. The IL-6 level produced from WI38VA-13 cells after 16-Gy irradiation continued to increase until 48 h of culture (data not shown). Whereas the stimulation of IL-6 production reached the maximum level after 24 h of 8-Gy irradiation in IMR-90 cells (Fig. 1B), an additive effect was not observed even if the
cells were cultured until 48 h. On the other hand, 10⁻⁶ M ATRA reduced IL-6 production from WI38VA-13 cells irradiated with 16 Gy and cultured for 48 h (Fig. 1C), whereas it had a marginal effect on the cells without irradiation. ATRA at 10⁻⁷ M showed a minor effect compared with 10⁻⁶ M, and dose escalation of ATRA to 10⁻⁵ M had no additive effect (data not shown). Although this effect in WI38VA-13 cells was also observed at 24 h, it remained a minor change. Moreover, when cells were preincubated in the presence of ATRA for 24 h before irradiation and then cultured another 24 h, the decrease of IL-6 production as the effect of ATRA was very small (data not shown). Similar results were obtained in IMR-90 cells at 24 h after 8-Gy irradiation (Fig. 1D). Production of IL-6 was not observed in supernatant of BEAS-2B, a human lung epithelial cell line as a control, even after irradiation (up to 24 Gy, until 48 h, data not shown).

Effect of ATRA on the expression of IL-6R and gp130 after irradiation. To investigate cell surface expression of IL-6R and gp130 in WI38VA-13 cells, we performed flow cytometric analysis using antibodies specific to these receptor components. Confluent WI38VA-13 cells were irradiated and cultured with or without ATRA (10⁻⁶ M). At 24 h after the incubation, cell surface IL-6R was not detectable in cells with or without the irradiation treatment, whereas IL-6R was clearly observed on a positive control RPMI8226 cells, a human myeloma cell line (36) (data not shown). Addition of ATRA had no effect on the expression (Fig. 2, A–C). On the other hand, gp130 was equally expressed on the cells with or without irradiation at 24 h, and the treatment of ATRA hardly affected the gp130 expression. There were no differences in mean fluorescence intensity (Fig. 2, D–F). We found a decrease in the cell surface expression of gp130 after 48-h culture (data not shown).

To evaluate the levels of cytoplasmic IL-6R protein, we examined the expression of IL-6R in WI38VA-13 cells by immunostaining following Triton X treatment. The cytoplasmic IL-6R expression was increased after 16 Gy of irradiation, and it was reduced by the addition of ATRA (Fig. 3).

Evaluation of mRNA IL-6 and its receptors by real-time RT-PCR analysis. To study the mechanism by which ATRA reduced the production of IL-6 from the irradiated lung fibroblasts, we then investigated the effect of ATRA on the expression of IL-6 mRNA by real-time RT-PCR analysis. Figure 4A shows that IL-6 mRNA/18s rRNA ratio increased in WI38VA-13 cells by the irradiation at 24 h approximately twofold compared with that of the control cells. With addition of ATRA to the culture just before irradiation, the level of mRNA decreased to almost the control level.

We considered it important to study the influence of cytokine/cytokine receptor system and “autocrine mechanism” to the cytokine production. Although the surface expression of IL-6R was not detected under any conditions with or without irradiation or ATRA, the changes in cytoplasmic IL-6R expression were determined as mentioned above. No changes were also observed in cell surface expression of gp130 by flow cytometric analysis. We then examined the mRNA levels of the receptors for IL-6 and found that the expression of IL-6R mRNA/18s rRNA ratio in the irradiated cells was increased about threefold compared with that of the control cells, and ATRA blocked the effect (Fig. 4B). Irradiation also increased gp130 mRNA/18s rRNA ratio, and ATRA attenuated the effect (Fig. 4C).

In IMR-90 cells, the IL-6 mRNA/18s rRNA ratio increased by the irradiation at 8 h about threefold compared with the control cells, and the addition of ATRA also reduced the mRNA to the control level (Fig. 4D).

Contribution of PKCδ to the effect of ATRA on IL-6/IL-6R system. To study the biological effect of ATRA on the irradiated WI38VA-13 cells, we evaluated the contribution of PKC activity to the inhibitory function of ATRA on IL-6 production. PKC kinase activity from WI38VA-13 cellular extracts was increased by irradiation; however, the treatment with ATRA inhibited the irradiation-mediated activation (Fig. 5A). We next examined which subclass of PKC was related to this effect, especially PKCδ, which is considered to be unique for its inhibitory effect to cell proliferation. We observed that rot-
Rottlerin, an inhibitor of PKCδ, restored IL-6 production, which was reduced by the addition of ATRA (Fig. 5B). On the other hand, Go6976, a PKCα inhibitor, had no effect on ATRA-dependent decrease in IL-6 production. We next examined the involvement of PKCδ in IL-6 mRNA expression. As a result, rottlerin recovered the ATRA-mediated mRNA decline when it was added to the culture 30 min before the administration of ATRA (Fig. 5C). The viability of WI38VA-13 cells was not affected at 48 h of culture by addition of ATRA, rottlerin, or Go6976 in various combination with or without 16 Gy irradiation (Fig. 5D).

**Activation of NF-κB p65 associated with increase in IL-6 mRNA after irradiation.** It is well known that the activation of NF-κB is involved in the expression of IL-6 mRNA. Thus we investigated NF-κB p65 amounts in WI38VA-13 cells by ELISA. The activated form of NF-κB p65, which was detected

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**Fig. 2. Flow cytometric analysis of IL-6R and gp130 expression on WI38VA-13 cells.** WI38VA-13 cells were cultured with (B, C, E, F) or without (A, D) irradiation (16 Gy) in serum-free medium for 24 h and stained for IL-6R (A–C) or gp130 (D–F). In some experiments, cells were cultured in the presence of 10^{-6} ATRA (C, F). The expression of IL-6R or gp130 is shown in a solid line and that of isotype control in a broken line. The representative result is shown from 3 independent experiments.

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**Fig. 3. Cytoplasmic expression of IL-6R in WI38VA-13 cells by immunostaining.** WI38VA-13 cells were cultured with (C, D) or without (A, B) 16-Gy irradiation in the presence (D) or absence (A–C) of ATRA, fixed, and treated with Triton X as described in MATERIALS AND METHODS. The cells were stained for isotype control (A) or IL-6R (B–D) (original magnification ×400). The representative result is shown from 3 independent experiments. E: fluorescence intensity was assessed at 5 levels. F: the proportion of positive cells was counted. *P < 0.05.
in the nuclear fraction, increased 8 h after irradiation compared with the control cells. However, at 24 h of culture, no difference was observed between the cells with or without irradiation, whereas its cytoplasmic activities were hardly affected by irradiation (data not shown). We then examined the effect of ATRA in combination with rottlerin on NF-κB p65 amounts 8 h after irradiation. ATRA reduced the activated form of NF-κB p65 in the nuclear fraction of the cells, although

Fig. 4. Effects of ATRA on the expression of transcripts for IL-6 and its receptors. Real-time RT-PCR was performed to determine the changes in mRNA levels for IL-6, IL-6R, and gp130 as described in MATERIALS AND METHODS. A–C: WI38VA-13 cells were cultured in the presence (hatched bars) or absence (solid bars) of 10^{-6} M of ATRA for 24 h, with or without 16-Gy irradiation. The levels of mRNA for IL-6 (A), IL-6R (B), and gp130 (C) are represented as the ratio to 18S rRNA, an endogenous control. D: IMR-90 cells were cultured with or without 8-Gy irradiation in the presence (hatched bars) or absence (solid bars) of 10^{-6} M ATRA for 8 h. The level of mRNA for IL-6 (D) is represented as the ratio to 18S rRNA. The results are indicated as means ± SD of 3 separate experiments in duplicate. *P < 0.05.

Fig. 5. Involvement of PKC in ATRA-induced decrease of IL-6 production. A: WI38VA-13 cells were stimulated with 16-Gy irradiation, cultured in the presence of 0.1% DMSO with or without ATRA (10^{-6} M) for 8 h, and the PKC kinase activities were analyzed as described in MATERIALS AND METHODS. B: WI38VA-13 cells were stimulated with 16-Gy irradiation and cultured in the presence of 0.1% DMSO with or without ATRA (10^{-6} M), rottlerin (5 μM), and Go-6976 (2.5 nM) in various combination for 48 h, and IL-6 concentrations in culture supernatants were measured. Rottlerin and Go-6976 were added 30 min before treatment with ATRA and ATRA was added just before the irradiation. C: mRNA of IL-6 from WI38VA-13 cells cultured in the presence of 0.1% DMSO with or without ATRA (10^{-6} M) and rottlerin (5 μM) for 24 h. The values of IL-6 mRNA were presented as a ratio to that of the control (only irradiation without ATRA) cells. D: viability of the cells cultured with (solid bars) or without (open bars) 16-Gy irradiation and cultured in the presence or absence of DMSO, ATRA, rottlerin, and Go-6976 in various combination. Cell viability was calculated by staining with trypan blue and counting over 200 cells. The results are indicated as means ± SD of 3 separate experiments in duplicate. *P < 0.05.
rotterlin reversed the level of activated NF-κB to the control level (Fig. 6A). On the contrary, the inactive form of NF-κB, present in the cytoplasmic fraction, was not affected by the addition of ATRA with or without rottlerin (Fig. 6B). However, the amounts of p50 from nuclear or cytoplasmic extracts were not affected by addition of ATRA (Fig. 6C and D).

Effect of ATRA on the IL-6-mediated proliferation of WI38VA-13 cells. To clarify the involvement of IL-6 in the development of fibrous tissue, we studied the effect of IL-6 on the proliferation of WI38VA-13 cells. At 96 h of culture, the addition of IL-6 stimulated cell growth in a dose-dependent manner and reached a plateau at the concentration of 1,000 pg/ml, which was a similar value produced by the irradiated (16 Gy) WI38VA-13 cells (Fig. 7A). The IL-6-mediated proliferation was blocked in the presence of neutralizing antibody against IL-6. ATRA also inhibited the IL-6-mediated proliferation equally to the neutralizing antibody (Fig. 7B). An additive effect was observed when the neutralizing antibody and ATRA were simultaneously added to the culture. Similar examinations were studied at 48 and 72 h, but the apparent cell proliferation was not seen by the addition of IL-6 (data not shown).

Fig. 6. Contribution of NF-κB activity to ATRA-induced inhibitory effects on irradiation. The activities of NF-κB were analyzed as described in MATERIALS AND METHODS. WI38VA-13 cells were pretreated with or without ATRA (10⁻⁶ M) and rottlerin (5 μM), irradiated (16 Gy) cells were cultured for 8 h, and p65 (A, B) and p50 (C, D) amounts in nuclear (A, C) and cytoplasmic (B, D) protein extracts were analyzed. Each level of p65 and p50 amounts was corrected to protein concentration of each sample. The values of NF-κB were presented as a ratio to that of the control (with only irradiation) cells. The results are indicated as means ± SD of 3 separate experiments in duplicate. *P < 0.05.

Fig. 7. IL-6-dependent proliferation of WI38VA-13 cells and inhibitory effect of ATRA on proliferation of WI38VA-13 cells after irradiation. A: WI38VA-13 cells were cultured in 96-well flat-bottomed culture plates for 4 days in serum-free medium with indicated (0–10,000 pg/ml) concentrations of IL-6 and cell proliferation was assayed as described in MATERIALS AND METHODS. *P < 0.05 compared with control cells. B: cells were cultured in the presence (solid bars) or absence (open bars) 1,000 pg/ml IL-6 with or without mouse anti-human IL-6R MAb (20 ng/ml), which can inhibit the binding of IL-6 to its receptor and/or ATRA (10⁻⁶ M). *P < 0.05. C: WI38VA-13 cells were irradiated (16 Gy) and cultured in the presence or absence of anti-IL-6R MAb (20 ng/ml) and/or ATRA (10⁻⁶ M) in serum-free medium for 4 days, and cell proliferation was assayed as described in MATERIALS AND METHODS. The results are indicated as means ± SD of 3 separate experiments in duplicate. *P < 0.05 compared with control cells.
**Inhibitory effect of ATRA on the proliferation of irradiated WI38VA-13 cells.** We then evaluated the effect of ATRA on the proliferation of fibroblasts after irradiation. The proliferation of WI38VA-13 cells treated with 16-Gy irradiation was inhibited by either IL-6 neutralizing antibody or ATRA. An additive inhibitory effect was not observed in the presence of both reagents (Fig. 7C).

**Chronic activation of IL-6 system and its inhibition by ATRA in vivo.** Finally, we investigated whether the activation of IL-6 system could be observed in vivo. After a single dose (20 Gy) of irradiation to the thorax, mice were injected intraperitoneally with 0.5 mg of ATRA dissolved in 0.1 ml of cottonseed oil or 0.1 ml of cottonseed oil alone as controls three times a week. ATRA administration did not produce noticeable morbidity and mortality. At 2 mo after irradiation, IL-6 mRNA increased in the whole lung of mice ~20-fold compared with that of controls. The systemic administration of ATRA significantly inhibited the effect (Fig. 8).

**DISCUSSION**

We showed the increase of IL-6 production from lung fibroblastic cell lines, WI38VA-13 and IMR-90, by irradiation in a dose-dependent manner, and furthermore, ATRA reduced this stimulant effect of irradiation on IL-6 production. The stimulatory effect of irradiation on IL-6 production was not observed in human lung epithelial cell line, BEAS-2B, suggesting that such an effect was seen in lung fibroblasts, but not in the cells transformed by SV40. The temporal increase of IL-6 production by irradiation, peaking at 9 h, was previously reported by Brach et al. and was not irradiation dose dependent. The current results showed sustained and dose-dependent stimulatory effect of irradiation on IL-6 production and further indicated the activated autocrine/paracrine loop of IL-6 and IL-6R as discussed below. Although the reason why our results differ from theirs is not clear, the different culture conditions such as serum starvation may be related to the results. The maximum effect was observed in WI38VA-13 cells after 48 h of culture, somewhat later than the usual cytokine production observed after other stimuli. To rule out the indirect effect by other irradiation-induced cytokines on IL-6 production, we screened cytokine levels in the culture supernatant after irradiation at 24 and 48 h. With ELISA assays, TNF-α and IL-1β, both of which are cytokines reported to stimulate IL-6 production, were undetected in both 16 Gy-treated and untreated culture supernatants (data not shown). Although the production of TGF-β was stimulated by irradiation, the effect was already observed after only 0.5 Gy without dose dependency (data not shown). Therefore, we considered that it was a direct effect of ATRA by downregulating the irradiation-induced IL-6 production. Both irradiation-induced IL-6 production and ATRA-dependent inhibitory effect were shown to be regulated at the pretranslational level by real-time RT-PCR for IL-6 mRNA. We could not examine the further culture of WI38VA-13 cells after 48 h because the viability of the cells cultured without FCS gradually declined after 48 h in preliminary experiments. In IMR-90 cells, the maximum effect on IL-6 production was seen at 8-Gy irradiation, and the stimulatory effect was fully observed already at 24 h of the culture. We consider that these differences in the effective irradiation dose and time course between WI38VA-13 and IMR-90 cells are caused by their disparity in proliferation speed. Indeed, the doubling time of IMR-90 cells is about a half of that of WI38VA-13 cells.

The IL-6R complex is composed of two different subunits, a ligand-binding 80-kDa IL-6R (gp80, CD126) and a signaltransducing 130-kDa gp130. When IL-6 binds to cell surface IL-6R, two molecules of gp130 make a homodimer, which leads to activation of the Janus kinase/STAT signal transduction pathway (16). In WI38VA-13 cells, flow cytometric analysis showed no detectable surface IL-6R even after irradiation, whereas gp130 expression was not affected by either irradiation or ATRA. Both cytoplasmic IL-6R expression and the levels of mRNA for IL-6R and gp130, however, were increased after irradiation, and their increase was inhibited by coexisting ATRA after 24 h of culture. This discrepancy between mRNA levels and cell surface expression must be discussed. Soluble forms of cytokine receptors have been shown to exist in culture supernatant or in serum of some patients. The membrane-bound receptor may be truncated and transferred into a soluble form, which results in a decrease in the number of cell surface receptors. We examined the concentration of sIL-6R in the supernatant by ELISA, but no detectable levels of sIL-6R were observed in any of the culture with or without irradiation and ATRA (data not shown), which contradicts the disappearance of cell-bound IL-6R by change to a soluble form. After binding to the receptor, IL-6 is rapidly internalized and degraded. This internalization causes the decrease in the cell surface IL-6R (49) and gp130 (44), namely downregulation by its ligand. Here, we showed the increase in mRNA for both IL-6 and two components of its receptor by irradiation in WI38VA-13. This suggests the IL-6/IL-6R autocrine and/or paracrine system is accelerated in the cells stimulated by irradiation. Thus we consider the possibility that cell surface IL-6R and gp130 on IL-6-producing cells themselves or the neighboring cells immediately decline by internalization because of accelerated IL-6 production after irradiation and that some IL-6/IL-6R complex may be formed in the cytoplasm promptly after production of each protein without secretion into culture supernatant. Finally, to clarify whether cell proliferation is observed as the result of accelerated IL-6/IL-6R autocrine/paracrine system, we then studied the direct effect of IL-6 on WI38VA-13 cell proliferation. IL-6 showed a dose-dependent stimulation on cell proliferation, which was proved to be an effect of IL-6 itself by the inhibition with its neutralizing antibody. Furthermore, 10⁻⁶ M ATRA, consistent with

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**Fig. 8.** Chronic activation of IL-6 system and its inhibition by ATRA in the lungs of mice. Real-time RT-PCR of IL-6 obtained from the lungs of mice were treated with either ATRA or cottonseed oil 2 mo after 20-Gy irradiation. The levels of IL-6 mRNA are represented as the ratio to 18S rRNA. The results are indicated as means ± SD of 3 separate experiments in duplicate. *P < 0.05.
phosphorylation, and the association of PKC with several recent reports showed the important roles of PKC (6), and insulin-like growth factor I receptor (25). In fact, associated with the p60 TNF receptor (22), the insulin receptor on IL-6/IL-6R system. In the presence of rottlerin, a PKC inhibitor, the effect of ATRA on IL-6 production disappeared, whereas G6-6976, a PKCα inhibitor, had no effect. PKCβ is a member of a heterogeneous multifamily of lipid-regulated serine/threonine kinases. Activated PKCβ was found to be associated with the p60 TNF receptor (22), the insulin receptor (6), and insulin-like growth factor I receptor (25). In fact, several recent reports showed the important roles of PKCβ in IL-6 signaling. PKCβ is required for IL-6-induced STAT3 phosphorylation, and the association of PKCβ with gp130 via STAT-3 enhances STAT3-gp130 interaction (20, 31, 35).

The promoter and enhancer regions of human IL-6 contain binding sites for several transcription factors, including NF-kB, NF-IL-6, and activator protein 1. Among these factors, NF-kB was demonstrated as the major inducers of IL-6 transcription by ionizing radiation in HeLa cells and human fibroblasts (3, 4). NF-kB transcription factors comprise a group of protein dimers, which are composed of various combinations of members of the NF-kB/Rel protein family. In mammals, there are at least five members, NF-kB1 (p50 and its precursor p105), NF-kB2 (p52 and its precursor p100), c-Rel, RelA (p65), and RelB, and the p65/p50 dimer is the most abundant. Only certain members possess a transcriptional activation domain, such as RelA (p65). In an inactive state, NF-kB/Rel family members are constitutively present in the cytosol and associated with IκB family members. When the cells are stimulated by various stimuli, IκB protein is phosphorylated and degraded, then NF-kB translocates into the nucleus where it binds to specific DNA sequences in the promoters of target genes and stimulates their transcription. Here, we showed nuclear NF-kB p65 was increased by irradiation, ATRA diminished the irradiation-induced activity, and moreover, the effect of ATRA was inhibited by rottlerin. Whereas the amount of NF-kB p50 was hardly affected by addition of ATRA, which suggested the specific inhibitory effect of ATRA on NF-kB p65/p50 dimer related to “trans-activation.” These findings are summarized in Fig. 9. We think that ATRA can inhibit the irradiation-mediated proliferation of lung fibroblasts, which eventually progresses to pulmonary fibrosis.

In conclusion, irradiation stimulated IL-6 production and accelerated transcription of its receptors and increased cell proliferation by a predictable IL-6/IL-6R autocrine/paracrine system in human lung fibroblastic cell lines. ATRA inhibited irradiation-induced production of both IL-6 and its receptors and IL-6-dependent cell growth. We propose that ATRA reduced NF-kB activity through PKCβ-specific pathways. Moreover, we showed that ATRA directly inhibited the proliferation of lung fibroblasts after irradiation as well as anti-human IL-6R antibodies. Current clinical approaches to the radiotherapy to the lung field for lung cancer, breast cancer, malignant lymphoma, and other thoracic malignancies focus mainly on the choice of irradiation dose with maximum antitumor effect without serious lung injury. Recent clinical trials in radiotherapy for lung cancers (18, 30) recommend a single massive dose of irradiation ~12–26 Gy instead of daily administration of small doses. The 16 Gy used in this study is useful to study the effects of such doses of irradiation on lung fibroblasts. The in vitro results in this study showed activation of the IL-6 system during the short period after irradiation. However, according to a previous report, the production of IL-6 in the lung was observed 3 mo after single 20-Gy irradiation in vivo (14). Therefore, the inhibitory effect of ATRA on IL-6 production may be beneficial in rather chronic development of lung fibrosis in vivo. In this study, we could show our preliminary data at 2 mo after irradiation. However, in this short period, we were able to show that IL-6 transcripts were upregulated and that the effect was blocked by the administration of ATRA. We are currently investigating the long-term influences of ATRA in vivo in irradiated mice. Antitumorogenic activities of ATRA reported would make the advantage of ATRA for radiotherapy to the lung field for the malignancies in thoracic regions concerning both the antitumor effects and prevention of radiotherapy-induced pulmonary injury. We propose that ATRA is a new strategy for the prevention of pulmonary fibrosis in the treatment involving lung field radiation.

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Fig. 9. Mechanism of the inhibition of irradiation-induced IL-6 production by ATRA. The dose- and time-dependent induction of IL-6 by irradiation is induced through NF-kB p65 activity. The effect is blocked by ATRA through PKCβ-dependent pathway, resulting in reduced NF-kB p65 activity.
GRANTS

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