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Effect of Continuous Gamma Ray Exposure on Human Cell Growth and Functions

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ABSTRACT

To evaluate biological effects of the radioactive wastes in the environment, we continuously exposed mammalian cells to low dose rate ionizing radiation. Growth of a human osteosarcoma was partially inhibited by the continuous irradiation with 0.08 Gy/h for 40 h, although apoptosis was not detected even at 6.12 Gy/h. Protein expressions of p53 and its down stream target p21, was increased above 0.08 Gy/h. On the other hand, transcription activity of p53 was significantly increased at 0.01 Gy/h. Continuous irradiation enhanced transcription activities of E2F and PPAR, and cytokine productions of T cells and macrophages, while suppressing AP-1 activity. These results suggest the possible way to utilize low dose rate-radiation for biological reactions involved in human diseases.

INTRODUCTION

Nuclear power is nowadays an indispensable tool for human beings, without which we cannot live even for a day, while atomic bombs at Nagasaki and Hiroshima, and several accidents of nuclear power stations have been threatening people that nuclear power is extremely dangerous. The legal restriction now carried out in the world is based on the hypothesis that the risk caused by irradiation is linear to dose, and thus, radioactive materials and wastes of background level to which ordinary people on the earth are continuously exposed, are also the subject for the legal restrictions. As a result, we have spent a big amount of money for the background level radioactive materials, and have been using a big amount of energy and resources on the earth, which we should reserve for progeny. It is urgently required to evaluate biological effects of low dose radiation in the appropriate methods.

Recent progress of molecular biology has revealed precise molecular mechanisms for biological reactions. The biological effects of irradiation on the mammalian cells and whole body, have also been studied in molecular levels, and the regulatory mechanisms for gene expression in response to irradiation have been elucidated. Ionizing radiation influences the biological responses mainly through the reaction with intracellular or intercellular water, which generates reactive oxygens. Reactive oxygens subsequently impair DNA, or induce so called stress responses. DNA injury induces expression of series of genes required for the DNA repair, which keeps the intact gene structure but simultaneously produces mutations through the inaccurate repair. Accumulation of such mutations leads to malignant transformation of cells. Thus, cells prepare the second phase protection system which protects the human beings from cancer through the suicide of such malignant cells. The key molecules determining the cell fate are tumor suppressor genes such as pRb and p53. pRb binds E2F, a family of transcription factors promoting S phase entry, and regulates the progress of cell cycle of mammalian cells (1). p53 arrests the cell growth or induces apoptosis by transcription regulation of its down stream targets involved in cell cycle progression and apoptosis such as p21 and Bax (2).

Moreover external stress induces the other set of genes which regulates growth and functions of mammalian cells (3). Heat shock proteins are family of proteins induced by the stress responses including heat, oxidative stress, or irradiation. They regulate intracellular distribution and degradation of the proteins through the conformational change of the proteins (4). Alternatively, they regulate the intracellular oxidation reactions which provide secondary regulatory molecules for cell functions, such as CO and NO. Stress response also activates SAPK/JNK, which phosphorylates c-Jun, a transcription factor involved in the regulation of cell growth and differentiation (5). The biological effects of low dose irradiation should be studied in the context of such progress of modern molecular biology in order to evaluate the legal restrictions properly.

The effect of irradiation is conventionally evaluated with the total amount of irradiation but not by the dose rate. The X-ray irradiation with high dose rate and short time exposure is usually used to study the effect of low dose irradiation on the biological activity, but such evaluation is inappropriate to evaluate the biological effects of the radioactive materials in the environment, which irradiate us continuously with low dose rate. In this study, we evaluated the biological effects of low dose irradiation with a 60Co irradiator for the source of ionizing radiation, which enable us to expose cells continuously to low dose rate-radiation for a long period. Our results suggest that low dose radiation has distinct effect on activity of transcription factors and cellular functions.

MATERIALS AND METHODS

Cell Culture and Irradiation.

The methotrexate-resistant Chinese hamster ovary cell line CHOC 400, which bears an amplified

DNA region containing reiterated dhfr genes (6) and a human osteosarcoma, U2OS, were cultured at 37 oC in a humidified 5 % CO2 atmosphere using D-MEM supplemented with 50 mg/ml kanamycin and 5% FBS (Gibco). Cells were irradiated in the room with 50,000 Ci 60Co source.

Plasmids and Antibodies.

A plasmid b-galactosidase (pCMV-b-gal) was a kind gift from Dr. Brooke Mosman (University of Vermont, Burlington, VT). AP-1 reporter (TRE-luc,-79-+170-luc), p53 reporter (pBL53-CAT, ref. 7), PPAR reporter (pBL-DR1x3-Tk-basic-CAT, ref.8) and E2F reporter (pGL3-E2Fx4-TATA basic-luciferase) were provided by Dr. Yvonne Janssen-Heininger (University of Vermont, Burlington,VT), Dr. Kaoru Segawa (Keio University, Tokyo), Dr. Shigeaki Kato (University of Tokyo, Tokyo) and Dr. Kristian Helin (European Institute of Oncology, Milan, Italy). Following antibodies were used in Western blotting. E2F-1 (KH20), kind gift from Dr. Chin-Li Wu (MGH Cancer Center, Boston, MA); cyclin A, kind gift from Dr. Nicholas H. Heintz (University of Vermont, Burlington, VT); c-Jun, and cyclin B, Transduction Lab; HSP72/73 (W-27), p21 (C-19) and E2F-3 (C-20), Santa Cruz; p53 (DO-1), Calbiochem..

Western Blotting.

Experiments were performed as described previously (9). In brief, cells were lysed in 2x SDS sample buffer containing 100 mM dithiothreitol, and uniform amounts of each sample were resolved by electrophoresis in denaturing polyacrylamide gels. The proteins in the gel were transferred to Immobilon-P membranes (Milipore). Blots were blocked in 5 % non-fat dry milk in TBS-T (Tris-buffered saline: 20 mM Tris, 0.8 % NaCl, 0.1 % Tween 20, pH 7.5), probed with the indicated primary antibodies, and then with the appropriate horse radish peroxidase-coupled secondary antibody (Amersham). Signals were detected by the ECL system (Amersham).

DNA Fragmentation Assays.

One million cells were suspended in 0.1 ml of lysis buffer (10 mM Tris, 10 mM EDTA, 0.5 % Triton X-100, pH 7.4) for 15 min on ice, centrifuged at 15,000 rpm for 10 min. The supernatant was mixed with 25 ml 5 M NaCl and 250 ml isopropanol, kept at -20 oC overnight, centrifuged at 15,000 rpm for 10 min. The precipitate was washed with 80 % ethanol once and suspended in 20 ml TE (10 mM Tris, 1 mM EDTA, pH 8.0). The sample was analyzed on 2% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA).

Cell Cycle Analysis by Flow Cytometry.

Flow cytometry was used for cycle analysis as described previously (10). To analyze DNA content, cells were washed with PBS, suspended in 4 mM sodium citrate containing 0.1% Triton X-100, 32 mg/ml RNAase A, and 50 mg/ml propidium iodide. DNA content was analyzed by flow cytometry (Epics XL, Coulter).

Reporter Assays.

Cells (3 x 105 cells/plate) were cotransfected with reporter plasmid and pCMV-b-gal by the calcium precipitation method as described previously (11). Sixteen hours after the transfection, cells were washed with PBS three times, further cultured in fresh medium for 24 hr, and the scraped cells were suspended in 0.1 ml lysis buffer (Boehringer). Commercial kits (Boehringer) were used to determine reporter enzyme activities in cell lysates according to the instructions of supplier.

Cytokine Production.

Thioglycollate-elicited peritoneal cells and single cell suspension of spleen cells were prepared from C3H/He mice (6-wk, female, SLC Ltd., Shizuoka, Japan) as described previously (12,13). Cytokine were induced from peritoneal cells by incubating with or without 10 mg/ml lipopolysaccharide (LPS, Sigma) or from splenic T cells by incubating with concanavalin A (con A, Sigma). Twenty hours after the initiation of the culture, cytokine amount in the supernatant was measured by commercial kits for enzyme-linked immune solvent assay (ELISA, Endogen).

RESULTS

Effect on Cell Growth.

A human osteosarcoma, U2OS, was continuously exposed to ionizing radiation from 60Co. The cell growth was inhibited partially at 0.08 Gy/h, and completely at 6.12 Gy/h. The inhibitory effect on 0.08 Gy/h was similar to actinomycin D at 2 ng/ml (Fig. 1A). Irradiation with 0.01 Gy/h did not affect the growth and viability of cells during 144 h-culture (Fig. 1B). The effect on cell cycle progression was analyzed by flow cytometry. Continuous irradiation with 0.08 Gy/h increased G2/M phase cells 16 h after the initiation of the irradiation and the profile of cellular DNA content was reversed after 48 h. Irradiation with 6.12 Gy/h increased S and G2/M

phase cells after 16 h, and G2/M phase Cells were accumulated after 48 h (Fig. 2A). These cells were in G2 phase because no mitotic figure was observed. Actinomycin D arrested cells in G1 phase. Continuous exposure of the cells on 0.01 Gy/h did not affect the cell cycle progression during the 144 h-culture (Fig. 2B).

Although it is known that ionizing radiation induces apoptosis of cells, irradiation as much as 300 Gy did not induced the apoptosis of U2OS cells which has wild type p53. We compared the effect of high dose rate irradiation on different kinds of cells (Fig. 3). Irradiation at 100 Gy/h for 60 min and 1,000 Gy/h for 6 min (total 10 Gy) markedly induced DNA fragmentation of Jurkat cells, a human T cell lymphoma. Less fragmentation was detected in K562 cells, a human promyelocytic leukemia, but not in U2OS cells. Thus, apoptosis induced by irradiation is dependent on cell property. WI-38, a normal human fibroblast, was as resistent to apoptosis as U2OS cells (data not shown).

Effect on Protein Expressions.

Radiation affects the expression of proteins which regulates cell cycle and functions. Continuous irradiation with low dose rate increased expression of p53 above 0.08 Gy/h (Fig. 4A). p21, one of the downstream target of p53, concomitantly increased above 0.08 Gy/h at 48 h. Actinomycin D also increased p21 and p53 expressions. Expression of cyclin A and cyclin B were also increased by the irradiation whereas actinomycin D did not affect the expressions. Continuous exposure of U2OS cells on ionizing radiation induced expression of E2F-1 and -3. pRb expression is also increased by the irradiation. The majority of these pRb was hyperphosphorylated form, while actinomycin D dephosphorylated pRb, consistent with the G1 arrest demonstrated in cell cycle analysis (Fig. 2A). Among stress-related proteins, c-Jun increased after 48 h. HSP70 was slightly increased at the dose rates above 0.08 Gy/h. Continuous irradiation with 0.01 Gy/h did not have significant effect on the protein expressions (Fig. 4B). The expression was similarly regulated, depending on the growth phase of the cells. It should be noted that expressions of E2F-1, E2F-3 and c-Jun were slightly decreased in the later phase of the culture after 72 h. Accumulated irradiation might affect the cell growth if these cells were allowed to grow in the fresh medium.

Effect on the Activity of Transcription Factors.

The effect of continuous irradiation on transcription activity was determined by introducing reporter plasmids into Chinese hamster cells which efficiently expresses exogenously transfected genes. Continuous irradiation with 0.08 Gy/h, or actinomycin D, significantly increased the transcription activity of E2F and p53, whereas AP-1 activity decreased (Fig. 5). Increase of p53 was detected at 0.01 Gy/h, which affected neither cell growth nor p53 expression. Peroxisome proliferator activated receptor (PPAR) is the nuclear receptor involved in the regulation of lipid and energy metabolism . Continuous irradiation and actinomycin D-treatment also increased PPAR activity as much as the level increased by pioglitazone, a potent ligand for PPAR-g (14). It should be noted that actinomycin D but not irradiation had additive effect with pioglitazone on PPAR activity.

Enhancement of Cytokine Production by Low Dose Irradiation.

The expression of cytokines is regulated by various transcription factors including AP-1, NFkB and PPAR (15-17). To evaluate the effect on cellular functions, thioglycollate-elicited macrophages and normal splenocytes were continuously exposed to radiation for 20 h, and cytokine production was determined by ELISA. Irradiation with 6.12 Gy/h increased the TNF-a production induced by LPS in as much as 8.7 folds (Table 1). Significant increase was also observed at 0.01 Gy/h. Irradiation increased IL-10 production in less degree, and did not increase IL-1a production. It also increased IL-2 production of splenocytes in the presence or the absence of stimulators, phorbol myristate acetate (PMA) and ionomycin (Table 2). Like the effect on TNF-a production, irradiation at 0.01 Gy/h was enough for significant enhancement of IL-2 production. Though IL-4 production was increased in the absence of stimulators, the enhancement was not observed in the presence of stimulators, suggesting that low dose irradiation affects Th1/Th2 conversion in activated T cells.

DISCUSSION

Because radioactive materials in the environment continuously irradiate us with low dose rate, we should construct an appropriate experimental system to evaluate their biological effects. An X-ray irradiator is conventionally used for the studies of low dose radiation, but it is not a proper experimental system to generate the continuous irradiation with low dose rate . As a results, we have poor information for the biological effects of continuous low dose rate-radiation, although effects of high dose rate radiation are extensively studied. To overcome this problem, we utilized 60Co-irradiation room. In this study we evaluated biological effects of continuous low dose-rate-radiation, as an experimental model for irradiation with environmental radioactive substances.

For U2OS cells which has wild type p53 and pRb, irradiation with 6.12 Gy/h for 40 h (total 240 Gy) completely suppressed the growth, and that with 0.08 Gy/h for 40 h (total 4 Gy h) only partly suppressed the growth. Because irradiation of 10 Gy with high dose rate for short time is enough to suppress the growth of the

cells completely, or to induce apoptosis (data not shown), the continuous irradiation seems less toxic, compare to short time-irradiation. Among the proteins known to be affected by the high dose rate radiation, expressions of p53 and p21 were elevated with continuous radiation, whereas that of HSP70 was hardly affected. Transcription activity of p53 was activated while that of AP-1 was suppressed. These results demonstrate that the biological effects of continuous low dose rate irradiation were not always the same as high dose rate irradiation for short time, even if the total radiation dose is the same. Thus, the biological effects of radiation are not simply proportional to the doses, and both irradiation intensity and exposure time should be taken into account.

Although actinomycin D, like radiation, generates DNA break, cell cycle analysis demonstrated that the inhibitory effect was entirely different. Actinomycin D arrested cells in G1 phase and pRb was dephosphorylated, while radiation arrested cells in G2 phase, and pRb was hyperphosphorylated, although both treatments increased the expression of p53. The results suggests the possibility that actinomycin D induced the G1 arrest and p53 accumulation with the mechanism other than DNA break. Alternatively, radiation might arrested the cells with the mechanism other than DNA break, such as generation of reactive oxygens and induction of heat shock proteins, although it is less likely because the expression of superoxide dismutase (data not shown) and HSP-70 were only negligibly affected even with the 6.12 Gy/h which dramatically induced p53. It is also interesting that expression and activity of E2F were elevated both in G1-arrested cells treated with actinomycin D, and in G2-arrested cells irradiated with 6.12 Gy/h for 40 h.

Activation of p53 by radiation is assumed to be resulted from the stabilization of the protein (18). p53 is degraded by ubiquitin/proteasome system. Oncogene, Mdm-2 promotes degradation of p53 as a ubiquitin-ligase for p53, or by regulating intracellular localization of p53. ATM kinase stabilizes p53 by the phosphorylation of serine 15, which prevents the association of Mdm-2 with p53. Consistent with these reports, continuous irradiation induced accumulation and activation of p53. At 0.01 Gy/h, however, irradiation activated p53 without increase of p53. Growth inhibition and accumulation of downstream target of p53 were not detected at this dose-rate. This result suggests that activation of p53 with low dose radiation is not mediated by the stabilization of p53 and that the activation regulates the expression of gene(s) other than regulators of cell cycle and apoptosis such as p21 and Bax. It is possible that p53 regulates cell functions in the non-toxic low dose rate. Activity of p53 is also regulated by modification including acetylation and phosphorylation, or association with p53 itself and other proteins. It is important to know biochemical properties of p53 in the cells irradiated continuously with low dose rate.

Continuous irradiation enhanced cytokine production of macrophages stimulated with LPS. The most profound effect was observed with TNFa production. IL-10 production was slightly enhanced, while IL-1a production was hardly affected. These results are consistent with the previous reports from the other groups, which demonstrated that the enhancement of cytokine production and tumoricidal activity of macrophages by g-irradiation (19-21). Continuous irradiation also enhanced IL-2 production of T cells. The enhancement might be mediated by the activation of macrophages because g-irradiation enhanced the proliferation of T cells stimulated with concanavalin A through the activation of macrophages (22). IL-2 production was selectively enhanced by low dose rate-irradiation with 0.01 Gy/h without affecting IL-4 production, suggesting that low dose rate-radiation converts Th2 phenotype into Th1 phenotype. Because the Th1/Th2 conversion is one of the causes of immunological disorders including AIDS (23), our results suggest that low dose rate-radiation could be used for the treatment of these human diseases.

We also found PPAR was activated by continuous irradiation. PPAR is a family of nuclear receptors regulating lipid and energy metabolism. It is suggested that PPAR-g plays important roles in metabolic diseases such as diabetes, hypertension and atherosclerosis (24-26). Although the activation is also observed with actinomycin D-treatment, irradiation, unlike actinomycin D-treatment, did not have additive effect with pioglitazone, a potent activator of PPAR-g. This result suggests that irradiation but not actinomycin D induced endogenous activators of PPAR-g , such as prostaglandin J2, saturated fatty acids and oxidized LDL (27-30), which are synthesized through the enzyme reactions involving reactive oxygens. g -irradiation might affect the production of endogenous PPAR-g ligands through the generation of reactive oxygens.

In conclusion, our results suggest that continuous irradiation with low dose rate has biological properties distinct from those induced by short time-irradiation with high dose rate. Our results demonstrate that further studies of the biological effects through an appropriate experimental system are necessary in order to evaluate the effect of irradiation from environmental materials. Our results also suggest that continuous irradiation with non-toxic dose rate could be used for the treatment of incurable diseases including immunological disorders, diabetes and hypertension.

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LEGENDS FOR FIGURES AND TABLES

Figure 1. Effect of continuous irradiation on the growth of U2OS cells.
A. U2OS cells (7x105 cells/dish) were cultured under 6.12 Gy/h (open square), 0.08 Gy/h (open triangle) or in the presence of actinomycin D (2 ng/ml, open circle). Cell number was counted at each time point. Untreated control, closed circle. B. U2OS cells (1x106 cells/dish) were cultured under 0.01 Gy/h (open symbols). Viability as judged by trypan blue exclusion test (circles) and cell number (triangles) were counted at each time point. Untreated control, closed symbol.

Figure 2. Effect of continuous irradiation on the cell cycle progression of U2OS cells.
Cells used in Fig.1 were stained with propidium iodide, and their DNA amount was analyzed by flow cytometry as described in Materials and Methods.

Figure 3. Apoptosis induced by irradiation. One million cells were irradiated as indicated in the Figure. Cells were further incubated for 20 h. DNA fragmentation at the termination of the culture was analyzed as described in Materials and Methods.

Figure 4. Effect o continuous irradiation on the protein expression.
Protein expression of the cells used in Fig.1, was analyzed by Western blotting as described in Materials and Methods.

Figure 5. Effect of continuous irradiation on the transcription activity.
CHOC400 cells were transfected with a reporter plasmid for the indicated transcription factors and pCMV-b-gal. Forty hour after the transfection, cells sere harvested and the corresponding enzyme activity in the cytoplasm was determined. Each bar represents stimulation index of enzyme activity normalized with galactosidase activity, compared with the value of untreated culture. Treatment with radiation or with pioglitazone (10 mM) started 20 h after the transfection until the termination (40 h after the transfection) of the culture .

Table 1. Effect of continuous irradiation on cytokine production of macrophages.
Thioglycollate-elicited peritoneal cells were cultured with or without 10 g.ml LPS for 20 h under the indicated radiation dose rate, or in the presence of actinomycin D. Cytokine amount in the supernatant at the termination of the culture was measured with ELISA. Values in the parenthesis, stimulation index.

Table 2. Effect of continuous irradiation on cytokine production of T cells.
Spleen cells were cultured with or without stimulators (PMA 10 ng/ml, ionomycin 1 mg/ml) for 20 h under the indicated radiation dose rate, or in the presence of actinomycin D. Cytokine amount in the supernatant at the termination of the culture was measured with ELISA. Values in the parenthesis, stimulation index.