Increase of Intracellular Glutathione by Low-Dose γ-Ray Irradiation Is Mediated by Transcription Factor AP-1 in RAW 264.7 Cells

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The mechanism of the elevation of intracellular glutathione induced by low-dose γ-rays was examined in RAW 264.7 cells. The expression of mRNA for γ-glutamylcysteine synthetase (γ-GCS) increased soon after γ-ray (0.5 Gy) irradiation, and peaked between 3 h and 6 h post-irradiation. A dose of 0.25 to 0.5 Gy was optimum for induction of γ-GCS mRNA expression at 3 h post-irradiation. The effect of inhibitors of activator protein-1 (AP-1) and nuclear factor κB (NF-κB) on the radiation-induced γ-GCS gene expression was then examined. The induction of γ-GCS mRNA expression was significantly suppressed when AP-1 DNA binding, but not NF-κB DNA binding, was inhibited. Finally, electrophoretic mobility shift assay showed that the low-dose radiation markedly increased the DNA binding of AP-1, but not NF-κB, soon after irradiation. These results suggest that the increase of glutathione levels in RAW 264.7 cells by low-dose γ-ray irradiation is mediated by transcriptional regulation of the γ-GCS gene, predominantly through the AP-1 binding site in its promoter.

Key words low-dose radiation; glutathione; gamma-glutamylcysteine synthetase (GCS) gene; AP-1

Radiation is injurious to living things, and any dose of ionizing radiation has been believed to be detrimental, even extremely low doses. This has been inferred by extrapolation from the known deleterious effects of high doses. It has recently been shown, however, that inhabitants of areas with high background levels of radiation in Guangdong Province, China, have lower cancer mortality than those in control areas.1) Also, epidemiological research on the people who live in the Misasa radon (222Rn) spa area of Japan suggested that they have significantly lower cancer mortality rates than those in the non-spa areas or control areas.2) These interesting phenomena are often called radiation hormesis.3)

Several kinds of effects of low-dose radiation on living organisms have been recognized; these include stimulation of the growth rate,3) tumor progression,5) activation of immune function,6) suppression of reactive oxygen species (ROS)-related diseases,7) resistance to high-dose irradiation,8) and prolongation of life span.9)

However, the mechanisms underlying these responses remain obscure. It has recently been postulated that some of the effects could result from activation of the antioxidant defense system.10,11) Cells have enzymatic and non-enzymatic defense systems against ROS, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST), reduced form of glutathione (GSH), thiolredoxin (TRX), etc. Induction of these antioxidant systems in vitro and in vivo by low-dose ionizing radiation has recently been reported.10—15)

Glutathione is the most abundant cellular thiol-containing peptide, and is present in millimolar concentrations in most prokaryotic and eukaryotic cells. This peptide is an important cellular antioxidant, and has a critical role in maintenance of the balance of cellular reduction and oxidation (redox). It also has many biological functions, being involved in protein and DNA synthesis, amino acid transport, activation of metabolism, catalysis (as a coenzyme), and detoxication of electrophiles either through direct reaction with reactive intermediates or via conjugation reactions catalyzed by GST.14)

GSH is synthesized from its three constituent amino acids, glutamate, cysteine, and glycine by two sequential ATP-dependent reactions catalyzed by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS), respectively; γ-GCS catalyzes the rate-limiting step in glutathione synthesis and is also the site of feedback inhibition by GSH.14,15) In another pathway, the oxidized form of glutathione (GSSG) is catalytically regenerated by glutathione reductase (GR).14)

We previously found that low doses (0.25—0.5 Gy) of radiation elevated the glutathione level in mouse organs, such as liver, pancreas and brain, and that these elevations were accompanied by increased activities of γ-GCS and GR.16,17) More recently, it has been revealed that glutathione induced by low-dose irradiation is involved in the enhancement of immune function.6) However, the mechanisms of the elevation of glutathione biosynthesis-related protein mRNAs induced by low-dose radiation exposure have yet not been fully elucidated.

The central issue concerning the signal transduction pathways activated by changes of intracellular redox status is the transcription factors. It is well known that activator protein-1 (AP-1) and nuclear factor κB (NF-κB) rapidly respond to a wide range of agents, factors, and chemicals that alter cellular redox status, and that both factors regulate the expression of a variety of genes.18—22) Furthermore, the γ-GCS gene has binding motifs for both AP-1 and NF-κB in the 5′ regulatory region.23,24) The induction of γ-GCS mRNA by several stimuli that cause redox disorder was found to be regulated by stimulus-dependent transcription factors.25—29) It has recently been reported that γ-GCS mRNA expression was mediated by NF-κB in T98G cells exposed to high doses of γ-rays.30) However, there have been no studies on this gene expression in low-dose γ-rays. We examined here the association between the elevation of γ-GCS gene expression in RAW 264.7 cells exposed to low-dose γ-rays and the activity of the transcription factors AP-1 and NF-κB.

MATERIALS AND METHODS

Materials Curcumin (CURCM) and N-α-tosyl-L-phenyl-

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at a density of 0.5—1.0
extracts were isolated in the following manner: the cells
nary experiment.
incubation period were determined on the basis of a prelimi-
solved in ethanol. The concentration of each inhibitor and the
buffer (pH 7.5) composed of 10 mM Tris–HCl, 130 mM NaCl,
GCCATGAGGCCACCAC-3
mouse liver total RNA, using oligo DNA primers for
verse transcription polymerase chain reaction (RT–PCR) from
products were subcloned into the pGEM-T vector (Promega
formation with \[
\text{a -32P}\] dCTP (111 TBq/mmol, NENTM Life Sci-
 Tata Diagnostic Co., Ltd.). This labeling reaction was termi-
label binding activity of each nuclear extract
were loaded on a 4% poly-acrylamide gel and subjected to
the competitive binding study, an excess (100-fold) of non-
the pGEM-T vector (Promega
were amplified (35 cycles, 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min) and PCR
and immediately incubated at 37 °C for the appropriate pe-
were seeded in 100 mm dishes at a density of 0.5—1.0×10^6 cells/ml and incubated for 18 h. After a 30 min pre-incubation in fresh medium containing 10% FBS under conventional conditions, cells were irradiated over the dose range from 0.1 Gy to 2.0 Gy at a dose rate of 1.02 Gy/min using a 131Cs γ-ray source (GAMMACELL 40, Nordin International Inc., Canada) at room temperature, and immediately incubated at 37 °C for the appropriate period.

Northern Blot Hybridization Analysis Total RNA was isolated from cells using TRIZOL reagent (GIBCO BRL, U.S.A.). Mouse γ-GCS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were synthesized by reverse transcription polymerase chain reaction (RT-PCR) from mouse liver total RNA, using oligo DNA primers for γ-GCS heavy subunit (5'-CACATCTACCAGCGATCA-3' and 5'-TTCCGTTCTTTCAAATCTCGA-3') and GAPDH (5'-TGAAGTCCGAGTTAAGGATTTGGC-3' and 5'-CATGTAGGACATGAGGCCACAC-3'). cDNAs were amplified (35 cycles, 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min) and PCR
were subcloned into the pGEM-T vector (Promega Co., U.S.A.) for amplification. cDNA probe labeling was performed with [α-32P]dCTP (111 TBq/mmol, NEN™ Life Science Products, Inc., U.S.A.), using a Random Primed DNA Labeling Kit (Roche Diagnostics Co., Germany). To remove unincorporated nucleotides from the labeling reaction mixture, the labeled probes were purified with a Sephadex G-50 Micro Column (Pharmacia Biotech, Co., Sweden).

Quantitation was done with a Bio-imaging Analyzer System (Fujix BAS 2000, Fuji Photo Film Co., Ltd., Japan), using an imaging plate (BAS-IP MS 2040, Fuji Photo Film Co., Ltd.). The relative amounts of both RNAs were judged by re-hybridization with a housekeeping gene (GAPDH) cDNA probe.

Treatment with Transcription Factor Inhibitors RAW 264.7 cells were incubated with AP-1 and NF-κB inhibitors for 30 min under conventional conditions, after pre-incuba-
tion in fresh medium containing 10% FBS for 30 min. Then, the cells were exposed to 0.5 Gy of γ-rays, as described above, and incubated for another 3 h at 37 °C. The inhibitors used here were an inhibitor of AP-1, CURCM (1 μM), and an inhibitor of NF-κB, TPCK (1 μM), both of which were dissolved in ethanol. The concentration of each inhibitor and the incubation period were determined on the basis of a prelimi-
nary experiment.

Electrophoresis Mobility Shift Assay (EMSA) Nuclear extracts were isolated in the following manner: the cells (1.0×10^7 cells) were washed twice with ice-cold washing buffer (pH 7.5) composed of 10 mM Tris–HCl, 130 mM NaCl, 5 mM KCl, and 8 mM MgCl_2, and then harvested in Epplen-dorf tubes. They were then homogenized with hypotonic buffer (pH 7.9) composed of 20 mM HEPES-KOH, 5 mM KCl, 0.5 mM MgCl_2, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonylfluoride (PMSF), and 0.05% Nonidet P-40 (Nakalai Tesque, Inc., Japan), and centrifuged at 5000×g for 3 min. The supernatant was removed, and the precipitated nuclear fraction was suspended in extraction buffer (pH 7.9) composed of 20 mM HEPES–KOH, 0.2 mM EDTA–NaOH, 500 mM NaCl, 1.5 mM MgCl_2, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, and 0.5 μg/ml peptatin A (Sigma Chemical Co., U.S.A.), and stirred for 30 min at 4 °C. The suspension was centrifuged at 15000×g for 15 min. The supernatant was divided into small volumes and stored at −80 °C as the nuclear extract, after dialysis against binding buffer (pH 7.9) composed of 20 mM HEPES–KOH, 0.5 mM EDTA–NaOH, 50 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 10% glycerol, for 24 h. Aliquots of the extracts were removed for protein assay using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories Co., U.S.A.).

Oligonucleotides containing the consensus sequence for AP-1 (5'-CGCTTGTGAGTGCACCCGGA3'-3') and NF-κB (5'-AGTGGAGGATCTCCCGG3'-3') were labeled with [γ-32P]ATP (111 TBq/mmol, NEN™ Life Science Products, Inc., U.S.A.), using the T4 polynucleotide kinase (Takara Shuzo Co., Ltd., Japan). This labeling reaction was terminated with 50 mM EDTA. The 32P-labeled oligonucleotides were purified using a Sephadex G-50 micro column.

The binding reaction proceeded in a 22 μl reaction mixture containing 12 μg of nuclear extract, 2 μg of poly(dI–dC) as a non-specific competitor DNA, and 32P-labeled oligonucleotides including either AP-1 or NF-κB specific binding domain (2.0×10^5 cpm), at room temperature for 30 min. For the competitive binding study, an excess (100-fold) of non-labeled consensus oligonucleotide was added. The samples were loaded on a 4% poly-acrylamide gel and subjected to electrophoresis in TAE buffer (pH 7.5) composed of 6.7 mM Tris–HCl, 3.3 mM NaOAc, and 1 mM EDTA, at 4 °C for 100 min. The DNA-binding activity of each nuclear extract was represented as the amount of 32P-labeled DNA-transcription factor complex measured with the bio-imaging analyzer system, using an imaging plate.

Statistical Analysis Results were expressed as the mean±S.D. Differences between values were compared using the Student’s t-test.

RESULTS

Changes in Expression Levels of γ-GCS mRNA Induced by Low-Dose γ-Ray Irradiation Changes in the expression of mRNA for γ-GCS, the rate-limiting enzyme of de novo GSH synthesis, were examined by Northern blot hybridization analysis. The time-dependent change in γ-GCS mRNA expression was first examined. As shown in Fig. 1, the γ-GCS mRNA expression was elevated soon after 0.5 Gy γ-ray irradiation, peaked between 3 h and 6 h post-irradiation, and thereafter decreased. Next, the dose-dependent induction of γ-GCS mRNA expression was tested at 3 h post-irradiation. As shown in Fig. 2, the mRNA expression markedly increased at the dose of 0.25 Gy to 0.5 Gy of γ-rays.
Effects of Transcription Factor Inhibitors for AP-1 and NF-κB on the Elevated γ-GCS mRNA Expression Induced by Low-Dose γ-rays

Involvement of the transcription factors, AP-1 and NF-κB, in the elevation of γ-GCS mRNA expression induced by low-dose γ-ray irradiation, was examined based on the effects of transcription factor inhibitors at the fixed dose and time of 0.5 Gy and 3 h, respectively. Inhibitors used here were CURCM and TPCK, inhibitors of AP-1 and NF-κB, respectively.31,32) As shown in Fig. 3, CURCM markedly blocked the elevation of γ-GCS mRNA expression induced by 0.5 Gy of γ-rays, while TPCK was less effective. These results suggest the involvement of AP-1 activation in the elevation of the γ-GCS mRNA expression induced by low-dose γ-rays.

Effect of Low-Dose γ-rays on DNA-binding Activities of AP-1 and NF-κB

To test whether activation of AP-1 and NF-κB mediates the induction of γ-GCS mRNA in RAW 264.7 cells exposed to low-dose γ-rays, changes in DNA-binding activity of both factors were assessed by EMSA. As shown in Fig. 4, the DNA-binding activity of AP-1 increased within a short period after exposure to 0.5 Gy of γ-rays, reached a maximum at 2 h, and returned almost to the...
control level at 6 h. The unlabeled oligonucleotide completely blocked this binding reaction. The γ-ray dose-dependency of AP-1 DNA-binding activity was then investigated at the constant time point of 2 h after irradiation. A dose of 0.5 Gy most strongly enhanced the binding activity, while doses of 0.25 Gy and 1.0 Gy only slightly affected the activity (Fig. 5).

In a similar manner, the binding activity of NF-κB with DNA was examined. No significant time- or dose-dependent changes in the activity were observed (data not shown).

Finally, determination of the effect of CURCM on the activation of AP-1 binding with DNA induced by 0.5 Gy of γ-rays was made. As shown in Fig. 6, CURCM suppressed the DNA-binding activity of AP-1, but TPCK did not, suggesting that low dose γ-ray irradiation-induced γ-GCS mRNA expression might be mediated by AP-1.

DISCUSSION

We previously showed that, in contrast to high doses, low doses (0.25–0.5 Gy) of radiation can increase the cellular GSH level in mice in vivo. The ability to induce cellular defense mechanisms in response to environmental changes is a fundamental characteristic of eukaryotic and prokaryotic cells. Increased intracellular glutathione participates in the protective mechanisms against radiation, one effect of which is to increase oxidative stress. Cellular levels of GSH are influenced by multiple factors, including the activities of enzymes in the γ-glutamyl cycle, the availability of precursors such as cysteine, and the rate of consumption or efflux of GSH. In this case, the elevation of intracellular GSH seems to be mediated by signal transduction pathways which participate in the regulation of glutathione biosynthesis-related molecules.

GSH biosynthesis from its constituent amino acids involves two ATP-requiring enzymes, γ-GCS and GS. γ-GCS catalyzes the rate-limiting step of GSH biosynthesis. The induction of γ-GCS gene expression and activity by various oxidative stimuli, such as hydrogen peroxide (H₂O₂), menadione, okadaic acid, a tumor necrosis factor-α, β-naphthoflavone, and heat shock has been well documented. In this regard, regulation of γ-GCS gene expression has been a topic of extensive research. Thus, in order to determine whether the radiation-induced elevation of GSH level is a post-transcriptional event, the induction of γ-GCS mRNA by low-dose radiation was first examined in RAW 264.7 cells, which showed a high glutathione productivity in response to low-dose radiation in our preliminary experiments, by analysis of γ-GCS mRNA expression. The γ-GCS mRNA expression was increased soon after γ-ray irradiation, and peaked between 3 h and 6 h post-irradiation. A dose of 0.25 and 0.5 Gy was most effective. The induction of transcription observed after exposure to low dose ionizing radiation has generally been though to be part of a complex response by cells which acts to protect them from additional oxidative and other injuries. In this regard, doses between 0.25 Gy and 0.5 Gy of γ-rays may provide a maximal signal to induce γ-GCS mRNA expression. From another standpoint, mechanistically different signaling pathways may be involved in the different dose ranges. Detailed exploration of the dose-dependent effect of low doses of γ-rays on γ-GCS mRNA expression is now under way.

Radiation might modulate the redox-signaling pathway through perturbation of the intracellular redox state, based on the finding that induction of γ-GCS gene by low-dose irradiation in RAW 264.7 cells is post-transcriptional. The redox pathway may undergo cross-talk with other pathways, and may be involved in various biological phenomena. The inducible transcription factors AP-1 and NF-κB are activated in response to a wide variety of exogenous and endogenous agents, including oxidative stress in various tissues.

The promoter (5'-flanking) region of the γ-GCS gene contains a consensus AP-1 and NF-κB binding site. Though several oxidative stimuli induce γ-GCS gene expression through the regulation of both transcription factors, there has been no information available on characteristics of the transcriptional regulation in response to low-dose γ-ray irradiation. To elucidate the involvement of transcription factors in the signaling pathways for intracellular glutathione induction caused by low-dose γ-rays, the effect of inhibitors of both factors on the radiation-induced γ-GCS gene expression was examined in RAW 264.7 cells. CURCM and TPCK are inhibitors of AP-1 and NF-κB, respectively. CURCM in-
hibits both AP-1 and NF-xB at high concentrations, but inhibits only AP-1 at low concentrations (ca. 10 μM). In this experiment, the induction of γ-GCS mRNA expression was significantly blocked only when AP-1 DNA binding was inhibited at a low concentration of CURC (1 μM). In EMSA, low-dose radiation activated the AP-1 DNA binding soon after irradiation, but not NF-xB DNA binding. It is known that γ-GCS mRNA is induced through the mediation of NF-xB at a high dose (30 Gy) of γ-rays. However, the present report is the first to show that AP-1 mediates the elevation of γ-GCS gene expression by low-dose radiation. Cells may recognize different doses of radiation as distinct stimuli, resulting in selective cellular responses mediated by different pathways. Induction of intracellular glutathione in response to low-dose γ-ray irradiation is considered an adaptive response.

The next problem is the mechanism of the elevation of AP-1 DNA-binding activity induced by the radiation. One possible mechanism for this is the induction of c-fos and c-jun gene expression. AP-1 consists of heterodimers of members of the Fos and Jun family. The proto-oncogenes c-fos and c-jun that encode DNA binding or interacting proteins, known as regulators of transcription, are highly induced by low-dose radiation exposure. Prasad et al. found that low-dose radiation (0.25—0.5 Gy) exposure induced mRNAs for c-fos and c-jun at between 1 h and 4 h post-irradiation in 244B cells. This is in good agreement with our finding that the elevation of AP-1 DNA-binding activity was observed at 2 h post-irradiation with 0.5 Gy of γ-rays. The induction of c-fos and c-jun genes is related to phosphorylation of epidermal growth factor receptor (EGFR), leading to activation of the mitogen-activated protein kinase (MAPK) pathway. EGFR activation is also one of the early responses in the cells, and low-dose radiation-induced tyrosine phosphorylation of EGFR has been reported. A study to examine the signaling pathway related to AP-1 activation induced by a low-dose of γ-ray irradiation is still required.

In conclusion, low-dose γ-ray radiation increases intracellular GSH levels through transcriptional regulation of the γ-GCS gene, probably at the AP-1 binding site in its promoter.

REFERENCES