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Regulation of γ-Glutamylcysteine Synthetase Expression in Response to Oxidative Stress

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Glutathione (GSH) is synthesized by the activity of two ATP-requiring GSH synthesizing enzymes. Gamma-glutamylcysteine synthetase (γ-GCS) is the rate limiting enzyme for the GSH synthesis. γ-GCS is a heterodimer of heavy, catalytic subunit and light, regulatory subunit and responsive to many stresses, such as heat shock, oxidative stress or cytokines. To know the regulation of the expression of γ-GCS gene, in the present study, we show evidences that γ-GCS heavy subunit is upregulated by oxidative stress by ionizing radiation and TNF-α mediated by nuclear factor-κB (NF-κB), and impairment of the expression of γ-GCS by TNF-α in diabetic condition. Furthermore we describe the importance of GSH in the regulation of NF-κB subunits.

Keywords: NF-κB, ionizing radiation, γ-glutamylcysteine synthetase, TNF-α, diabetes mellitus

INTRODUCTION

Glutathione (γ-glutamylcysteinylglycine, GSH), a tripeptide found one hundred years ago, participates in many biological processes, such as in the cellular defense system against oxidative stress, by reducing the disulfide linkage of proteins and other cellular molecules, or by scavenging free radicals and other reactive oxygen species (ROS).[1] GSH and its related enzymes function as ROS scavengers, and the physiological role of GSH as an antioxidant has been described in numerous disorders reflecting increased oxidation as a result of an abnormal GSH metabolism. Another important function of GSH is to maintain the redox potential within cells.

GSH is synthesized in most mammalian cells by the activity of two ATP-requiring GSH-synthesizing enzymes, γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase.[2] γ-GCS catalyzes the rate-limiting step of GSH synthesis. γ-GCS is a heterodimer of heavy (h) and light (l) subunit. γ-GCSh possesses a catalytic site and γ-GCSl regulates the kinetic properties. This enzyme has been purified and the structure of the cDNA and the promoter from rat and human

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has been determined.[3–5] GSH synthesis is also thought to be an important factor in cellular defense against stresses such as radiation and drug treatment. We provided evidence that the concentration of GSH and the activity of γ-GCS affect oxidative stress in endothelial cells and that the expression of γ-GCS mRNA is sensitive to stresses.[5] In the present report, data are presented indicating that induction of γ-GCS is regulated by NF-κB.

INDUCTION OF γ-GCS BY IONIZING RADIATION IS MEDIATED BY NF-κB

Glioblastoma is one of the most malignant forms of neoplasm, and often shows resistance to chemotherapy and radiation therapy.[6] This resistance may be due to several direct factors, such as a low intrinsic radiation sensitivity, a high recovery capacity, an increased number of clonogens and a high hypoxic fraction.[6,7]

Irradiation produces physical and chemical damage in tissues that may lead to cell death. Irradiation increases the formation of superoxide radicals ($O_2^{\bullet -}$) in the presence of oxygen.[7] These radicals react with DNA to break its strands, lipids to cause lipid peroxidation, or proteins to modify their structure and function. In response to irradiation-induced stresses, cells induce the synthesis or activation of proteins with protective capacities.[8]

It has been reported that ionizing radiation induces the expression of particular genes, such as TNF-α, *c-fos/c-jun*, *c-myc*, gadd 45, and platelet-derived growth factor. Furthermore, ionizing radiation activates transcription factors, such as nuclear factor kappa B (NF-κB).[9] Although the precise mechanisms responsible for the specific gene expression are still nuclear, transcriptional modulation is known to play a major role in the repair of DNA damage, proliferation and other cellular functions.[10] NF-κB is part of a family of dimeric transcriptional factors and through its dissociation from its inhibitor, IκB, transcriptionally activates various cellular genes involved in immune response, inflammation, oxidative stress, and embryonic development. NF-κB is characterized as a heterodimer with two subunits, p50 and p65.[10]

Characterization of antioxidants in cells The data shown in Table I indicate that the level of GSH was 14-times higher in T98G cells than NB9 cells, the radiation sensitive cells. The activity of γ-GCS was correlated to the levels of GSH. When cells were treated with various doses of radiation, it was found that radiation from 10 to 50 Gy did not have a cytotoxic effect on T98G cells.

Induction of GSH by Radiation The time course of the effect of radiation on the levels of GSH in T98G cells was studied. T98G cells were treated with 30 Gy of radiation and changes in levels of GSH were estimated for 46 h. The data in Figure 1 show that radiation increased the levels of GSH with a peak at 6 h after the radiation followed by a decline to the basal levels within 48 h.

Figure 2 shows change in the levels of GSH and the activity of γ-GCS by ionizing radiation in T98G cells. Levels of GSH, and the activity of γ-GCS were estimated after 6 h of radiation at various doses. The levels of GSH increased dependent on the radiation dose from 10 Gy (1.1-fold) to 30 Gy (2-fold). The activity of γ-GCS increased in a dose-dependent manner from 10 Gy (1.1-fold) to 30 Gy (1.9-fold).

Expression of γ-GCS To further study the mechanisms by which activities of antioxidants are stimulated by radiation, expression of γ-GCS mRNA was estimated after 6 h of radiation at

TABLE I Levels of GSH

Cells	GSH (nmol/10^6 cells)	γ-GCS (milliunits/10^6 cells)
T98G	85.3 ± 3.1	25.3 ± 3.2
NB9	5.97 ± 0.25	2.2 ± 0.35

Data are mean ± SD of four independent analyses.

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10 and 30 Gy. The data in Figure 3 show that concomitant stimulation of the expression of γ-GCS mRNA was observed in T98G cells at 30 Gy of radiation. The increase in the levels of γ-GCS mRNA induced by 30 Gy of radiation was 2.7-fold the control.

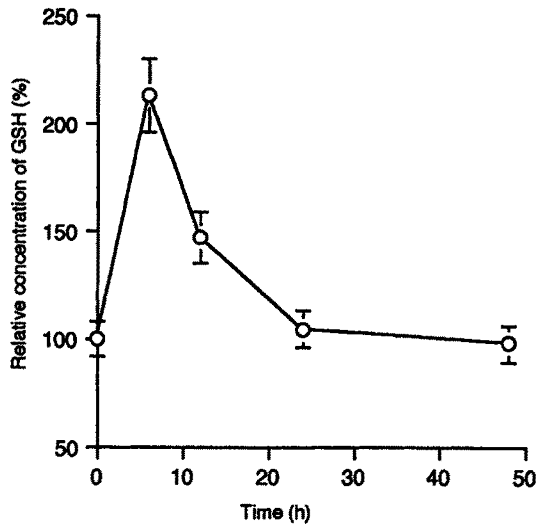

FIGURE 1 Time course study on the effect of radiation on the concentration of GSH. T98G cells were irradiated at 30 Gy and changes in the intracellular concentrations of GSH were estimated for 48 h. Values are the mean ± SD of four experiments.

Effect of buthionine sulfoximine Next, we studied the effect of buthionine sulfoximine (BSO), a specific inhibitor of γ-GCS, on the expression of γ-GCS mRNA. Since there is a NF-κB binding site on the regulatory region of γ-GCS gene, expression of γ-GCS was estimated at 3–40 Gy of radiation with or without pre-treatment with BSO. As shown in Figure 4, in control T98G cells, the induction of γ-GCS mRNA was observed at 20 Gy with a peak at 30–40 Gy. Treatment of T98G cells with 0.1 mM BSO sensitized the induction of γ-GCS mRNA caused by radiation. The induction of γ-GCS was observed in BSO-treated cells at a peak of 3 Gy of radiation.

Luciferase activity of γ-GCS promoter To elucidate whether the activation of NF-κB–DNA binding activity relates to the induction of γ-GCS and the increase in the levels of GSH, the luciferase activity of the γ-GCS promoter was estimated. We constructed chimeric genes containing various regions of the γ-GCS gene promoter and the coding region for luciferase.

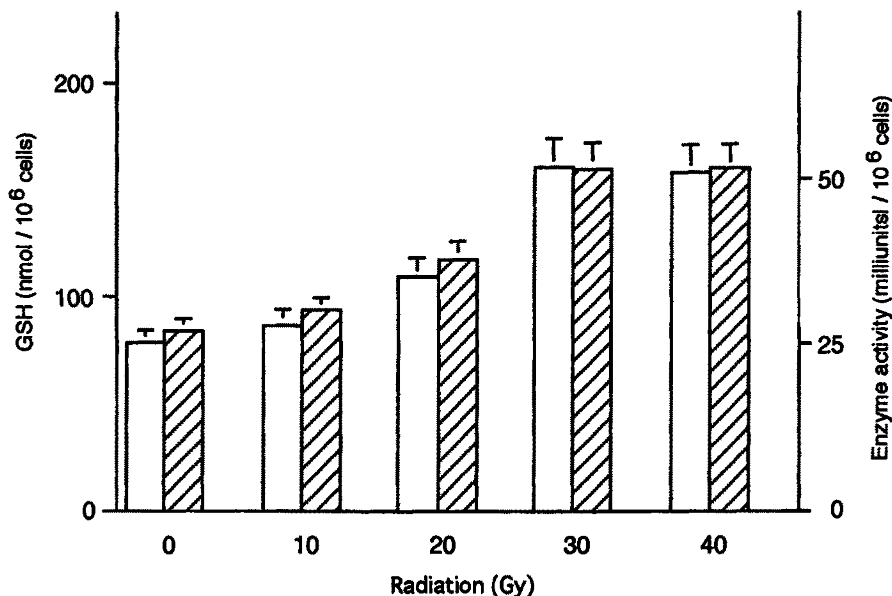

FIGURE 2 Changes in the concentration of GSH and the activity of γ-GCS induced by radiation in T98G cells. T98G cells were treated with radiation (10–40 Gy), and the effect of irradiation on the concentration of GSH (□), and the activities of γ-GCS (▨) were estimated after 6 h of radiation. Values are the mean ± SD of four experiments.

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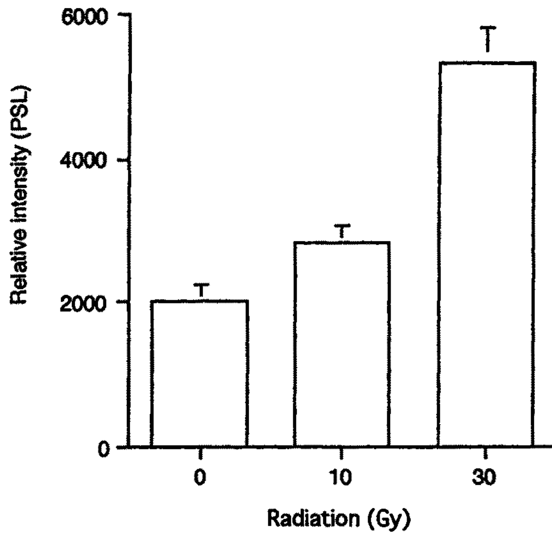

FIGURE 3 The expression of γ-GCS in T98G cells. Induction of γ-GCS mRNA by radiation in T98G cells was estimated by Northern blots. T98G cells were irradiated at 10 and 30 Gy, and after 6 h, the expression of mRNA were estimated. Northern blot analysis of γ-GCS. Lane 1, 0 Gy; lane 2, 10 Gy; and lane 3, 30 Gy. Values are expressed as relative intensity (PSL). The data are the mean ± SD of three independent analyses.

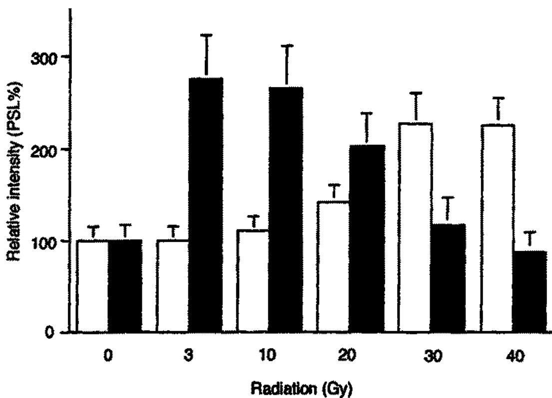

FIGURE 4 Effect of buthionine sulfoximine on the radiation-induced expression of γ-GCS mRNA. Effect of BSO on the expression of γ-GCS mRNA was studied by Northern blots. T98G cells were radiated at various doses and 6 h after the radiation, the expression of γ-GCS mRNA was estimated. (■), T98G cells pre-treated with 0.1 mM BSO for 24 h. (□) cells without treatment with BSO. The data are the mean ± SD of three independent analyses expressed as relative intensity (PSL%).

T98G cells transiently transfected with a plasmid containing the γ-GCS promoter-luciferase construct showed increased luciferase activity when treated with ionizing radiation. Figure 5 shows the results of the luciferase assay. Incubation of T98G cells with 30 Gy of ionizing radiation increased the luciferase activity of the γ-GCS promoter with −3848 base pairs including AP-1 like element, TRE and NF-κB sites (first lane). Deletion of AP-1 like element and TRE site resulted in no apparent change in the stimulation of luciferase activity (2nd and 3rd lanes). Deletion of NF-κB site to −1099 base pairs lost the stimulation of luciferase activity (4th lane). Site directed mutagenesis of NF-κB site lost the stimulation of luciferase activity (6th lane). Another oxidant response element, AP-1, had no apparent stimulatory effect in radiation (5th lane). This strongly suggests that the luciferase activity stimulated by ionizing radiation was in the γ-GCS promoter containing the NF-κB binding site and the induction of γ-GCS mRNA by ionizing radiation is mediated by NF-κB.

GLUTATHIONE DOWNREGULATES THE AUTOLOOP REGULATION OF NF-κB BY TNF-α

NF-κB is part of a family of dimeric transcriptional factors. NF-κB is present in the cytosol of unstimulated cells in a complex with its inhibitor (IκB).[11]

NF-κB is characterized as a heterodimer with two subunits, p50 and p65.[10] Cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) activates NF-κB. Through dissociation of IκB, NF-κB subunits transcriptionally activate various genes. In the process of the dissociation of IκB, phosphorylation, ubiquitination, and proteolysis are involved. Phosphorylation of IκB-α by IκB kinase has been shown to dissociate NF-κB–IκB-α complexes.[12] Phosphorylations of IκB-α on two serine/threonine residues are necessary for the rapid degradation by the proteasome, although the signaling pathway leading to activation of NF-κB is not fully understood.

It has been clarified that IκB mRNA is upregulated by TNF-α caused by the presence of NF-κB binding sites on the promoter region of the IκB

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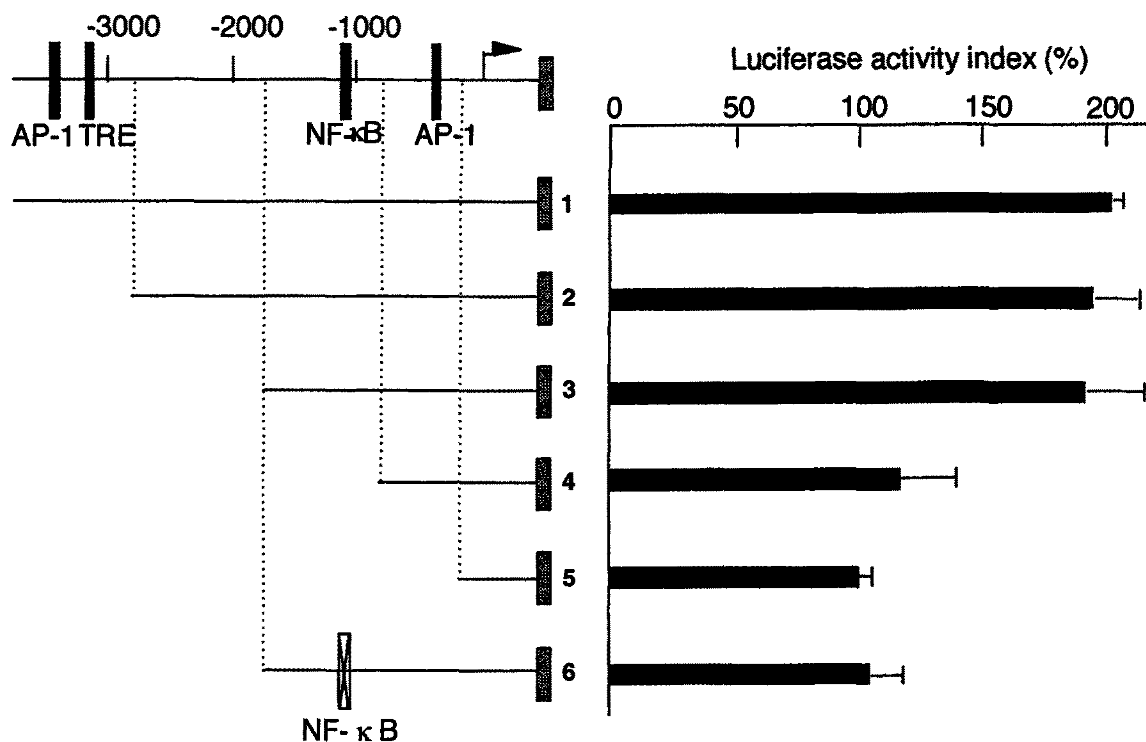

FIGURE 5 Luciferase activity of γ-GCS promoter. T98G cells were transfected with pGL-3 luciferase expressing vector containing various regions of the 5′-flanking sequence of γ-GCS heavy subunit gene and cotransfected with β-galactosidase. Twenty four hour after transfection, one-half of the transfectants were treated with 30 Gy of irradiation. After 8 h, the cells were lysed and assayed for luciferase activity and β-galactosidase. The luciferase activity of each individual transfectant was subsequently normalized by to β-galactosidase activity. The luciferase activity stimulated by radiation was divided by that of control cells to give the induction rations illustrated in the upper left. Numbers indicate distance in base pairs from the start of transcription. Data are the mean ± SD of three independent analyses.

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gene.[13] The questions to be clarified are whether or not TNF-α regulates the expression of NF-κB subunits and if so, whether or not GSH participates in the regulation of the expression of NF-κB subunits. In this study, we examined the effect of GSH on the phosphorylation of IκB-α and expression of p65/p50, and IκB-α as well as the GSH synthesis by TNF-α.

GSH Inhibits the TNF-α-induced phosphorylation of IκB-α We studied the effect of GSH on the serine-phosphorylation of IκB-α by TNF-α. Treatment of MHE cells with 100 units/ml of TNF-α for 15 min resulted in stimulation of the phosphorylation rates of IκB-α (Figure 6, lanes 1 and 2). No stimulation of the phosphorylation rates of IκB-α was observed when the concentration of GSH increased to 2-fold (22.5 ± 1.1 versus 11.7 ± 1.3 nmol/10^6 cells) by the previous treatment of MHE cells with 10 mM GSH ester for 24 h (lanes 3 and 4).

GSH downregulates the expression of p65/p50, and IκB-α by TNF-α We studied the effect of TNF-α on the expression of p65/p50 and IκB-α on Northern blots. Figure 7 shows the results of the induction of p65/p50 and IκB-α mRNA by TNF-α. The TNF-α induced p65/p50 and IκB-α mRNA with a peak at the 6th hour.

Next, this effect of the intracellular concentrations of GSH on the expression of p65/p50 and IκB-α by TNF-α was studied. Previous treatment of MHE cells with 10 mM GSH ester for 24 h increased the concentration of GSH by 2 times and

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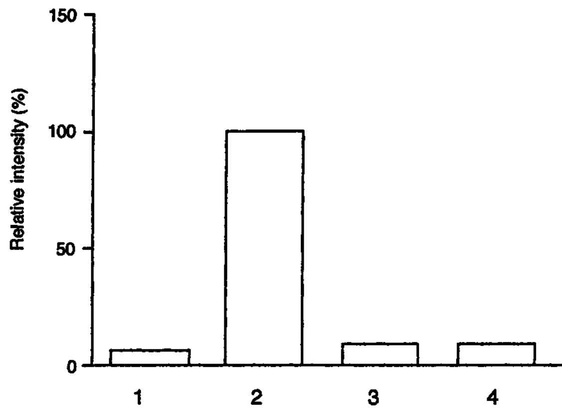

FIGURE 6 Serine-phosphorylation of IκB-α. The effect of GSH on the serine-phosphorylation of IκB-α was studied. Cells were treated with 100 units/ml of TNF-α for 15 min. Lysates (100 μg of total protein) were immunoprecipitated with anti-IκB-α antibody and then resolved by electrophoresis on 10% SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. Serine-phosphorylated proteins were detected using anti-phosphotyrosine antibody. Lane 1, control; lane 2 and 4, +100 units/ml of TNF-α; lanes 3 and 4, MHE cells previously incubated with 10 mM GSH ester.

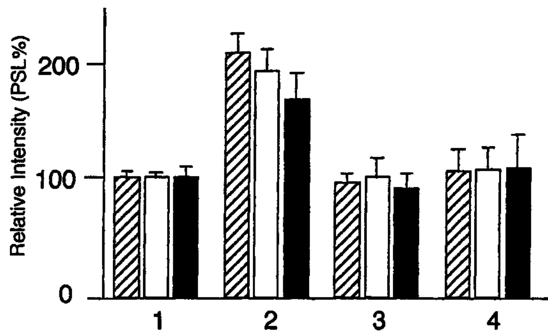

FIGURE 7 Northern blots of p65/p50 and IκB-α. After incubating MHE cells with or without TNF-α for 6 h, about 30 μg of each total RNA extracted from the cells was fractionated by electrophoresis through 1% agarose gel, transferred to nylon membranes and hybridized with ^{32}P-labeled nick-translated DNAs. Lanes 1 and 2, control MHE cells; lanes 3 and 4, MHE cells previously incubated with 10 mM GSH ester; lanes 2 and 4, +100 units/ml of TNF-α. The relative amounts of radioactivity are expressed as percentage of PSL. Dashed bars correspond to the expression of P65 mRNA, open bars that of p65 mRNA and closed bars that of IκB-α mRNA. Each numbered bar corresponds to the lane. Values are means ± SD of three independent analyses.

downregulated the TNF-α-induced expression of p65/p50 and IκB-α (Figure 8, lanes 3 and 4). Their *denovo* synthesis may not be for NF-κB to rapidly respond to extracellular stimuli.

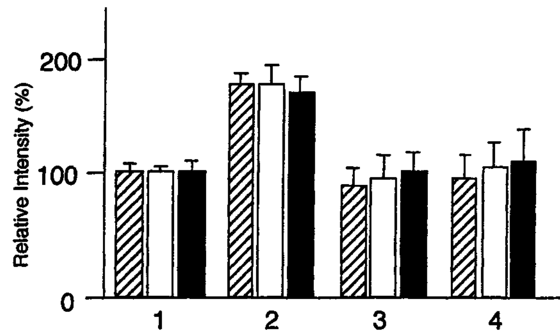

FIGURE 8 Western blots of p65/p50 and IκB-α. After incubating MHE cells with or without TNF-α for 12 h, about 50 μg of each total protein extracted from the cells was immunologically stained using rabbit anti-p65/p50 and IκB-α polyclonal IgG. Lanes 1 and 2, control MHE cells; lanes 3 and 4, MHE cells previously incubated with 10 μM GSH ester; lanes 2 and 4, +100 units/ml of TNF-α. The relative amounts of the immunoactivity are expressed as percentage of the control. Dashed bars indicate the expression of p65, open bars that of p50 and closed bars that of IκB-α. Values are mean ± SD of three independent analyses.

INDUCTION OF γ-GCS BY TNF-α MEDIATED BY NF-κB IS IMPAIRED IN HYPERGLYCAEMIC CONDITIONS

Endothelial cells play important roles in selective transport, anticoagulation, lipid metabolism, vascular tension, vascularization, and immunological regulation. The secretion of some cytokines or growth factors from endothelial cells and their binding to specific receptors mediates these functions.[14] In diabetes mellitus, endothelial cell damage is believed to be a significant contributing factor in the development of medical complications. Oxidative stress is a factor involved in cellular injury. Increases in the production of oxygen radical species or decreases in the scavenging activity against oxidative stress may play crucial roles in the development of pathological conditions.[15]

Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are cytokines initially produced by monocyte-macrophages in response to endotoxin and various mitogens.[16] These cytokines mediate multiple immunological and inflammatory events, such as increases in endothelial cell surface procoagulant and plasminogen activator

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TABLE II Concentration of glutathione and the activities of its related enzymes

Condition	GSH (nmol/ 10^6 cells)	γ-GCS (milliunits/ 10^6 cells)	Cu,Zn-SOD (milliunits/ 10^6 cells)
5.5 mM glucose	11.7 ± 1.3	2.53 ± 0.35	11.7 ± 0.8
28 mM glucose	6.5 ± 0.7[a]	1.28 ± 0.51[a]	8.1 ± 0.7[a]

Values are means ± SD of four experiments.
[a]$p < 0.01$ versus 5.5 mM glucose.

inhibitor, or vasodilatory and angiogenic effects.[17] The effect of IL-1β is potentiated by TNF-α, which activates NF-κB formation. To understand the regulatory mechanisms for GSH synthesis under diabetic conditions, we studied changes in the responsiveness of γ-GCS to TNF-α in mouse endothelial (MHE) cells cultured with a high concentration of glucose. We also investigated how the activations of NF-κB and γ-GCS expression by TNF-α are linked.

Concentration of glutathione and the activity of its related enzymes Incubation of MHE cells with increasing concentrations of glucose resulted in a corresponding decrease in the concentration of GSH after 7 days. The concentration of GSH in 11, 22, and 28 mM glucose (Table II) was 78%, 59%, and 56% of that with 5.5 mM glucose, respectively. As shown in Table I the activity of γ-GCS in cells incubated with 28 mM glucose was 51% of the activity in cells incubated with 5.5 mM glucose.

Effects of cytokines on the concentration of GSH Incubating MHE cells with TNF-α resulted in a marked increase in the concentration of GSH which reached a maximum after 3 h of 547% and 474%, respectively, followed by a gradual decline (Figure 9). The following experiments to determine the effects of the cytokines were performed using a 3-h incubation. No stimulatory effect of TNF-α on the concentration of GSH was observed after the cells had been incubated with 28 mM glucose for 7 days (0.9 ± 1.9 nmol/10^6 cells with TNF-α) (Figure 10A). The impaired responses to cytokines were dependent on the concentration

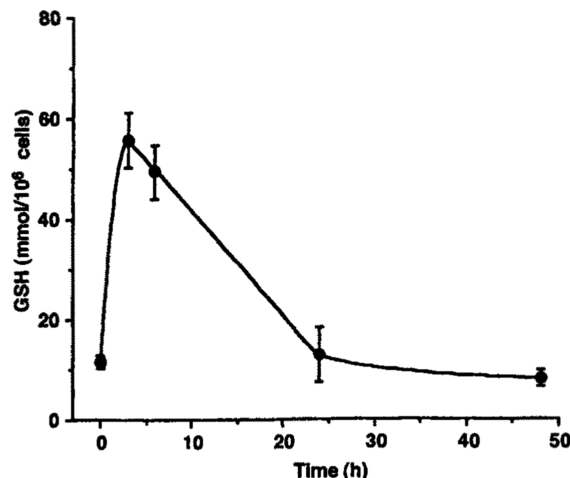

FIGURE 9 Time course study on the effect of a cytokine on the concentration of GSH. MHE cells were incubated with 100 units/ml of TNF-α, and changes in intracellular GSH were estimated. Values are mean ± SD of four experiments.

of glucose, increases in the concentration of GSH in response to TNF-α were about 300% with 11 mM glucose, 147% with 22 mM glucose, and 138% with 28 mM glucose with respect to the control. There was no shift of the peak with these high concentrations of glucose (data not shown).

TNF-α also had stimulatory effects on the level of activity of γ-GCS in cells grown in 5.5 mM glucose (172% and 192% increase compared with the control, respectively) but no effect on the level of activity of γ-GCS in MHE cells incubated with 28 mM glucose for 7 days (Figure 10B). A TNF-α receptor binding assay showed no apparent change in the characteristics of the receptor between the two cell groups (data not shown).

Activity of NF-κB The effect of TNF-α on the expression or NF-κB was estimated using a gel-shift assay. The concentration of NF-κB was increased 5-fold when MHE cells with 5.5 mM glucose were incubated with TNF-α, whereas NF-κB concentration did not increase in the cells incubated with 28 mM glucose. These results indicated that the high concentration of glucose inhibited cytokine-dependent NF-κB activation in MHE cells. The endothelial cells exposed to high concentrations of glucose for 7 days

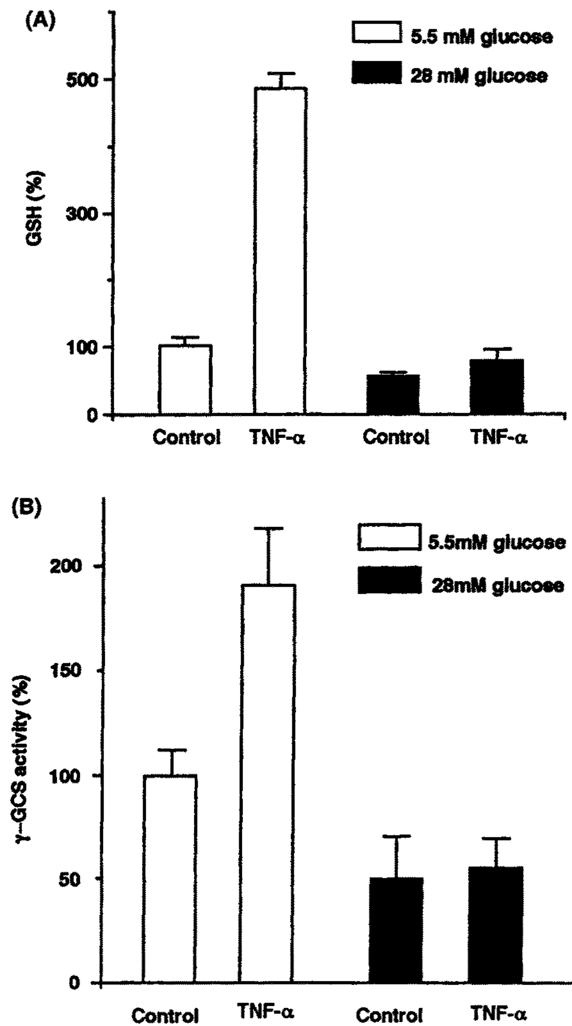

FIGURE 10 Effect of high glucose concentration on the concentration of glutathione and the activity of γ-glutamylcysteine synthetase stimulated by TNF-α in endothelial cells. MHE cells were incubated with 5.5 or 28 mM glucose for 7 days, then the effects of TNF-α (100 units/ml) after 3 h on the GSH concentration (A), and the γ-GCS activity (B) was estimated. Values are mean ± SD of four experiments.

showed a decrease in the expression of γ-GCS mRNA and its response to TNF-α. NF-κB is regulated by oxidoreductive control mechanisms, and the oxidoreductive condition of NF-κB regulates its DNA binding activity.

Luciferase activity of γ-GCS promoter The luciferase activity of the γ-GCS promoter was estimated to further examine whether the stimulation of the cells by TNF-α is mediated by NF-κB and the NF-κB–DNA binding activity relates to the induction of γ-GCS and the increase in the levels of GSH. We constructed chimeric genes containing various regions of γ-GCS-heavy subunit promoter gene and the coding region for luciferase. MHE cells transiently transfected with a plasmid containing the γ-GCS promoter-luciferase construct showed increased luciferase activity when treated with TNF-α. Figure 11 shows the results of the luciferase assay. Incubation of MHE cells with TNF-α increased the luciferase activity of the γ-GCS promoter including NF-κB site (A, the first lane). Deletion and mutagenesis of NF-κB site lost the stimulation of luciferase activity (A, the 2nd and 3rd lanes). When MHE cells were previously treated with 28 mM glucose for 7 days. The stimulation of the luciferase activity was not found (B). This strongly suggests that the luciferase activity stimulated by TNF-α was in the γ-GCS promoter containing the NF-κB binding site and the induction of γ-GCS mRNA by TNF-α is mediated by NF-κB.

DISCUSSION

Firstly, the physiological significance of the induction of the expression of γ-GCS followed by the increase in the levels of GSH produced by ionizing radiation seems to be to scavenge free radicals. This induction of γ-GCS was mediated by NF-κB. Since NF-κB is an oxidative stress-responsive transcription factor, the induction of γ-GCS and increase in the levels of GSH may act as an emergency signal in the cells. While cells can react to ionizing radiation by activating NF-κB or other transcription factors to enhance defense systems, when they are unable to respond to oxidative stress induced by ionizing radiation, DNA damage occurs. Induction of γ-GCS in response to higher doses of radiation may be a factor in the resistance of T98G glioblastoma cells to ionizing radiation. GSH synthesized in response to ionizing radiation downregulates the

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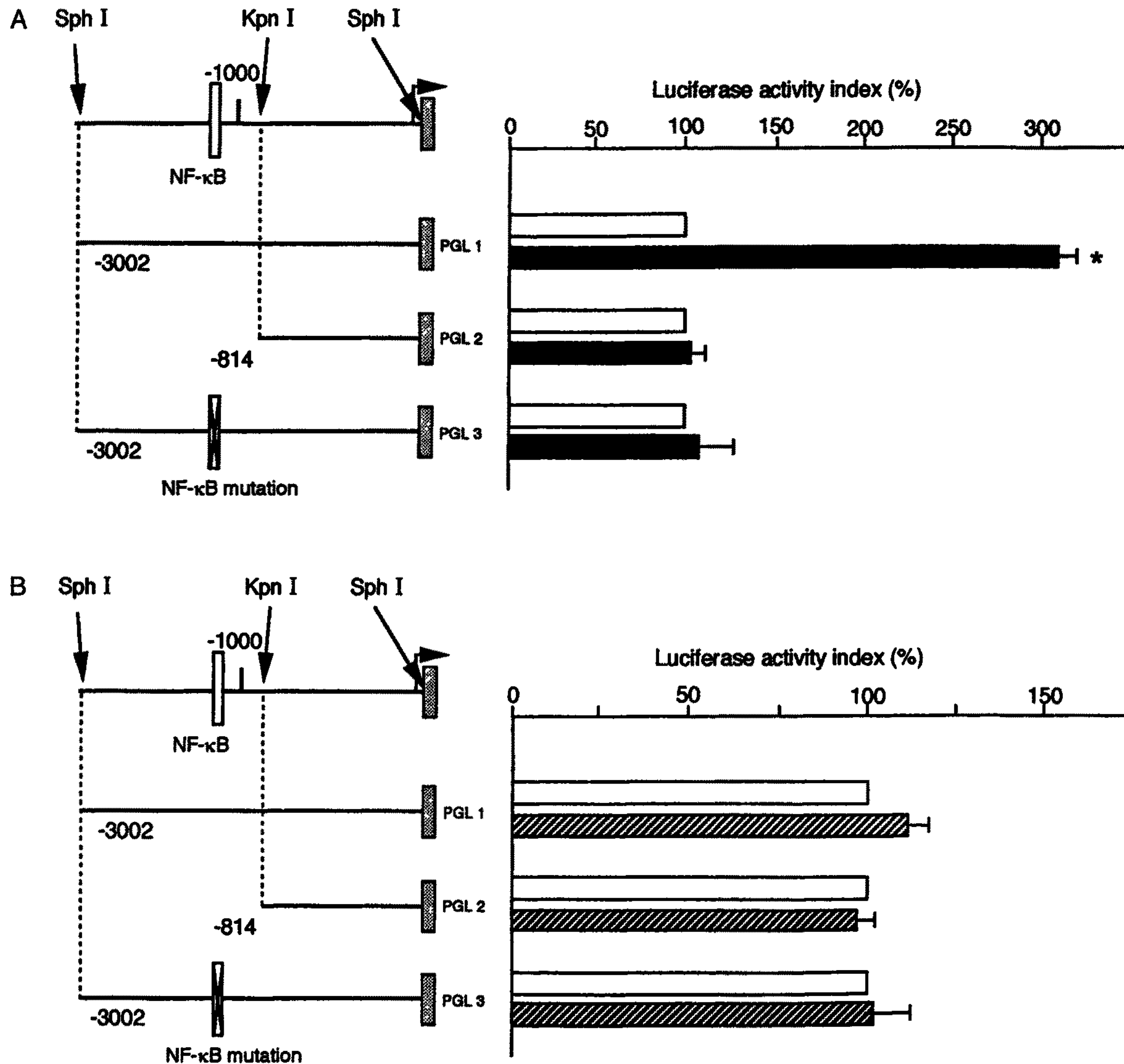

FIGURE 11 Luciferase activity of γ-GCSh promoter. MHE cells were transfected with pGL-3 luciferase expression vector containing γ-GCSh promoter regions and cotransfected with β-galactosidase. After the transfection, the cells were treated with 100 units/ml of TNF-α for 3 h, and the luciferase and the β-galactosidase activities were estimated in the lysates. Effect of deletion (PGL2) and mutagenesis of NF-κB binding site (PGL3) on the luciferase activity was estimated. Numbers indicate distance in base pairs from the start of transcription. (A), MHE cells incubated with 5.5 mM glucose for 7 days. open bar −TNF-α, closed bar +TNF-α (B) MHE cells incubated with 28 mM glucose for 7 days. open bar −TNF-α, closed bar +TNF-α Values are the mean ± SD of three experiments.

activation of NF-κB. This may enhance the poor responses of T98G cells to oxidative stresses.

Secondly, rapid responses to external signals are often mediated by inducible activation of transcriptional factors by post-transcriptional mechanisms, as opposed to their *de novo* synthesis. In case of NF-κB, it has been reported that the induction of NF-κB transcription in the pre-B cell line does not require new protein synthesis. Sen and Baltimore demonstrated that the previous

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treatment of pre-B cells with cycloheximide does not inhibit the activation of NF-κB, suggesting that the activation of the NF-κB, by the DNA alkylating agents does not require the induction of the synthesis of the NF-κB subunits.[18] The ability of activated NF-κB (p65/p50) to stimulate the expression of NF-κB–IκB complexes could result in the reaccumulation of these complexes in the cytosol. Such an autoregulatory mechanism may be important to control the cellular localization of the p50/p65 dimer and/or to control the specificity of the NF-κB-mediated responses. Since NF-κB is an oxidative stress-responsive transcriptional factor, GSH which is a potent antioxidant may work to diminish oxidative stress and to suppress the activity of NF-κB. In addition to GSH, a role of GSH peroxidase on the redox regulation of NF-κB has been reported.

Thirdly, our results present evidence that TNF-α stimulates the expression of γ-GCS, resulting in an increase in the levels of GSH. The stimulation of the expression of γ-GCS by TNF-α was found to be mediated by NF-κB. Prolonged exposure of endothelial cells to high glucose levels impairs GSH synthesis stimulated by TNF-α, which apparently weakens defense systems against oxidative stress in diabetes mellitus. Endothelial cells face the circulating blood. A weakened defense system against oxidative stress is thought to contribute to the development of diabetic complications. The results of luciferase activity of γ-GCS-heavy subunit promoter showed that the NF-κB-dependent luciferase activity in response to TNF-α was lost in endothelial cells exposed to high concentration of glucose for 7 days, however, the mechanisms of the loss of transcriptional activity in diabetic condition is not clarified.

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