

resident chaperones or proteins, such as CRT, calnexin, Grp94, and Erp57, was examined by immunoblot analysis using specific antibodies, and compared between the cell lysate samples. The level of CRT was apparently higher in H4 cells than U251MG or T98G cells. On the other hand, the expression levels of calnexin, Grp94, and Erp57 did not show a significant difference among the cell types.

Establishment of CRT gene-overexpressing U251MG cells. To investigate the biological significance of the level of CRT to the radiosensitivity of glioma cells, a CRT gene expression vector was constructed and introduced into U251MG cells as described in Materials and Methods. U251MG cells were chosen because they expressed a relatively low level of CRT compared with H4 cells (Fig. 1B). After screening by culturing with G418, the expression level of CRT was characterized immunologically in the G418-resistant transfectants. Two transfectants (CRT-M5 and CRT-M6) expressing high levels of CRT were established and used in subsequent experiments. Figure 2A shows that the expression of CRT increased in CRT-M5 and CRT-M6 cells compared with the parental and mock-transfected (Vector8) U251MG cells. The transfection had no apparent effect on the expression of other endoplasmic reticulum chaperones, such as calnexin and Grp94 (data not shown). Next, the intracellular distribution of CRT was examined by indirect immunofluorescence microscopy, as shown in Fig. 2B. The immunoreactivity for CRT distributed in a perinuclear granular pattern in all cases, including the control and gene-transfected cells, although the signal intensity was increased in the transfectants compared with the control cells (arrows).

Effect of overexpression of CRT on radiosensitivity of U251MG cells. To evaluate the effect of overexpressed CRT on radiosensitivity in U251MG cells, colony-forming ability was examined after 14 days of γ -irradiation in control (Vector8) and CRT-overexpressing (CRT-M5 and CRT-M6) cells (Fig. 2C). The colony-forming ability markedly decreased in CRT-overexpressing cells compared with control cells. These results indicate that overexpression of CRT enhances the radiation-induced cell damage in U251MG cells. To further characterize the enhanced radiation-induced cell damage in CRT-overexpressing cells, apoptotic characteristics were examined by TUNEL assay as described in Materials and Methods (Fig. 2D). Among control cells (Vector8),

TUNEL-positive cells bearing DNA-strand breaks appeared after 72 hours of γ -irradiation, and then decreased after 96 hours. In contrast, among CRT-overexpressing cells (CRT-M5), the TUNEL-positive cells appeared after 72 hours of γ -irradiation, and were still detectable 96 hours later. In the assay, some damaged cells were detached from culture plates and removed during the washing step, and the remaining cells attached to the plates were examined, especially after 96-hour exposure to irradiation. As a result, the population of less-damaged surviving clonogens (TUNEL-negative) might be increased in controls than in CRT-overexpressing cells after 96 hours. These results suggest that overexpression of CRT enhances radiation-induced apoptosis, resulting in the enhanced suppression of the ability of U251MG cells to form colonies after γ -irradiation.

Overexpression of CRT suppresses Akt/protein kinase B activity after γ -irradiation. The Akt pathway is known as a pivotal cell survival signal in the cell (28). We previously reported that overexpression of CRT suppressed Akt activity during the cardiac differentiation of H9c2 cells (20). Therefore, we also focused on the Akt pathway in CRT-overexpressing cells treated with irradiation. In Fig. 3A, the phosphorylation status of Akt was examined in control and CRT-overexpressing cells treated with γ -irradiation by immunoblot analysis using the antibodies against phosphorylated Akt. In control cells, the levels of Akt phosphorylated at both Ser⁴⁷³ and Thr³⁰⁹ were increased at 3 hours after the irradiation. On the other hand, in CRT-overexpressing cells, levels of phosphorylated Akt were unchanged after the irradiation. To confirm the functional link between phosphorylation status and activity in Akt, the cells were treated with or without γ -irradiation (5 Gy), and Akt activity was examined after 3 hours, by assessing the phosphorylation of GSK-3 α/β , a substrate of Akt kinase, as described in Materials and Methods (Fig. 3B). Akt was not activated by γ -irradiation in CRT-overexpressing cells, in spite of the marked activation of Akt in control cells treated with γ -irradiation. These results are consistent with the results for the phosphorylation status of Akt in control and CRT-overexpressing cells treated with γ -irradiation.

Constitutive activation of Akt protects against radiation-induced apoptosis in U251MG cells. To investigate whether the activation of Akt contributes to cellular protection against irradiation in U251MG cells, an expression vector for myristoylated

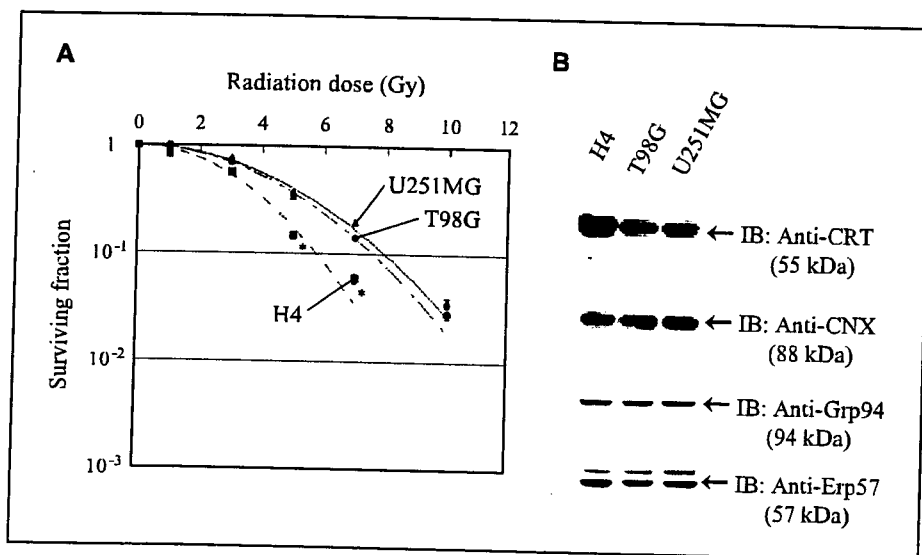
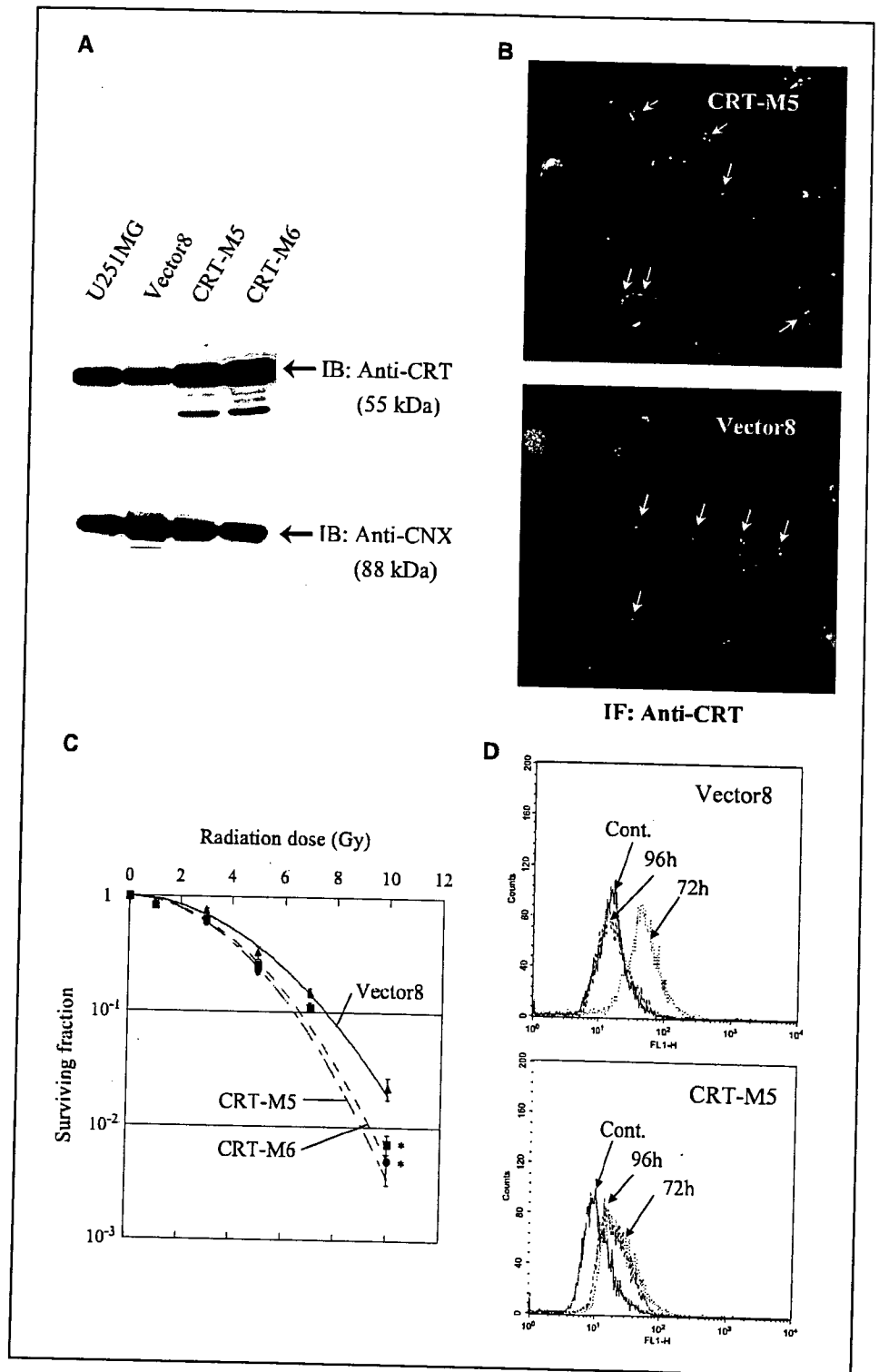


Figure 1. The expression of CRT is up-regulated in radiosensitive neuroglioma H4 cells. A, radiosensitivity was evaluated in human neuroglioma (H4) and glioblastoma (T98G and U251MG) cells by colony-forming assay after γ -irradiation (5 Gy) as described in Materials and Methods. ■, H4; ●, T98G; ▲, U251MG. Points, mean of four experiments; bars, SD. *, $P < 0.01$ versus same dose of irradiation for U251MG and T98G. B, expression levels of endoplasmic reticulum chaperones [i.e., CRT, calnexin (CNX), Grp94, and Erp57] were examined in H4, T98G, and U251MG cells by immunoblot analysis using specific antibodies as described in Materials and Methods.

Figure 2. Overexpression of CRT enhances radiation-induced cell death in U251MG cells. **A**, U251MG cells were transfected with the expression vector for the CRT gene, and antibiotic-resistant cell lines were established as described in Materials and Methods. The expression level of CRT was examined by immunoblot analysis in parental cells and cells transfected with a mock vector (Vector8) and the CRT gene expression vector (CRT-M5 and CRT-M6). **B**, the intracellular distribution of CRT was examined by indirect immunofluorescence (IF) microscopy using a specific antibody in control and gene-transfected cells. **C**, radiosensitivity was evaluated based on colony-forming ability after γ -irradiation (5 Gy) as described in Materials and Methods in control and CRT gene-transfected cells. \blacktriangle , Vector8; \bullet , CRT-M5; \blacksquare , CRT-M6. Points, mean of at least four experiments; bars, SD. *, $P < 0.01$ versus same dose of irradiation (10 Gy) for Vector8. **D**, control (Vector8) and gene-transfected (CRT-M5) cells were irradiated (5 Gy) for 10 minutes. Then, at 72 and 96 hours postirradiation, DNA double-strand breaks were detected by the TUNEL method as described in Materials and Methods.



Akt (Myr-Akt1) was introduced into the CRT-overexpressing cells as described in Materials and Methods, to generate cells in which Akt is constitutively activated. After 24 hours of transfection, the expression of Myr-Akt1 was detected by immunoblot analysis using the anti-myc antibody (Fig. 3C). Then, the cells were treated with γ -irradiation (5 Gy), and apoptosis was estimated after 72 hours, by the TUNEL assay as described above. As shown in

Fig. 3D, although TUNEL-positive cells were detected among CRT-overexpressing cells, numbers were diminished in the cells transfected with the Myr-Akt expression vector, indicating that activation of Akt plays an important role in cytoprotection against irradiation. This also suggests that a suppressed Akt pathway is a cause of the enhanced susceptibility to radiation-induced apoptosis in CRT-overexpressing cells.

PP2A is up-regulated in CRT-overexpressing U251MG cells. Ionizing radiation is known to trigger the Akt pathway through the activation of epidermal growth factor receptor families and phosphatidylinositol 3-kinase (PI3K; refs. 29, 30). To establish whether overexpression of CRT affects the activity of PI3K, a signaling molecule upstream of Akt, we examined PI3K activity in control and CRT-overexpressing cells treated with γ -irradiation (5 Gy). However, PI3K activity was not suppressed in CRT-overexpressing cells after the irradiation, but rather was slightly increased, compared with that in control cells (data not shown). This suggests that the suppression of the radiation-induced

activation of Akt in CRT-overexpressing cells is due to enhanced inactivation of Akt by PP2A (20, 31). In fact, the phosphorylation of Akt was apparently up-regulated in CRT-overexpressing cells treated with okadaic acid (100 nmol/L), a specific inhibitor of PP2A (data not shown). To investigate whether PP2A is influenced by overexpression of CRT, PP2A activity was assayed with cell lysates from control and CRT-overexpressing cells treated with or without γ -irradiation (5 Gy). In Fig. 4A, PP2A activity was always greater in CRT-overexpressing cells rather than controls, although the activity was slightly suppressed by irradiation in both cells. Next, the expression of phosphatases was examined at the level of

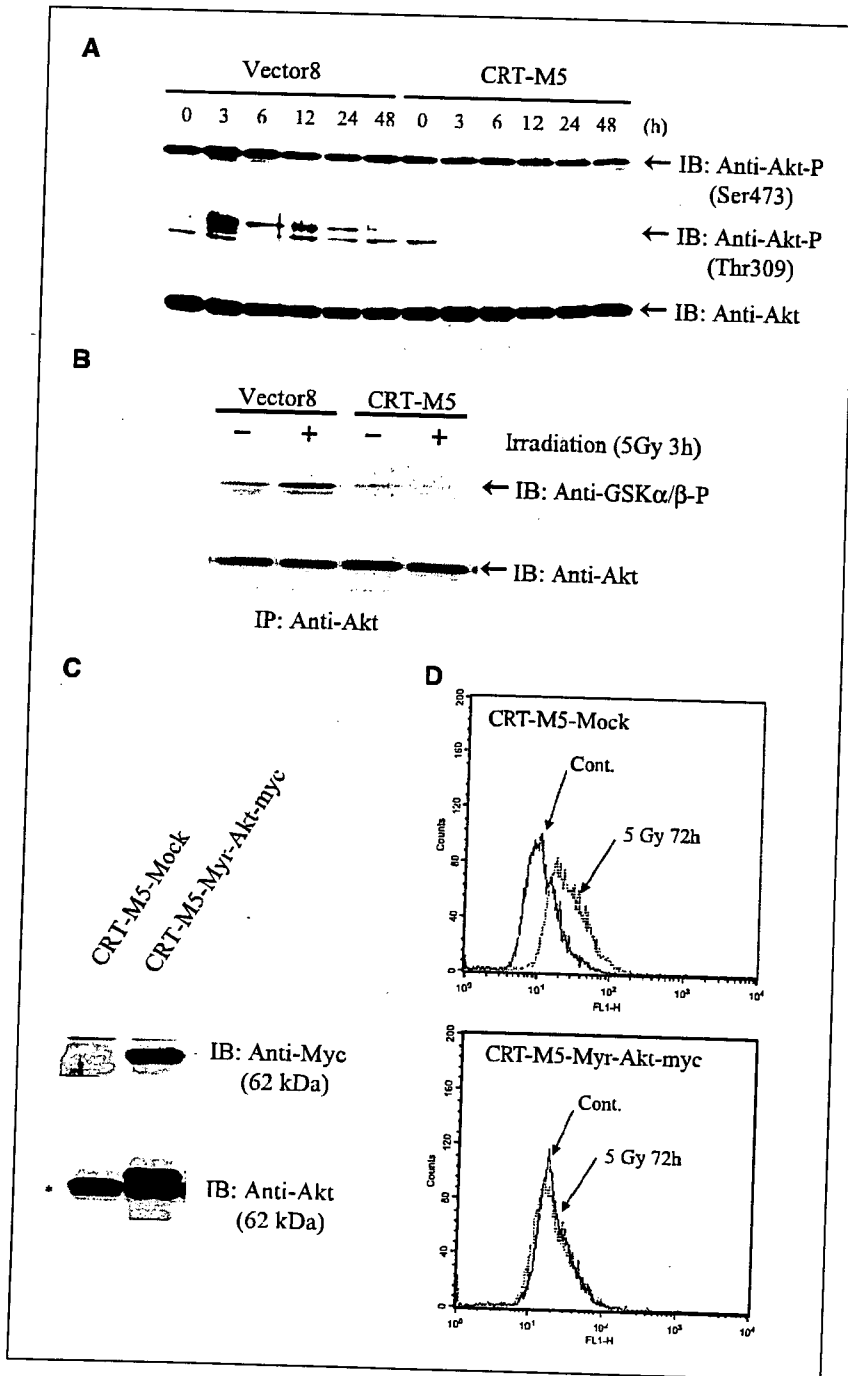
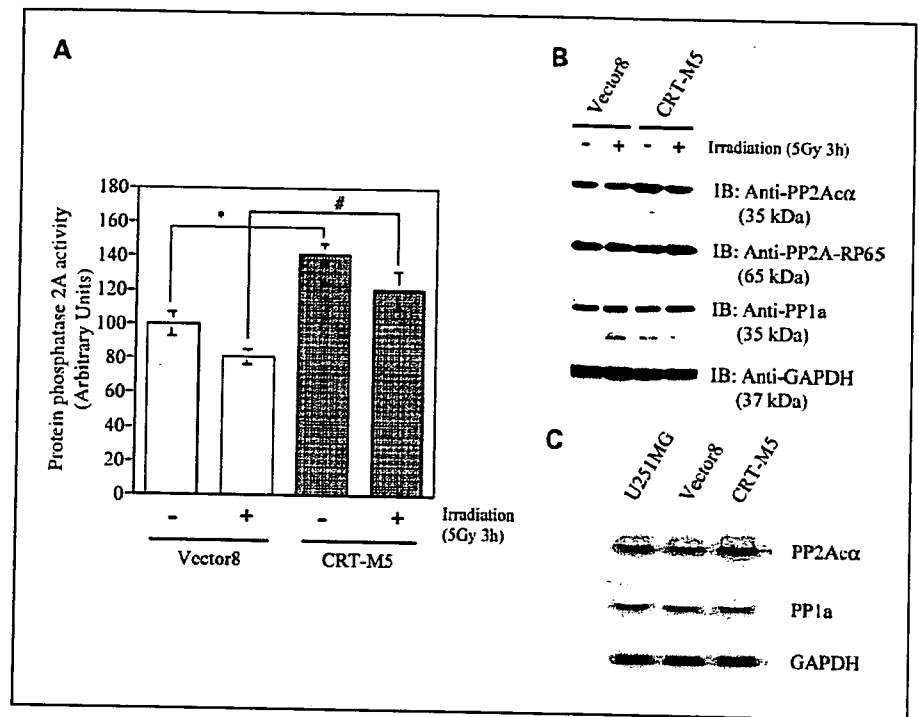


Figure 3. Radiation-induced activation of Akt is suppressed in CRT-overexpressing cells. **A**, the phosphorylation status of Akt was examined in control (Vector8) and CRT-overexpressing (CRT-M5) cells treated with γ -irradiation (5 Gy) by immunoblot analysis using antibodies against Akt phosphorylated at Ser⁴⁷³ and Thr³⁰⁹ as described in Materials and Methods. **B**, Akt activity was examined in control and CRT-overexpressing cells at 3 hours after the treatment with or without γ -irradiation (5 Gy) by estimating the phosphorylation of GSK-3 α/β as described in Materials and Methods. **C**, CRT-overexpressing cells (CRT-M5) were transiently transfected with a mock vector or the expression vector for the myristoylated Akt gene (*Myr-Akt1*) to overexpress active Akt as described in Materials and Methods. The expression of myristoylated Akt was examined by immunoblot (IB) analysis using the anti-myc antibody in cells transfected with the mock vector (CRT-M5-Mock) and Myr-Akt1 (CRT-M5-Myr-Akt-myc). *, endogenous Akt. **D**, after 24 hours of transfection with the mock or Myr-Akt1, the cells were irradiated (5 Gy); then, at 72 hours postirradiation, cell damage was estimated by the TUNEL method as described above.

Figure 4. The expression of PP2A is up-regulated in CRT-overexpressing cells. **A**, the activity of PP2A was assayed in control (Vector8) and CRT-overexpressing (CRT-M5) cells at 3 hours after the treatment with or without γ -irradiation (5 Gy). Columns, mean of at least three experiments; bars, SD. *, $P < 0.05$ versus untreated Vector8 cells. #, $P < 0.05$ versus Vector8 cells treated with irradiation. **B**, the protein levels of protein Ser/Thr phosphatases (i.e., PP2A α , PP2A-RP65, and PP1a) and GAPDH were examined by immunoblot analysis using specific antibodies in control and CRT-overexpressing cells at 3 hours after the treatment with or without γ -irradiation (5 Gy). **C**, transcriptional expression of PP2A α , PP1a, and GAPDH was examined in control and CRT-overexpressing cells by Northern blot analysis as described in Materials and Methods.



the protein of transcription by conducting immunoblot and Northern blot analyses, respectively. In Fig. 4B, the protein level of PP2A α increased in CRT-overexpressing cells, compared with controls. However, no significant change was observed in the levels of PP2A regulatory protein 65 (PP2A-RP65), PP1a, and GAPDH between control and CRT-overexpressing cells. Furthermore, the expression of PP2A α was up-regulated at the transcriptional level in CRT-overexpressing cells (Fig. 4C). Together, these results indicate that PP2A α is transcriptionally up-regulated in CRT-overexpressing cells. They also support that PP2A activity is increased by the up-regulated expression of PP2A α in the CRT-overexpressing cells.

Ca²⁺ homeostasis and responses to irradiation are altered in CRT-overexpressing U251MG cells. We previously reported that the gene expression of PP2Ac was controlled by altered Ca²⁺ homeostasis in myocardial H9c2 cells (27). To investigate the effect of CRT overexpression on cellular Ca²⁺ homeostasis, intracellular Ca²⁺ pools were characterized in control and CRT-overexpressing cells. After 48 hours of loading with ⁴⁵Ca²⁺, the cells were washed and resuspended in Ca²⁺-free buffer. Unidirectional fluxes to the extracellular medium after stimulation with several Ca²⁺ modulators were then measured as described in Materials and Methods. Thapsigargin, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA), ionomycin, a Ca²⁺ ionophore, and monensin, another ionophore affecting acidic stores, were used to stimulate the cellular Ca²⁺ pools (Fig. 5A). The results showed that cellular Ca²⁺ contents were apparently increased mainly in the thapsigargin-sensitive Ca²⁺ pools of CRT-overexpressing cells, compared with controls, suggesting that Ca²⁺ stores in the endoplasmic reticulum were increased in CRT-overexpressing cells. We next examined [Ca²⁺]_i in control and CRT-overexpressing cells after treatment with irradiation (5 Gy). As shown in Fig. 5B, [Ca²⁺]_i was markedly higher in CRT-overexpressing cells than controls at 30 minutes after γ -irradiation (5 Gy), although it increased by γ -irradiation in both cells.

In U937 cells lacking inositol 1,4,5-triphosphate receptor (IP3R) 1, the irradiation-induced increase of [Ca²⁺]_i is significantly suppressed, suggesting that Ca²⁺ stores in the endoplasmic reticulum plays an important role in the irradiation-induced increase of [Ca²⁺]_i (32). To investigate whether the IP3R-dependent release of Ca²⁺ from the endoplasmic reticulum is involved in the mechanism, the effect of xestospongin C (5 μ mol/L), an inhibitor of IP3R (21) on the irradiation-induced increase of [Ca²⁺]_i in control and CRT-overexpressing cells was examined. As shown in Fig. 5C, with xestospongin C, the irradiation-induced increase of [Ca²⁺]_i was apparently suppressed in both control and CRT-overexpressing cells; however, the levels were still higher than those of nontreated cells. On the other hand, when the cells were pretreated with Ni²⁺ (5 mmol/L) to block Ca²⁺ influx from the extracellular space through Ca²⁺ channels and Na⁺/Ca²⁺ exchangers in the plasma membrane (33), the irradiation-induced increase of [Ca²⁺]_i was significantly suppressed to reach the levels of nontreated cells. To further investigate the effect of CRT overexpression on irradiation-induced Ca²⁺ influx from the extracellular spaces, ⁴⁵Ca²⁺ uptake was examined in control and CRT-overexpressing cells after treatment with irradiation as described in Materials and Methods. As shown in Fig. 5D, the uptake of ⁴⁵Ca²⁺ was apparently enhanced in CRT-overexpressing cells after 30-minute exposure to irradiation, compared with controls, although the uptake increased in both control and CRT-overexpressing cells. Together, these results indicate that the IP3R-sensitive release of Ca²⁺ and Ca²⁺ influx from the extracellular space both play important roles in the mechanism of the irradiation-induced increase of [Ca²⁺]_i in the cells, and were enhanced by the overexpression of CRT.

The gene promoter activity of PP2A α is up-regulated in CRT-overexpressing cells through cytoplasmic free Ca²⁺. In the PP2A α gene promoter, cyclic AMP response element (CRE) is a pivotal transcription site, through which the activation is regulated by [Ca²⁺]_i, although both the GC-box and CRE additively contribute

to the basal promoter activity (27). To investigate whether the CRE-dependent gene expression of PP2Ac α is influenced by the overexpression of CRT, the gene promoter activity was examined by assaying the luciferase activity as described in Materials and Methods. Control and CRT-overexpressing cells were transfected with various luciferase vectors [i.e., pGL3-pro-PP2Ac, which contains the entire promoter sequence of PP2Ac α (-1,209 to +258); pGL3-pro-PP2Ac (C3), which contains a CRE but no GC-box (-145 to +258); and pGL3-pro-PP2Ac (C3-Mut/C) in which the CRE is disabled by mutation (-145 to +258)]. After 24 hours of transfection, cell lysates were prepared and subjected to an assay for luciferase activity. As shown in Fig. 6A, the level of activity was higher in CRT-overexpressing cells than controls when either pGL3-pro-PP2Ac or pGL3-pro-PP2Ac (C3) was transfected. However, in the case of pGL3-pro-PP2Ac (C3-Mut/C), no activity was detected in the control or CRT-overexpressing cells. These results

indicate that the activity of the PP2Ac α promoter is up-regulated by overexpression of CRT through the CRE. Next, to investigate whether the enhancing effect of overexpressed CRT on the PP2Ac α promoter is regulated through the change in [Ca²⁺]_i, we examined the promoter activity in control and CRT-overexpressing cells treated with Ca²⁺ modulators such as thapsigargin and BAPTA-AM. Thapsigargin and BAPTA-AM, a cell-permeable Ca²⁺ chelator, were used to increase and decrease [Ca²⁺]_i in the cell, respectively (27). In Fig. 6B, control cells transfected with pGL3-pro-PP2Ac (C3) were treated with or without thapsigargin (5 μ mol/L for 2 hours), and then the luciferase activity was assayed as described above. The results showed that the activity of the PP2Ac α promoter was up-regulated in control cells by the increase of [Ca²⁺]_i with thapsigargin treatment. These findings are consistent with those in the case of myocardial H9c2 cells (27). In Fig. 6C, CRT-overexpressing cells transfected with pGL3-pro-PP2Ac (C3) were

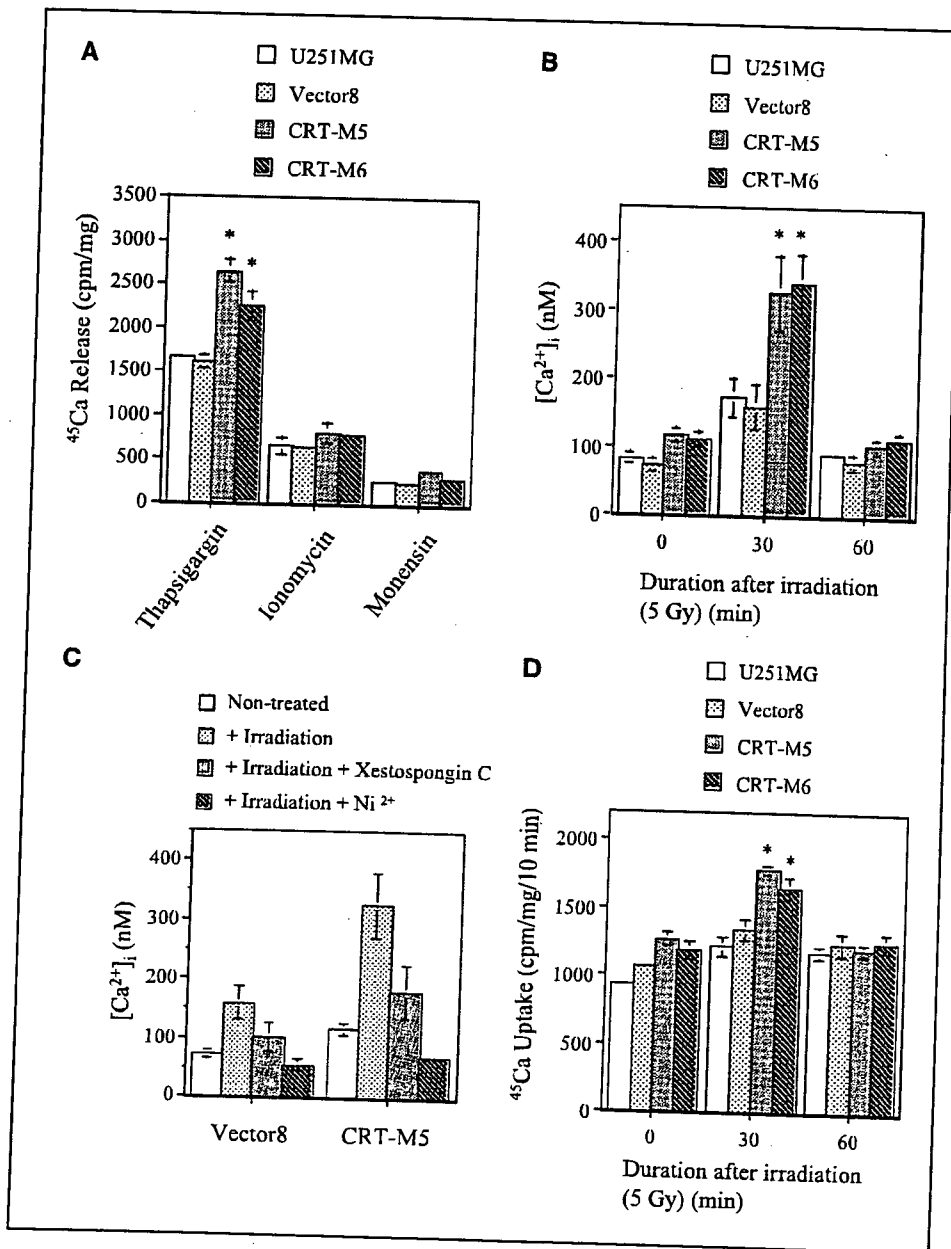
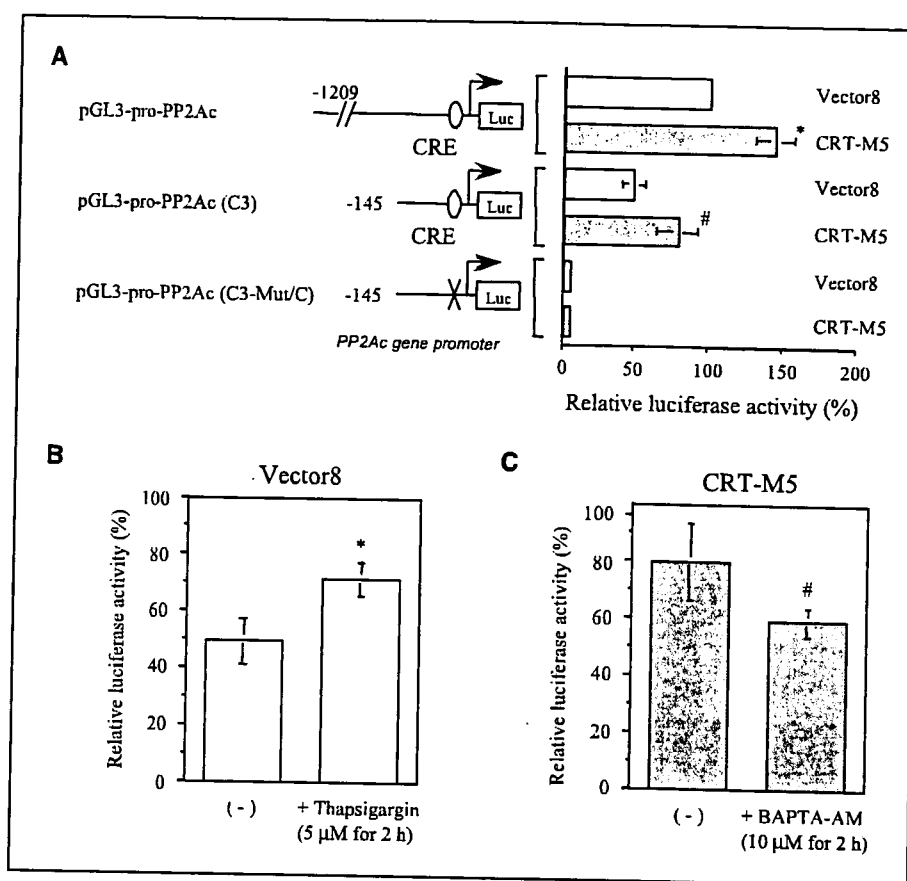


Figure 5. Effect of CRT overexpression on intracellular Ca²⁺ pools and Ca²⁺ responses to irradiation in U251MG cells. **A**, control (U251MG and Vector8) and CRT-overexpressing (CRT-M5 and CRT-M6) cells were cultured with ⁴⁵Ca²⁺ (1 μ Ci/mL) for 48 hours, then detached from the culture dish and resuspended in Ca²⁺-free EBSS. Cell suspensions were preincubated at 37°C for 3 minutes, and sequentially stimulated with thapsigargin (0.1 μ mol/L), ionomycin (2 μ mol/L), and monensin (2 μ mol/L). The cell suspensions were collected 5 minutes after the addition of each reagent and centrifuged. The radioactivity released from the cells was measured in the supernatant. Cell pellets were lysed and protein amounts were determined using a BCA assay kit (Pierce). Columns, mean of the cpm subtracted from those recovered in the preceding collection, and normalized to the protein in the corresponding cell pellets; bars, SD. *, *P* < 0.01 versus U251MG or Vector8 cells treated with thapsigargin. **B**, control and CRT-overexpressing cells were treated with γ -irradiation (5 Gy). [Ca²⁺]_i was measured after the periods indicated using Fura2-AM as described in Materials and Methods. Columns, mean of at least four experiments; bars, SD. *, *P* < 0.01 versus U251MG or Vector8 cells at 30 minutes after the irradiation. **C**, control (Vector8) and CRT-overexpressing (CRT-M5) cells were pretreated with or without xestospongine C (5 mmol/L) or Ni²⁺ (5 mmol/L) for 5 minutes, then treated with γ -irradiation (5 Gy). [Ca²⁺]_i was measured at 30 minutes after the irradiation using Fura2-AM as described above. Columns, mean of at least four experiments; bars, SD. *, *P* < 0.01 versus Vector8 cells at 30 minutes after the irradiation. **, *P* < 0.01 versus CRT-M5 cells at 30 minutes after the irradiation. **D**, control and CRT-overexpressing cells were treated with γ -irradiation (5 Gy). Then, cells were incubated for 10 minutes with ⁴⁵Ca²⁺ (5 μ Ci/mL) at the periods indicated after irradiation. After washing with EBSS, the cells were harvested and ⁴⁵Ca²⁺ uptake was measured as described in Materials and Methods. Columns, mean of three independent experiments; bars, SD. *, *P* < 0.01 versus U251MG or Vector8 cells at 30 minutes after the irradiation.

Figure 6. The promoter activity of the PP2A α gene is increased in CRT-overexpressing cells through CRE, and is regulated via altered Ca²⁺ homeostasis.

A, left, schematic representation of luciferase vector constructs for the rat PP2A α promoter [i.e., pGL3-pro-PP2Ac, pGL3-pro-PP2Ac (C3), and pGL3-pro-PP2Ac (C3-Mut/C)]. Each luciferase vector construct was generated as described in Materials and Methods. The CRE site (-26) was mutated in pGL3-pro-PP2Ac (C3-Mut/C). **Right,** luciferase activity of the vector constructs for the PP2A α gene promoter in control and CRT-overexpressing cells. The cells were transiently transfected with the PP2A α promoter-luciferase gene fusion plasmids. After 24 hours of transfection, luciferase activity was assayed with cellular extracts as described in Materials and Methods. **Columns,** mean of at least three experiments; **bars,** SD. *, *P* < 0.01 versus Vector8 cells transfected with pGL3-pro-PP2Ac. #, *P* < 0.01 versus Vector8 cells transfected with pGL3-pro-PP2Ac (C3). Control and CRT-overexpressing cells were transiently transfected with pGL3-pro-PP2Ac (C3). After 24 hours of transfection, control cells were treated with 5 μ mol/L thapsigargin for 2 hours (**B**) and CRT-overexpressing cells were treated with 10 μ mol/L BAPTA-AM for 2 hours (**C**). Then, luciferase activity was assayed with cell nuclear extracts as described in Materials and Methods. **Columns,** mean of at least three experiments; **bars,** SD. *, *P* < 0.05 versus untreated Vector8 cells. #, *P* < 0.05 versus untreated CRT-M5 cells.



treated with or without BAPTA-AM (10 μ mol/L for 2 hours), and then the luciferase activity was assayed as described above. The results showed that the activity of the PP2A α promoter was down-regulated in CRT-overexpressing cells by the decrease of [Ca²⁺]_i with BAPTA-AM treatment. Taken together, these results indicate that the activity of the PP2A α promoter was regulated by [Ca²⁺]_i through CRE, and overexpression of CRT up-regulates the promoter activity through the altered homeostasis of Ca²⁺ in U251MG cell.

Discussion

In the present study, we focused on functions of endoplasmic reticulum chaperones in malignant gliomas to obtain a new perspective for therapy. To evaluate the association between radiosensitivity and the expression of the molecular chaperones in the endoplasmic reticulum, we used three cultured glioma-derived cell lines, i.e., two glioblastoma cell lines (U251MG and T98G) and a neuroglioma cell line (H4). We found that H4 cells were more radiosensitive than U251MG and T98G cells, and the expression level of CRT was specifically higher in H4 than in U251MG or T98G cells (Fig. 1A and B). These findings suggested that the expression level of CRT seems to be related with radiosensitivity in gliomas. Although the expression of CRT is induced by radiation (34, 35), the function of CRT in radiosensitivity is not well understood. To investigate the significance of CRT in radiosensitivity, we established a CRT-overexpressing cell line using U251MG, and examined the effect of the overexpression on the radiosensitivity. It was found that CRT-overexpressing cells

showed greater radiosensitivity than control cells, and enhanced radiation-induced apoptosis.

Radiation-induced apoptosis is controlled by various mechanisms, such as the p53 status, the Bcl-2 gene family, the caspase pathways, and so forth (3). Zhao et al. (36) reported that the DNase activation pathway through p53 and Ca²⁺-mediated DNase γ pathway were involved in the regulation of radiation-induced apoptosis in Molt-4 cells. However, in U251MG cells, the enhancement of radiation-induced apoptosis by CRT may not be due to a p53-dependent mechanism, because of the mutation in the p53 gene (37). On the other hand, apoptosis is regulated by several signaling pathways, including the mitogen-activated protein kinases and Akt pathways (38). The Akt signaling pathway is an important cell survival and antiapoptotic signal in radiation-induced apoptosis (39, 40). In this study, we found that the pathway was significantly suppressed in the CRT-overexpressing cells after γ -irradiation. Moreover, we found that the expression of PP2A α was significantly increased in overexpressing cells compared with control cells. PP2A is known to modulate the activities of several kinases, and is responsible for the dephosphorylation and inactivation of Akt (20, 31). Therefore, these results suggest that Akt signaling was suppressed by the up-regulation of PP2A α expression in CRT-overexpressing cells treated with γ -irradiation.

The Ca²⁺ concentration of the endoplasmic reticulum or cytoplasm is thought to be a key determinant of radiation-induced apoptosis (9, 11). CRT is a Ca²⁺-binding molecular chaperone in the endoplasmic reticulum and is involved in the regulation of intracellular Ca²⁺ homeostasis and endoplasmic reticulum Ca²⁺ storage capacity (16). In this study, we found that,

in CRT-overexpressing cells, the thapsigargin-sensitive Ca^{2+} pool was increased, and the levels of $[Ca^{2+}]_i$ and Ca^{2+} influx from the extracellular spaces were both up-regulated, especially after ionizing irradiation. This indicated that CRT overexpression significantly influenced the regulatory mechanism of Ca^{2+} homeostasis in U251MG cells, although the precise mechanism of CRT action has not yet been fully clarified. In addition, we found that the gene promoter of PP2A α was regulated through the change in $[Ca^{2+}]_i$ (Fig. 6B and C). These results strongly suggest a mechanical link between down-regulated Akt signaling and altered Ca^{2+} homeostasis in CRT-overexpressing cells, and it is consistent with our finding that the antiapoptotic activity of Akt is down-regulated by Ca^{2+} in myocardial H9c2 cells (27).

Concerning CRT and apoptosis, Nakamura et al. (10) reported that overexpression of CRT resulted in an increase in the sensitivity of HeLa cells to both thapsigargin- and staurosporine-induced apoptosis. The authors suggested that overexpression of CRT affects the communication between the endoplasmic reticulum and mitochondria to increase the sensitivity to apoptosis via the altered Ca^{2+} homeostasis, and this has been supported by the study of Arnaudeau et al. (41). We also reported that overexpression of CRT influences the function of SERCA2a under oxidative stress, leading to an alteration of Ca^{2+} homeostasis (42) and to enhanced susceptibility to apoptosis (20, 21). These findings suggest that the expression level of CRT is well correlated with the susceptibility to

apoptosis. In contrast, overexpression of CRT provided resistance to oxidant-induced cell death in renal epithelial LLC-PK1 cells treated with iodoacetamide (43), *tert*-butylhydroperoxide (44), or hydrogen peroxide (45). In the neuroblastoma \times glioma hybrid cell line NG-108-15, suppression of CRT by an antisense oligonucleotide increased sensitivity to ionomycin-induced cytotoxicity (46, 47). The function of CRT in the regulation of apoptosis may differ in specific cell types, and is still controversial. Although almost all studies suggest regulatory functions of CRT in the susceptibility to apoptosis, further investigation is required to clarify the relevance to CRT in cancer biology.

In conclusion, we found that the expression level of CRT was well correlated with radiosensitivity in glioma cell lines, and CRT modulated the radiosensitivity of glioblastoma cell lines by affecting the cell survival pathway of Akt signaling through alterations of Ca^{2+} homeostasis and responses in the endoplasmic reticulum.

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SMOKING

Long term smoking with age builds up excessive oxidative stress in bronchoalveolar lavage fluid

K Nagai, T Betsuyaku, T Kondo, Y Nasuhara, M Nishimura



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See end of article for authors' affiliations

Correspondence to:
Dr T Betsuyaku, First
Department of Medicine,
Hokkaido University
School of Medicine, N-15,
W-7, Kita-ku, Sapporo,
Japan 060-8638;
bytomoko@med.hokudai.
ac.jp

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Background: Epithelial lining fluid plays a critical role in protecting the lung from oxidative stress, in which the oxidised status may change by ageing, smoking history, and pulmonary emphysema.

Methods: Bronchoalveolar lavage (BAL) was performed on 109 young and older subjects with various smoking histories. The protein carbonyls, total and oxidised glutathione were examined in BAL fluid.

Results: By Western blot analysis, the major carbonylated protein in the BAL fluid was sized at 68 kDa, corresponding to albumin. The amount of carbonylated albumin per mg total albumin in BAL fluid was four times higher in older current smokers and three times higher in older former smokers than in age matched non-smokers ($p < 0.0001$, $p = 0.0003$, respectively), but not in young smokers. Total glutathione in BAL fluid was significantly increased both in young ($p = 0.006$) and older current smokers ($p = 0.0003$) compared with age matched non-smokers. In contrast, the ratio of oxidised to total glutathione was significantly raised (72%) only in older current smokers compared with the other groups. There was no significant difference in these parameters between older smokers with and without mild emphysema.

Conclusions: Oxidised glutathione associated with excessive protein carbonylation accumulates in the lung of older smokers with long term smoking histories even in the absence of lung diseases, but they are not significantly enhanced in smokers with mild emphysema.

The lung is an organ constantly exposed to exogenous oxidants such as cigarette smoke and air pollutants. Epithelial lining fluid (ELF) acts as an interface between the airspace epithelium and the external environment and therefore affords protection against epithelial cell injury.¹ Because the constituents of ELF form the primary targets of inhaled oxidant pollutants and also inflammatory reactive oxygen species (ROS) generated within the alveolar space, the oxidative modifications of certain extracellular targets and their functional consequences have received considerable attention.² It is known that protein carbonylation reflects the oxidation of Lys, Arg, or Pro residues in proteins. The content of protein carbonyls is therefore by far the most commonly used marker for protein oxidation.³

On the other hand, there are various kinds of extracellular antioxidants in the lungs such as mucin, uric acid, ascorbic acid, α -tocopherol, glutathione, antioxidant enzymes and proteins that are capable of binding metals. Glutathione is a sulfhydryl-containing tripeptide (L-gamma-glutamyl-L-cysteinyl-glycine) that is present in high concentrations in all cells and protects against intracellular oxidants and xenobiotics.⁴ Extracellular glutathione can be augmented by cellular sources from respiratory tract epithelial cells and inflammatory cells such as macrophages. The levels of glutathione in ELF are 100-fold higher than in plasma, which may indicate its relative importance among the various antioxidants.^{4,5}

The elderly are particularly vulnerable to smoking associated respiratory diseases. Worldwide, the ratio of smokers is still high among the middle aged or elderly population, and they usually have long term smoking histories. Cigarette smoking is a major risk factor for the development of emphysema, and it normally affects patients in the fifth to sixth decade of life. Although an oxidant/antioxidant imbalance has been implicated in the development of emphysema,⁶ there is still a large gap in our understanding of the link between the cumulative effects of ageing and decades of continuous smoking on the oxidative stress and

antioxidant mechanisms in the lung. Total glutathione is known to be raised in the bronchoalveolar lavage (BAL) fluid of recent young smokers, and the majority (>95%) of the total glutathione is maintained in the reduced state.⁵ However, there is a lack of information on the levels and redox state of glutathione in the BAL fluid of older smokers with or without pulmonary emphysema.

We therefore investigated whether chronic smoking induced changes in protein carbonylation and glutathione redox status in BAL fluid differ by age, duration of smoking, or the presence of emphysema. BAL was performed to obtain ELF from lifelong non-smokers as well as young and older smokers with various smoking histories.

METHODS

Subjects

A total of 109 community based asymptomatic volunteers were recruited from the patients in our smoking cessation and non-pulmonary clinics and from the employees and students in our medical school. Most of the subjects had taken part in previous studies in our laboratory.^{7,8} The young group (age 20-29 years, $n = 39$) consisted of 16 lifelong non-smokers and 23 current smokers who had smoked for less than 3 years. The older group (age 37-77 years, $n = 70$) included seven lifelong non-smokers, 17 former smokers, and 46 current smokers with smoking histories of more than 20 years. Former smokers were arbitrarily defined as people who had quit smoking for at least 1 year. None of the subjects was on regular medication or had a history of asthma or other allergic disorders. All subjects had been free of clinically apparent respiratory infections for the preceding 2 months and had been evaluated by physical examination, chest radiography, and a blood test. After elimination of non-

Abbreviations: BAL, bronchoalveolar lavage; DNP, dinitrophenylhydrazine; ELF, epithelial lining fluid; FEV₁, forced expiratory volume in 1 second; GSH, reduced glutathione; GSSG, glutathione disulfide

eligible subjects, all older former and current smokers were screened for emphysematous changes by high resolution computed tomographic (HRCT) scanning as previously described.⁹ Pulmonary function tests were performed in accordance with the standard techniques of the American Thoracic Society. Forced expiratory volume in 1 second (FEV₁) from the flow-volume curve was expressed as a percentage of the predicted value according to the equations of Berglund *et al.*¹⁰

All of the subjects provided written informed consent and the study was approved by the ethics committee of Hokkaido University School of Medicine.

Sequential BAL

Sequential BAL was performed through a wedged flexible fiberoptic bronchoscope (Olympus BF-B3R, Tokyo, Japan) as described previously.⁹ The fluid returned from the first 50 ml aliquot was not used in the study. The remaining lavage fluid was pooled and used as the BAL fluid. Differential cell counts were performed as previously described.⁹ The level of total protein in the BAL fluid was determined using a Micro BCA Protein Assay Reagent kit (Pierce Biotech, IL, USA). The level of albumin was quantified by laser nephelometry as described previously.⁹ The level of transferrin was measured using the human transferrin enzyme linked immunosorbent assay quantification kit (Bethyl Laboratories Inc, TX, USA).

Assessment of protein carbonyls in BAL fluid

Oxidation of individual BAL fluid proteins was measured by analysis of Western blots according to the method of Shacter *et al.*¹¹ BAL fluid was derivatised with dinitrophenylhydrazine (DNP) using the OxyBlot Protein Oxidation Detection kit (Chemicon International, Temecula, CA, USA) with slight modification. Briefly, 16 µl of unconcentrated BAL fluid were denatured by adding 3 µl of 20% sodium dodecylsulfate (SDS)-polyacrylamide, derivatised by adding 1 µl 10X DNPH solution, and neutralised by adding 7.5 µl neutralization solution and 1.5 µl 2-mercaptoethanol. Samples were separated by electrophoresis on 10% SDS-polyacrylamide electrophoresis gels. Blots were performed using the anti-DNP antibody and scanned with a GT-9500 scanner (Epson, Nagano, Japan); the intensity of the bands was calculated using NIH Image software (version 1.62). On each blot the recorded total DNP intensity of all bands detected for each lane was divided by that of a standard sample from a representative young non-smoker. The total carbonyl content in the BAL fluid was referred as total DNP units/ml BAL fluid. The assay for the total carbonyl content had an intrabatch coefficient of variation of 12% (n = 7) and an interbatch coefficient of variation of 16% (n = 5).

The molecular masses of albumin and transferrin were determined by Western blotting on the same membrane used for the Oxyblot. Specifically, after the Western blot for carbonyl proteins, the anti-DNP antibody was removed, then incubated with 1:1000 peroxidase conjugated anti-human albumin antibody (DAKO, Glostrup, Denmark) or 1:1000 rabbit anti-human transferrin (Dakocytomation, Glostrup, Denmark) followed by 1:15 000 horseradish peroxidase conjugated goat anti-rabbit antibody (DAKO). Sites of antibody binding were visualised using the ECL^{Plus} Western blotting detection system (Amersham, Aylesbury, UK).

Measurement of total glutathione and glutathione disulfide in BAL fluid

Total glutathione and glutathione disulfide (GSSG), oxidised glutathione, were measured in all samples using a glutathione assay kit (Cayman Chemical Co, MI, USA) as described by Tietze and co-workers.¹² The assays for total glutathione and GSSG had intrabatch coefficients of variation of 3% (n = 12) and 3% (n = 10), respectively, and interbatch coefficients of variation of 6% (n = 10) and 13% (n = 8), respectively.

Statistical analyses

Differences between the two means were performed with a two tailed unpaired Student's *t* test using Statview Software (SAS Institute Inc, NC, USA). More than two means were compared by analysis of variance followed by the Games-Howell method using SAS software Version 8.2 (SAS Institute Inc). A value of *p* < 0.05 was considered to be statistically significant. The results are reported as standard error of the mean (SE).

RESULTS

Characteristics of subjects

Clinical characteristics and pulmonary function data for the subjects are summarised in table 1. Emphysema, as detected on CT scans, was less than 25% of the total lung area in most subjects categorised in the emphysema groups. There was no difference in the number of cigarettes smoked per day between young and older current smokers.

BAL findings

BAL findings are shown in table 2. The total protein, albumin, and transferrin levels in BAL fluid from young smokers were significantly lower than those from young non-smokers. There were no differences in the levels of total protein, albumin, and transferrin in BAL fluid among older groups. The numbers of total cells and macrophages in BAL fluid were significantly increased in current smokers

Table 1 Characteristics of subjects

	Young			Older			
	Non-smokers	Current smokers	Non-smokers	Former smokers		Current smokers	
				No emphysema	Emphysema	No emphysema	Emphysema
No of subjects	16	23	7	7	10	21	25
Age (years)	23 (1)	23 (0)	63 (3)	65 (2)	59 (3)	57 (2)	61 (2)
Cigarettes/day	0	20 (1)	0	0	0	27 (5)	25 (4)
Pack-years	0	5 (1)	0	35 (10)	41 (7)	55 (5)	45 (4)
VC (% pred)	97 (3)	96 (3)	122 (8)	125 (3)	110 (3)†	110 (4)	110 (4)
FEV ₁ (% pred)	83 (3)	88 (3)	124 (8)	129 (5)	97 (5)†	101 (5)	92 (4)‡
FEV ₁ /FVC (%)	91 (2)	88 (2)	84 (2)	80 (2)	73 (3)*	79 (2)	70 (2)‡

VC, vital capacity; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity.

Data are expressed as mean (SE).

*Significantly different from older non-smokers (*p* < 0.05).

†Significantly different from older former smokers without emphysema (*p* < 0.05).

‡Significantly different from older current smokers without emphysema (*p* < 0.05).

Table 2 BAL fluid findings

	Young		Older				
	Non-smokers	Current smokers	Non-smokers	Former smokers		Current smokers	
				No emphysema	Emphysema	No emphysema	Emphysema
Recovery rate (%)	74 (4)	64 (3)*	71 (4)	54 (5)	33 (5)†‡	50 (4)†	33 (5)†§
Albumin (mg/l)	47 (7)	33 (3)*	55 (9)	57 (11)	59 (11)	32 (2)	50 (10)
Total protein (mg/l)	116 (14)	92 (7)*	160 (22)	128 (28)	210 (44)	111 (10)	142 (13)
Transferrin (mg/l)	3.8 (0.4)	2.9 (0.2)*	7.4 (2.0)	4.3 (1.2)	4.5 (1.1)	3.9 (0.4)	3.6 (0.3)
Total cells ($\times 10^{-4}$ /ml)	8.1 (0.9)	20.1 (2.2)*	13.4 (1.3)	9.6 (2.4)	10.5 (3.4)	27.3 (4.3)†	36 (8.2)†
Macrophages ($\times 10^{-4}$ /ml)	6.9 (0.9)	19.5 (2.1)*	10.8 (1.3)	8.0 (1.9)	8.4 (2.4)	25.9 (4.1)†	32.9 (8.0)†
Neutrophils ($\times 10^{-4}$ /ml)	0.20 (0.05)	0.24 (0.08)	0.04 (0.01)	0.05 (0.01)	0.06 (0.02)	0.62 (0.45)	0.59 (0.25)

Data are expressed as mean (SE).

*Significantly different from young non-smokers ($p < 0.05$).

†Significantly different from older non-smokers ($p < 0.05$).

‡Significantly different from older former smokers without emphysema ($p < 0.05$).

§Significantly different from older smokers without emphysema ($p < 0.05$).

compared with non-smokers, both in young and older groups. The percentage of macrophages and neutrophils in BAL fluid did not differ between young and older current smokers.

Total protein carbonyls in BAL fluid

The immunoblot analysis demonstrated a major carbonyl protein band for all of the subjects with a molecular weight of 68 kDa (fig 1A and B) and a faint band of 80 kDa for most of the subjects. These bands were speculated to be albumin and transferrin, respectively, based on removal of the anti-DNP antibody followed by Western blotting with antibodies against human albumin or human transferrin (fig 2). As shown in fig 3, the total DNP in units/ml BAL fluid from older current and former smokers was significantly higher than in lifelong non-smokers of the same age (1.72 (0.18) and 1.81 (0.24) ν 0.7 (0.29) unit/ml; $p = 0.0028$ and $p = 0.0078$, respectively). In contrast, no difference was found in the total DNP units/ml BAL fluid between non-smokers and current smokers among the young subjects (1.05 (0.14) ν 1.0 (0.09) units/ml, $p = 0.82$). There was also no difference between former smokers with and without emphysema (2.16 (0.34) ν 1.32 (0.22) units/ml, $p = 0.08$) or between current smokers with and without emphysema (2.00 (0.31) ν 1.44 (0.14) units/ml, $p = 0.15$).

Albumin carbonylation in BAL fluid

To focus on the carbonylation of albumin, DNP units of the 68-kDa band (corresponding to albumin) were quantified. The value was normalised according to the concentration of albumin in the BAL fluid (fig 4). The ratio of carbonylated albumin per mg total albumin was four times higher in older current smokers and three times higher in older former smokers than in age matched lifelong non-smokers (fig 4). In contrast, there was no difference in the ratio of albumin carbonylation in BAL fluid between young smokers and non-smokers ($p = 0.21$). In both current and former smokers there was no difference in the ratio of albumin carbonylation between the subjects with and without emphysema.

Total glutathione and GSSG in BAL fluid

Total glutathione was detected in unconcentrated BAL fluid for all subjects (fig 5). The concentration of total glutathione in BAL fluid was significantly increased in smokers compared with non-smokers, even in young subjects (3.11 (0.4) ν 1.17 (0.33) $\mu\text{g/ml}$, $p = 0.006$). It was also significantly increased in older current smokers compared with age matched non-smokers and former smokers (2.64 (0.34) ν 0.89 (0.19) and 0.88 (0.29) $\mu\text{g/ml}$, $p = 0.0003$ and $p = 0.0014$, respectively). In contrast, the level of GSSG was markedly raised only in older current smokers (1.38 (0.2) $\mu\text{g/ml}$) and was mostly

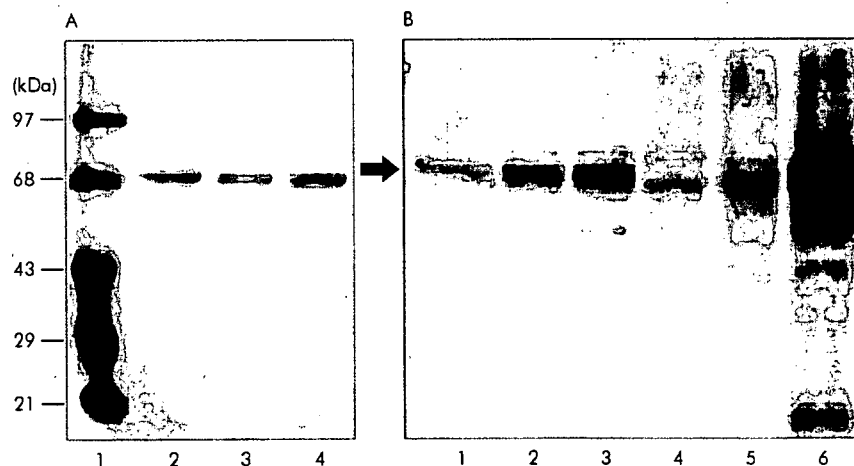


Figure 1 Representative Western blots of oxidised protein in BAL fluid. (A) Representative Western blots from young subjects. Lane 1, molecular weight protein standards in which the second band from the top (68 kDa) is bovine serum albumin; lane 2, a young non-smoker; lane 3, a young current smoker; lane 4, a young non-smoker which was used as a standard for each blot. (B) Representative blots from older subjects. Lane 1, standard BAL sample also shown in lane 4 of (A); lanes 2 and 3, older current smokers without emphysema; lane 4, older non-smoker; lanes 5 and 6, older current smokers with emphysema.

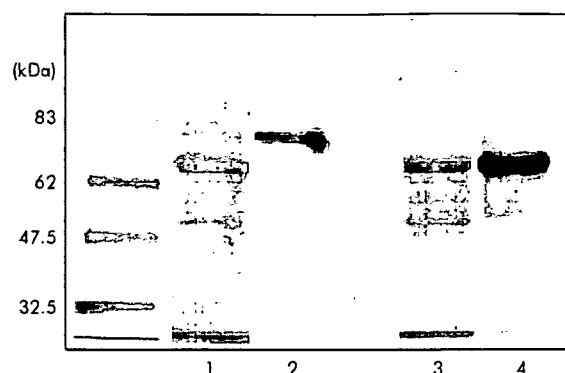


Figure 2 Immunoblots for transferrin and albumin in BAL fluid. After removal of the anti-DNP antibody, Western blotting was carried out on the same membrane. Lanes 1 and 3, anti-DNP antibody; lane 2, anti-human transferrin antibody; lane 4, anti-human albumin antibody. The positions of the molecular weight standards are shown on the left.

under the detection limit in the other groups (fig 6). No significant difference was observed between former or current smokers with and without emphysema either in the level of total glutathione in BAL fluid (former smokers: 1.15 (0.47) v 0.49 (0.12) $\mu\text{g/ml}$, $p = 0.23$; current smokers: 2.78 (0.55) v 2.46 (0.38) $\mu\text{g/ml}$, $p = 0.64$) or in the level of GSSG in BAL fluid (former smokers: 0.28 (0.13) v 0.09 (0.05) $\mu\text{g/ml}$, $p = 0.27$; current smokers: 1.48 (0.38) v 1.28 (0.26) $\mu\text{g/ml}$, $p = 0.67$, respectively). The ratio of GSSG per total glutathione in older current smokers was as high as 0.72 and was significantly higher than in the other groups (fig 7).

DISCUSSION

In this study we have shown that older smokers with a long term history of smoking have excessive protein carbonyls and accumulate GSSG in BAL fluid, indicating that endogenous antioxidant defences are overwhelmed. Based on the number of cigarettes smoked per day, the amount of exogenous ROS from cigarette smoke was similar for all current smokers regardless of their age; however, the young and older current smokers undoubtedly developed different levels of oxidative stress in the lungs. On the other hand, ageing alone did not affect the level of protein carbonyls, total glutathione, or GSSG in BAL fluid.

The reaction of ROS with proteins results in the formation of carbonyl groups on amino acid residues. Oxidative stress overwhelming the antioxidant defence of the lung may lead to lung injury through a variety of mechanisms including lipid peroxidation of epithelial cell membranes.^{13, 14} Total protein carbonyls are reportedly increased in the BAL fluid from patients with acute respiratory distress syndrome,¹⁵ idiopathic pulmonary fibrosis, sarcoidosis, asbestosis,¹⁶ and cystic fibrosis.¹⁷ Notably, we found in this study that the level of total protein carbonyls in BAL fluid was raised not only in older current smokers but also in older former smokers despite not smoking for at least a year, indicating that the mechanisms responsible for increased protein carbonyls associated with smoking persist after cessation.

To clarify how protein oxidation participates in pathogenic processes, it will be important to identify the organ and/or disease specific proteins that are most susceptible to oxidative modification and the functional effects of their oxidation. Albumin and transferrin in the serum of mice and albumin and α_1 -macroglobulin in the serum of rats accrue significant levels of carbonylation.¹⁸ In the plasma of humans, albumin, fibrinogen, and both fibrinogen γ -chain and α_1 -antitrypsin precursors are major targets for carbonylation in uraemia,¹⁹ lung cancer,²⁰ and Alzheimer's disease,²¹ respectively. We report here for the first time that the oxidation of albumin—the most abundant protein in BAL fluid—mostly accounts for excessive total protein carbonylation in older smokers. It is plausible that albumin in BAL fluid from older current smokers may lose its functional efficiency as a consequence of carbonylation because oxidative modifications of albumin reportedly attenuate its antioxidative capacity.²²

Glutathione also functions as an extracellular antioxidant of the lungs by preventing the oxidation of functional surfactants and antioxidant enzymes only in its reduced form (GSH).^{3, 23} GSH inhibits protein carbonyl formation in plasma following exposure to cigarette smoke.²⁴ Although the concentration of total glutathione in BAL fluid is known to vary between lung diseases, the presence of its oxidised form in BAL fluid has only been studied in asthma,²⁵ cystic fibrosis,²⁶ and acute respiratory distress syndrome.²⁷ In the BAL fluid of acute respiratory distress syndrome patients, a significant portion of the total glutathione is in the oxidised form (GSSG), which is likely to be due to the rapid extracellular oxidation of glutathione.²⁷

Although the regulation of glutathione in BAL fluid is poorly understood, we speculate that GSSG accumulates in

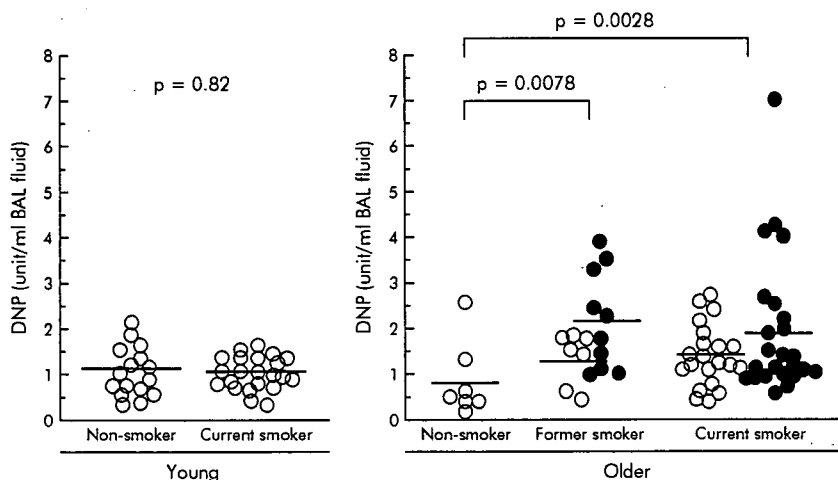


Figure 3 Total DNP units/ml in BAL fluid. Horizontal lines represent mean values of each group. Open circles represent subjects without emphysema; closed circles represent subjects with emphysema. DNP, dinitrophenylhydrazine.

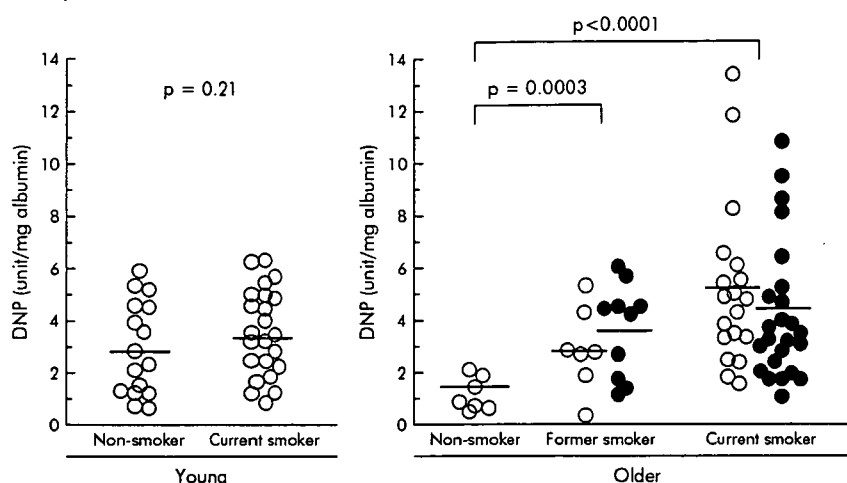


Figure 4 Carbonylated albumin/mg total albumin in BAL fluid. The horizontal lines represent mean values of each group. Open circles represent subjects without emphysema; closed circles represent subjects with emphysema. DNP, dinitrophenylhydrazine.

BAL fluid in older smokers by the following mechanisms. Firstly, the extracellular conversion of GSH into GSSG is accelerated as a result of excessive hydrogen peroxide generated in the ELF by extracellular glutathione peroxidase. Secondly, the efflux of GSSG from the lung cells is increased because of intracellular oxidative stress. Lung cells have many redox regulating enzymes and thiol containing proteins such as thioredoxins or peroxiredoxins that affect the intracellular GSSG/GSH balance.²⁸⁻³⁰ When the level of intracellular GSSG increases, the export might be accelerated by adenosine triphosphate binding cassette transporters such as multidrug resistant associated protein 1 (MRP 1).³¹ Thirdly, extracellular GSSG catabolism might be impaired due to the attenuation of membrane bound γ -glutamyl transpeptidase activity.³² Fourthly, protein reactive thiols, which react with GSSG to release GSH, might be reduced in ELF leading to protein mixed disulfide synthesis.³³ Taken together, it is likely that excessive oxidative stress in the lung is causally related to the high level of GSSG in BAL fluid.

Sampling of the ELF by BAL is a common means of studying proteins and investigating their changes in lung diseases. Because of the lack of an appropriate marker for the dilution of lavage fluids (to correct for variable recovery of epithelial lining fluid³⁴), we decided not to normalise the data to albumin or urea. Instead, the data were expressed as the concentrations in the lavaged fluid as in our previous studies.^{7,8} Despite the difference in the recovery rate of lavage fluid, the level of albumin in the BAL fluid did not differ between the groups (table 2). However, whether the variable rate of recovery may affect the results of comparisons between the groups cannot be determined.

The method and period of storage are also important for evaluating the oxidative status. We used frozen/preserved BAL fluid samples for the assays. Small aliquots of cell free supernatant of BAL fluid were made upon harvest and stored at -70°C to avoid repeated freezing and thawing. It is difficult to directly validate the stability of BAL fluid stored for long periods, but we did not find a significant correlation

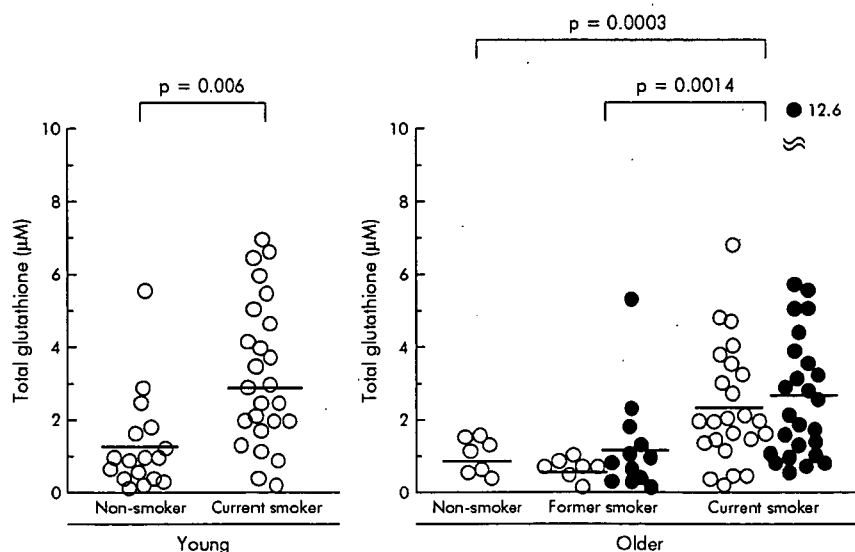


Figure 5 Levels of total glutathione in BAL fluid. The horizontal lines represent mean values of each group. Open circles represent subjects without emphysema; closed circles represent subjects with emphysema.

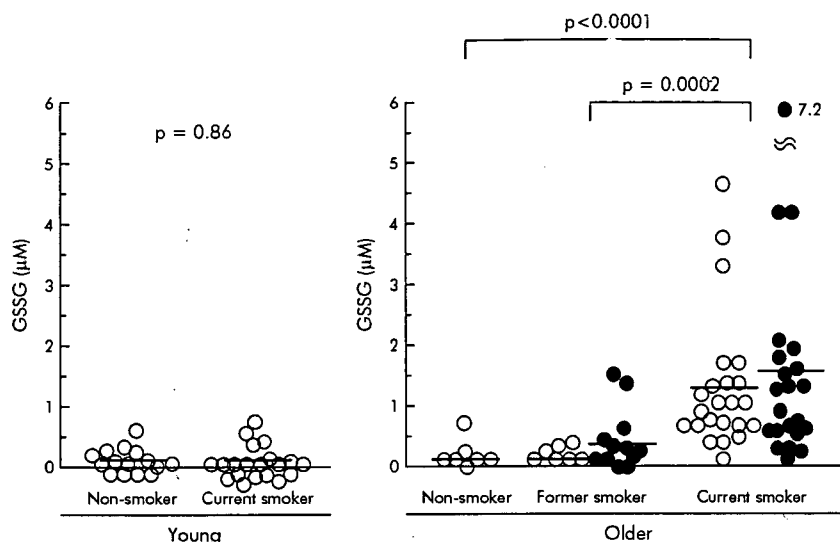


Figure 6 Levels of glutathione disulfide (GSSG) in BAL fluid. The horizontal lines represent mean values of each group. Open circles represent subjects without emphysema; closed circles represent subjects with emphysema.

between storage periods (1–11 years) and the values for each assay for older current smokers (for carbonylated albumin, $n = 40$, $p = 0.73$; for GSSG, $n = 40$, $p = 0.65$). These results imply that markers of oxidative status such as GSSG and carbonylated albumin in BAL fluid are not directly affected by long term storage at -70°C .

This study clearly shows that the effects of smoking on the extracellular redox system differ with age between recent and long term smokers. It should be noted that older smokers are exposed to cigarette smoke over the years while concurrently ageing. Following smoking, the content of glutathione and the ratio of GSH to total glutathione in the whole lung are significantly lower in senescence accelerated mice than in senescence resistant mice.³⁵ In humans, long term smoking leads to age related decreases in antioxidant activity in alveolar macrophages.³⁶ We also recently reported a decrease with age in surfactant proteins A and D in the BAL fluid of long term smokers compared with age matched non-smokers, whereas such differences were not observed among

young subjects.⁷ Increases in several markers of oxidative stress have been reportedly linked to pulmonary diseases such as chronic obstructive pulmonary disease.^{14 37–39} It is still not clear whether oxidative stress plays a direct role in the pathogenesis of chronic obstructive pulmonary disease or whether there is more oxidative stress as a result of ageing with long term chronic smoking. To address this issue, we first evaluated oxidative stress and glutathione redox status in BAL fluids collected from more than 100 subjects classified by age, smoking status, and the presence of mild emphysema in this study.

Although we did not observe a significant enhancement associated with the presence of emphysema according to CT scans, it should be noted that the severity of emphysema was classified as low and that the distribution was heterogeneous even in the older smokers classified as having emphysema. We cannot therefore rule out the possibility that the protein carbonyls and/or GSSG might be higher in BAL fluid from individuals with more advanced stages of emphysema.

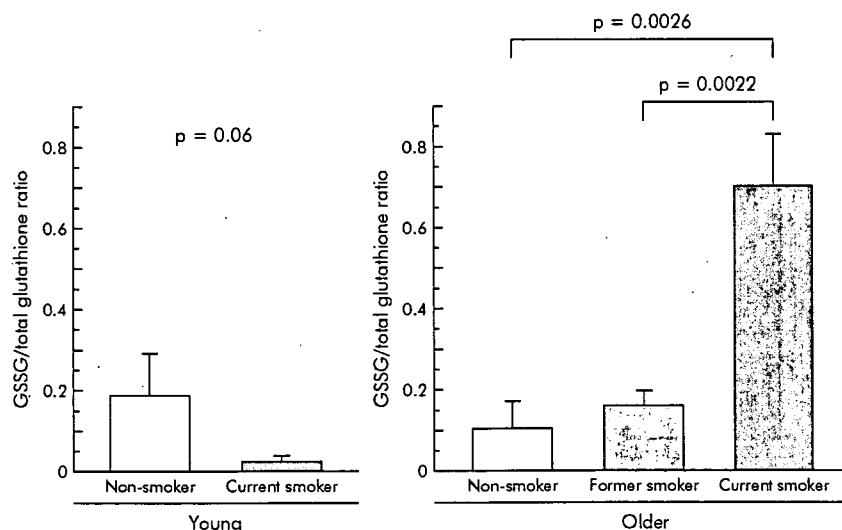


Figure 7 Glutathione disulfide (GSSG)/total glutathione ratio in BAL fluid. Data are mean (SE) values for each group.

Although smoking related diseases are often found in older individuals, it is important to keep in mind that the extracellular antioxidant systems in the lungs of older people have an impaired ability to resist damage from cigarette smoke. Further studies will investigate the functions of GSSG and oxidised proteins in BAL fluid and the potential benefits of antioxidant supplementation for middle aged or elderly smokers.

Authors' affiliations

K Nagai, T Betsuyaku, Y Nasuhara, M Nishimura, First Department of Medicine, Hokkaido University School of Medicine, Sapporo, Japan
T Kondo, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

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Serum Levels of S-Glutathionylated Proteins as a Risk-Marker for Arteriosclerosis Obliterans

Kazuki Nonaka, MD; Noriaki Kume, MD^{††}; Yoshishige Urata, PhD;
Shinji Seto, MD^{*}; Takaaki Kohno, BS^{*}; Sumihisa Honda, PhD^{*};
Soji Ikeda, MD^{*}; Takahiro Muroya, MD^{**}; Yoshihiko Ikeda, MD[†];
Yoshito Ihara, MD; Toru Kita, MD^{††}; Takahito Kondo, MD

Background Oxidative stress plays a role in the development of chronic peripheral arterial disease (PAD) because under these conditions redox regulation is impaired, inducing the S-glutathionylation of proteins. A method of estimating the levels of S-glutathionylated proteins has been developed using biotinylated glutathione S-transferase, which allows the study of their crucial role in the oxidative stress-related progression of PAD.

Methods and Results The serum levels of S-glutathionylated proteins were examined in 41 patients with arteriosclerosis obliterans (ASO) and 38 age-matched non-ASO patients using biotinylated glutathione S-transferase. The levels were higher in the patients with ASO, even early on, and positively correlated with the ankle/brachial index. In vitro, the levels of S-glutathionylated proteins were reduced in the presence of glutathione and glutaredoxin.

Conclusions Serum levels of S-glutathionylated proteins are a sensitive risk-marker for ASO at an early stage. (Circ J 2007; 71: 100–105)

Key Words: Arteriosclerosis obliterans; Oxidative stress; S-glutathionylation

The number of patients suffering from arteriosclerosis obliterans (ASO) is anticipated to increase, accompanying the increase in incidence of risk factors such as obesity, hypercholesterolemia, diabetes, and hypertension. Pathologically, ASO derives from atherosclerosis, and complete occlusions by fresh or old thrombi are often observed. Treatment includes anticoagulants, antiplatelet drugs, and vasodilators, and in the advanced stages percutaneous transluminal angioplasty, bypass surgery, and prosthetic arterial grafts have been used. New approaches include intravenous administration of prostaglandin E₂ or gene therapy with hepatocyte growth factor,³ both aimed at increasing peripheral blood flow. Most patients with ASO have no apparent clinical symptoms early on, but diagnosis at the early stage is essential for preventing progression. Unfortunately, there are currently no specific and sensitive markers for ASO, so the aim of this study was to find a new risk-marker for the diagnosis of ASO in the earlier stages.

The development of atherosclerosis is induced by severe damage to endothelial cells from various pro-inflammatory cytokines, adhesion molecules, or sheer stress, for example.^{4–6} Furthermore, oxidative stress is believed to play a

crucial role in the progression of peripheral arterial disease (PAD),⁷ because it induces modifications of cellular components such as proteins, lipids, and DNA, leading to cell dysfunction or apoptosis. Most of the risk factors for PAD, such as smoking, obesity, hypertension, diabetes, and hypercholesterolemia, create oxidants that damage endothelial cells.⁷ The cysteine thiols of proteins are easily modified by oxidative stress when the antioxidative systems are suppressed and under oxidative stress caused by reactive oxygen species or nitrogen oxide species, it is the sulfhydryl residues of proteins that are most susceptible. In response, the sulfhydryl groups are oxidized to form disulfides in a reaction with the reduced form of glutathione disulfide (GSSG) or converted irreversibly to sulfenic, sulfinic, and sulfonic acid derivatives.¹ S-Glutathionylated proteins reported to date include glyceraldehyde-3-phosphate dehydrogenase,² annexin A2,³ protein kinase C,⁴ and carbonic anhydrase III.⁵ The S-glutathionylation of proteins is initiated in the presence of GSSG.⁸ The S-glutathionylation of the sulfhydryl groups changes a protein's function, and the process is regulated by thioredoxin (TRX) or glutathione (GSH)/glutaredoxin (GRX). Such modifications of protein-thiols by oxidative stress are speculated to occur in patients with PAD; however, no data on changes in the levels of serum S-glutathionylated proteins have been reported for patients with peripheral or cardiovascular diseases such as stroke, coronary artery disease, and end-stage renal disease. We are interested in the S-glutathionylation of proteins in ASO patients and so the aim of the present study was to evaluate the serum levels in these patients, as a risk-marker for ASO in the early stages, because elevation supposedly reflects redox imbalance.

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Department of Biochemistry and Molecular Biology in Disease, *Department of Radiation Epidemiology, Atomic Bomb Disease Institute, **Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, †Tagawa Municipal Hospital, Tagawa and ††Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Mailing address: Takahito Kondo, MD, Department of Biochemistry and Molecular Biology in Disease, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. E-mail: kondo@net.nagasaki-u.ac.jp

Table 1 Characteristics of Patients With ASO

	Non-ASO patients	ASO patients (Fontaine)		
		Stage 1	Stage 2	Stage 3
Patients, n	38	9	22	10
ABI (mean±SD)	0.94±0.02	0.72±0.07	0.41±0.13	0.21±0.06
Age (mean±SD), years	66±11	73±8	72±7	71±5
Male sex, n (%)	15 (39)	6 (67)	16 (73)	8 (80)
Risk factors, n (%)				
Hypertension	17 (45)	7 (78)	19 (86) [†]	9 (90) [†]
Diabetes	23 (61)	3 (33)	6 (27) [‡]	3 (30)
Smoking	9 (24)	6 (67) [§]	15 (68) [§]	5 (50)
Hypercholesterolemia	16 (42)	4 (44)	9 (41)	5 (50)
Chronic renal failure on hemodialysis	0	0	0	0
Angina	4 (11)	2 (22)	4 (18)	3 (30)
Lipid profile (mean±SD)				
Total cholesterol, mg/dl	253±45	198±23*	205±28*	202±17*
LDL-C, mg/dl	153±46	111±24 [†]	119±23 [†]	116±14 [†]
hs-CRP (mean±SD), ng/ml	2.19±0.42	3.17±1.01	3.57±0.85 [#]	4.30±0.99 [#]

Values for hs-CRP were transformed in logarithm of 10. One-way ANOVA was followed up with Tukey-Kramer pairwise comparisons among means.

[†]*p*<0.05 for comparison with non-ASO patients; [‡]*p*<0.05 for comparison with non-ASO patients; [§]*p*<0.05 for comparison with non-ASO patients; **p*<0.005 for comparison with non-ASO patients; [#]*p*<0.05 for comparison with non-ASO patients; [†]*p*<0.05 for comparison with non-ASO patients.

ASO, arteriosclerosis obliterans; ABI, ankle/brachial index; LDL-C, low-density lipoprotein-cholesterol; hs-CRP, high-sensitivity C-reactive protein.

Methods

Patient Sample

We enrolled 41 patients diagnosed with ASO. All of them had the characteristic complaints of chronic limb ischemia, including intermittent claudication, rest pain, or non-healing ischemic ulcers (Fontaine I, n=9; Fontaine II, n=22; Fontaine III, n=10) as confirmed by angiography. Of the patients visiting hospital without apparent PAD, we recruited 38 age-matched controls. All participants gave written informed consent and prior to the commencement of the present study, the protocol was approved by the ethics committees of all the participating universities and hospitals.

Measurement of Ankle/Brachial Index (ABI)

Blood pressure, heart rate, and ABI were measured using the Form pulse wave velocity (PWV)/ABI non-invasive vascular screening device (Nihon Colin Inc, Tokyo, Japan) after the subject had rested supine for at least 20 min. ABI was calculated 2 or 3 times for both legs and averaged; an ABI <0.9 was considered to indicate the presence of disease.

Immunoblot Analysis

Unless otherwise indicated, 20 µg samples of serum was used. Protein concentrations were determined using a BCA assay kit (Pierce, Rockford, IL, USA). Samples were electrophoresed on 5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) in the absence of dithiothreitol (DTT) and the proteins in the gels were transferred onto nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (10 mmol/L Tris-HCl [pH7.5] and 0.15 mol/L NaCl; TBS) containing 0.05% Tween 20, v/v (TBST), and 5% (w/v) nonfat dry milk, then reacted with primary antibodies in TBST containing 3% (w/v) bovine serum albumin overnight with constant agitation at 4°C. After several washes with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-IgG antibodies. Proteins

in the membranes were then visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences) according to the manufacturer's instructions. Levels of high-sensitivity C-reactive protein (hs-CRP) were determined in the same serum samples used for sLOX-1, with a commercially available electrochemiluminescent immunoassay kit (F. Hoffman-La Roche Ltd).

Detection of S-Glutathionylated Proteins by Biotin-Glutathione S-Transferase (GST) on Blotted Membranes

Serum levels of S-glutathionylated proteins were estimated according to the methods described by Cheng et al⁸ using biotinylated GST. Serum samples were collected serially and stored at -80°C until assays were performed. Of each sample, 20 µg/lane were subjected to 5% SDS-PAGE under non-reducing conditions. The proteins in the gels were transferred onto nitrocellulose membranes, which were blocked in phosphate buffered saline (PBS) containing 0.1% Tween 20, v/v, and 5% nonfat dry milk, then treated with BSA containing 5% (w/v) bovine serum albumin for 2 h at room temperature and further incubated with 30 mg/ml biotin-GST overnight. After several washes with PBS, the membranes were incubated with HRP-conjugated streptavidin (1:1,000 dilution) for 1 h at room temperature. Peroxidase activity was detected after treatment with 2 mmol/L hydrogen peroxide and 0.6 mg/ml 4-chloro-1-naphthol in PBS.

Statistical Analysis

Statistical analysis was performed using Stat-View (version 4.5, Abacus Concepts Inc, Calabasus, CA, USA) and R. The 1-way ANOVA was used to compare continuous variables, with the Tukey-Kramer test for multiple comparisons, and 2-way cross-tabulation with the chi-square test was used for binary variables, when appropriate, to compare differences between groups. Statistically significant differences among groups were analyzed by the Kruskal-Wallis test with Dunn's test. When S-glutathionylated proteins

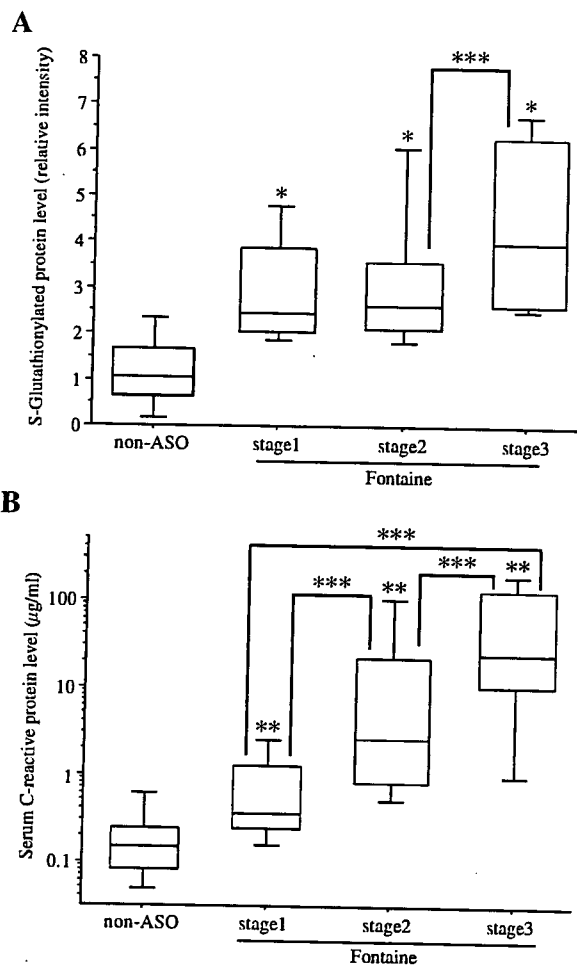


Fig 1. Serum levels of S-glutathionylated proteins and high-sensitivity C-reactive protein (hs-CRP). S-glutathionylated protein levels are expressed as relative intensity compared with non-ASO patients (A), and hs-CRP ($\mu\text{g/ml}$) levels (B) were determined in 41 ASO patients (9 at Fontaine stage 1, 22 at stage 2, and 10 at stage 3) and 38 non-ASO patients. Data are indicated in box plots. Center horizontal lines indicate median values; upper and lower edges of outer boxes, 25th and 75th percentiles; and lower and upper bars, 10th and 90th percentiles. Statistically significant differences among the 4 groups by Kruskal-Wallis test with Dunn's test. * $p < 0.0001$ vs non-ASO patients; ** $p < 0.05$ vs non-ASO patients; *** $p < 0.05$. ASO, arteriosclerosis obliterans.

were undetectable by immunoblot analysis, a score of 0 was assigned. Any association between S-glutathionylated proteins and hs-CRP, total cholesterol or low-density lipoprotein (LDL)-cholesterol (C) was evaluated with Spearman's rank correlation coefficient. Logarithmic values of hs-CRP were used as variables for statistical analyses. The effect of S-glutathionylated proteins on ABI was analyzed by using a multiple linear regression model with hyperlipidemia, hypertension, smoking, diabetes mellitus, and hs-CRP as covariates. The squared multiple correlation coefficient (R^2) was calculated as a goodness-of-fit measure. Values of $p < 0.05$ were considered statistically significant.

Results

Clinical Characteristics of the Study Group

Table 1 summarizes age, gender, conventional vascular risk factors, ABI, lipid profile, and levels of hs-CRP. Pa-

tient characteristics, including age and the incidence of hypercholesterolemia and angina, were comparable between the ASO and non-ASO groups. The ratio of males to females was higher in the ASO groups. Of the risk factors, the rate of hypertension was higher at Fontaine stages 2 and 3 in the ASO groups than in the non-ASO group ($p < 0.05$), and the rate of smoking was higher at Fontaine stages 1 and 2 in the ASO groups ($p < 0.05$). There was no difference in the rate of angina between the ASO and non-ASO groups. In this study, patients with chronic renal failure on hemodialysis were excluded. The serum concentrations of total cholesterol and LDL-C were lower in the ASO groups than in the non-ASO group ($p < 0.05$).

Serum Levels of S-Glutathionylated Protein

Fig 1A shows the estimated S-glutathionylated protein levels in serum samples from the ASO groups. Statistically significant differences were found among the 4 groups (Kruskal-Wallis test). The median level of S-glutathionylated proteins was 1.06 in non-ASO patients, 2.46 at stage 1, 2.62 at stage 2, and 3.97 at stage 3. The number of males in the non-ASO group was less than in the ASO groups; however, in a preliminary study, there was no difference in the levels of S-glutathionylated proteins between the sexes (data not shown). The levels were increased at every stage of ASO compared with the non-ASO patients ($p < 0.0001$). A significant difference in the levels of S-glutathionylated proteins was observed between stages 2 and 3 ($p < 0.05$). Table 1 and Fig 1B show the serum levels of hs-CRP in the ASO groups; they were higher than in the non-ASO patients ($p < 0.0001$) and increased as the disease developed ($p < 0.05$).

Fig 2 shows a typical result of the analysis of S-glutathionylated proteins using biotin-GST. SDS-PAGE profiles did not differ between sera from non-ASO patients and sera from ASO patients under reduced (Fig 2A, lanes 2, 3) or non-reduced (lanes 4, 5) conditions. S-glutathionylated protein bands were detected more in ASO patients than in non-ASO patients under non-reduced conditions (Fig 2B). In vitro, levels of S-glutathionylated proteins were reduced weakly in the presence of the GSH/GSSG system (Fig 2C, lane 2), and strongly in the presence of the GSH/GSSG system and GRX (lane 3). This suggests that the increase in the serum levels of S-glutathionylated proteins reflects a reduced redox regulation in ASO patients. Immunoprecipitation of proteins by anti-apolipoprotein B100 (apoB100) and treatment with biotin-GST revealed that apoB100 is S-glutathionylated in ASO (Fig 2D), which suggests that the S-glutathionylation of proteins in serum involves apoB100.

The serum levels of total cholesterol and LDL-C were higher in the non-ASO patients than in the ASO patients; however, there was no correlation between the levels of S-glutathionylated proteins and those of total cholesterol or LDL-C. Similarly, the levels of S-glutathionylated proteins did not relate to the levels of triglyceride in serum (data not shown).

Relationship Between S-Glutathionylated Proteins and ABI

The relationship between S-glutathionylated proteins and ABI was analyzed using a multiple linear regression model with covariates (Table 2). The coefficient, standard error, and p-value of S-glutathionylated proteins were -0.0455 , 0.0173 , and 0.0105 , respectively. Similarly, the p-value of both hypertension and smoking was less than 0.05. The data suggest that formation of S-glutathionylated

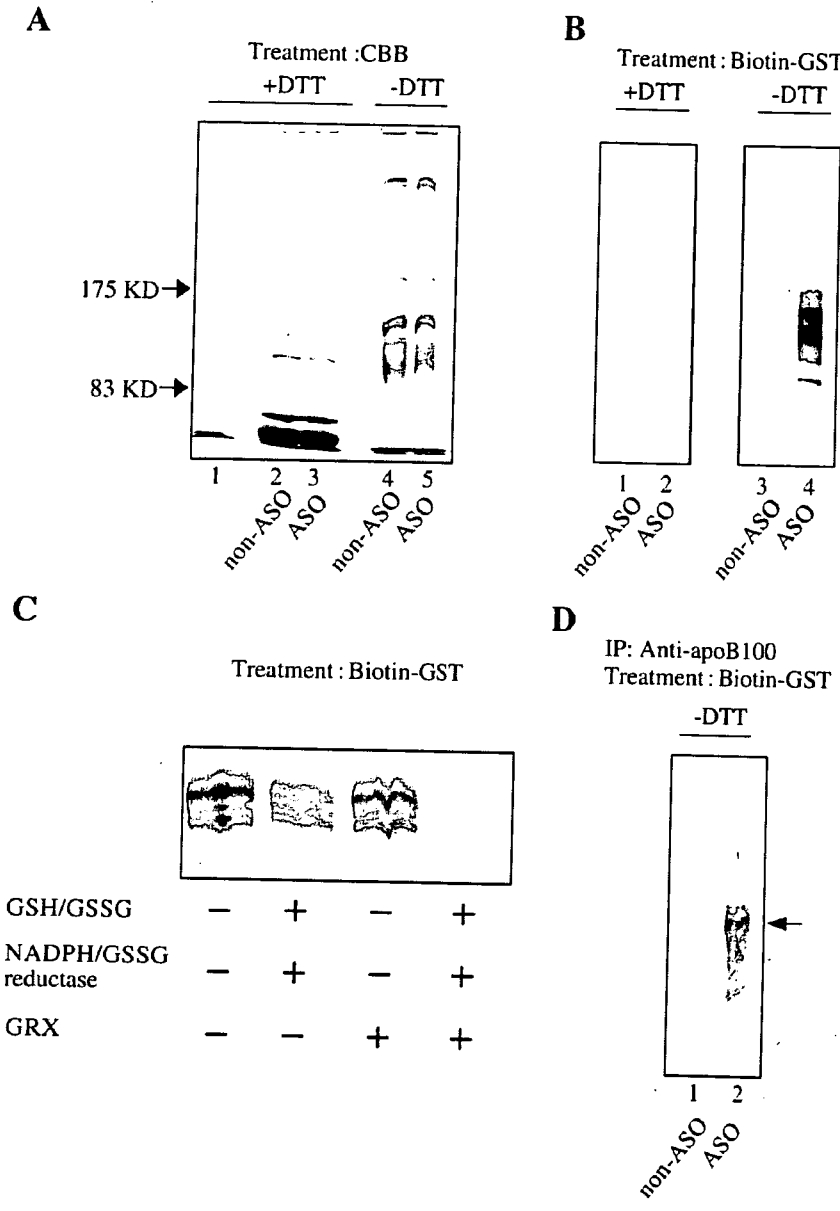


Fig 2. Characteristics of S-glutathionylated proteins. Serum samples from a patient with ASO and another with non-ASO was subjected to 5% SDS-PAGE and analyzed using biotin-GST. (A) CBB stain. Lane 1, molecular weight standard; lane 2, non-ASO with DTT; lane 3, patient with DTT; lane 4, non-ASO without DTT; lane 5, patient without DTT. (B) Staining using biotin-GST under reducing conditions (1 and 2) and non-reducing conditions (3 and 4). (C) Effect of the GSH/GRX system on the S-glutathionylated proteins. From the serum sample of a patient with ASO 1 μ l was treated in buffer containing components of the GSH/GRX system for 30 min at 30°C. GSH/GSSG, 1 mmol/L GSH and 0.05 mmol/L GSSG; NADPH/GSSG reductase, 1 mmol/L NADPH and 1.2 units of GSSG reductase; 1 μ g of GRX (16 samples were subjected to 5% SDS-PAGE under non-reducing conditions and analyzed using biotin-GST. (D) Serum was immunoprecipitated by anti-apoB100 antibody, subjected to 5% SDS-PAGE under non-reducing conditions, and analyzed using biotin-GST. Lane 1, non-ASO patient; lane 2, ASO patient. The arrow indicates a band corresponding to apoB100 protein. ApoB100, apolipoprotein B100; ASO, arteriosclerosis obliterans; CBB, coomassie brilliant-blue; DTT, dithiothreitol; GRX, glutaredoxin; GSH, glutathione; GST, glutathione S-transferase; GSSG, the reduced form of glutathione; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gels.

Table 2 Multiple Linear Regression Model for ABI

Variables	Coefficient	Standard error	p value
S-glutathionylated proteins (relative intensity)	-0.0455	0.0173	0.0105
Hypertension*	-0.189	0.0622	0.00338
Diabetes*	0.0275	0.0503	0.587
Hypercholesterolemia*	-0.0229	0.0514	0.657
Smoking*	-0.170	0.0512	0.00142
hs-CRP (ng/ml)**	-0.0513	0.0286	0.0774

*Positives are defined as 1. Negatives are defined as 0. **Values for hs-CRP were transformed in logarithm of 10. Abbreviations see in Table 1.

proteins in serum is involved in the progress of ASO.

Discussion

As to the diagnosis of ASO, several tests, such as angiography, estimations of ABI and PWV, and measurements of circulating levels of hs-CRP, have been used to

detect PAD. However, these estimations are not sufficient to predict the development of ASO in its earlier stages.

Oxidative stress is a principle cause of aging and the development of diseases such as inflammation, infection, cancer, and cardiovascular disorders^{9,10} Exogenous or endogenous sources of oxidative stress and weakened anti-oxidative defenses can damage macromolecules such as

DNA, lipids, and proteins. The levels of molecules modified by oxidative stress can be estimated; however, there are currently no sensitive and specific methods to evaluate the oxidative stress-induced development of cardiovascular diseases.

The redox system regulates certain protein functions and protects cells from H₂O₂-induced apoptosis.¹⁶ TRX is a protein that is ubiquitously expressed in all living cells and which fulfils a variety of biological functions related to cell proliferation and apoptosis.¹⁷ Increases in serum TRX levels have been found in patients with various coronary risk factors, such as smoking, hypertension, and hypercholesterolemia.¹⁸ Increases in S-glutathionylated proteins have been found in ischemic preconditioned hearts.¹⁹ Those reports suggest that chronic oxidative stress may be involved in the progression of the coronary diseases associated with risk factors. As to the role of GSH/GRX, we previously found that the anti-apoptotic activity of Akt is regulated by the GSH/GRX system inside the cell¹⁶ which led us to speculate that an imbalance of the redox state in serum reflects an impairment of circulatory compartments by oxidative stress, and we became interested in estimating the levels of S-glutathionylated proteins in serum as a marker for the risk of developing peripheral vascular damage. In the present study, serum levels of S-glutathionylated proteins were elevated in the earlier stages of ASO (Fig 1A). Levels of S-glutathionylated proteins in sera from ASO patients were reduced in the presence of the GSH/GRX system (Fig 2C). These results strongly suggest that during the development of ASO, chronic oxidative stress induces an imbalance of the redox state and protein thiols are oxidized in the serum of patients with ASO, although the mechanism of redox regulation to maintain the reduced form of cysteine thiols is not well understood. The application of anti-oxidant therapies, such as α -tocopherol,¹⁸ statins²⁰ or exercise²¹ may improve the redox imbalance and reduce the levels of S-glutathionylated proteins in ASO. If so, estimation of S-glutathionylated proteins is useful as a marker for the success of therapies and trials may be warranted.

Redox-active cysteine residues in the albumin of human serum have been reported;²² however, under the experimental conditions used in the present study, we could not identify S-glutathionylated albumin (data not shown). We found that apoB100 protein is S-glutathionylated (Fig 2D). At present, it is unclear if the thiol-modification of apoB100 affects its function.

The method we used for the estimation of S-glutathionylated proteins used biotinylated GST. However, methods using electrophoresis are neither simple nor sensitive. Attempts have been made to detect S-glutathionylated proteins by a proteomic approach using ³⁵S-labeled GSH in vitro, but this is not a convenient method.²³ Further development of a widely applicable method, such as enzyme-linked immunosorbent assay, is required for use with clinical samples.

ASO is an atherosclerotic peripheral occlusive disease. Oxidized LDL (ox-LDL) appears to play a key role in atherogenesis.²⁴ A circular Ox-LDL, a product of oxidative stress, has been reported in patients with hyperlipidemia.^{25,26} In the present study, there was no relation between lipid metabolism and levels of S-glutathionylated proteins (data not shown), and a pathological comparison of S-glutathionylated proteins with ox-LDL was not conducted.

The relationship between various risk-markers and the development of ASO was analyzed with a multiple regression model (Table 2). The data suggested that levels of

S-glutathionylated proteins in serum are a risk-marker for ASO. Similarly, cigarette smoking was found to correlate with a decrease in ABI, which is consistent with a report that smoking induces low-grade inflammation and thrombogenicity,²⁷ as well as chronic obstructive pulmonary disease.²⁸ It should be taken into account that there are many other factors influencing the progress of ASO, such as drugs, duration of accompanying diseases, genetic background, etc and the analysis here is not sufficient to rule out other risk factors; however, the increase in the serum levels of S-glutathionylated proteins may, at least in part, reflect progression of ASO.

In summary, the S-glutathionylation of proteins in serum may reflect the redox imbalance induced by oxidative stress and play a role in the development of ASO.

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