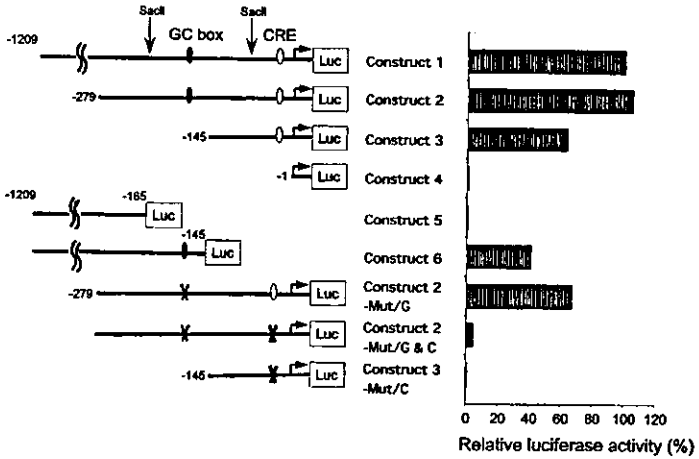

FIG. 6. **Both the GC box and CRE are important for basal activity of the PP2Acα promoter in H9c2 cells.** *Left*, schematic representation of luciferase vector constructs for the rat PP2Acα promoter. Each luciferase vector construct was generated as described under "Materials and Methods." A putative GC box site (−155) was mutated in Constructs 2-Mt/G and 2-Mt/G&C. The CRE site (−26) was mutated in Constructs 3-Mt/C and 2-Mt/G&C. *Arrows* show the transcription initiation site reported by Kitagawa *et al.* (23). *Right*, luciferase activity of the vector constructs for the rat *PP2Acα* gene promoter in H9c2 cells. The cells were transiently transfected with the *PP2Acα* promoter-luciferase gene fusion plasmids. After 24 h of transfection, luciferase activity was assayed with cellular extracts as described under "Materials and Methods." Each value represents the mean of at least three independent experiments, and the S.D. was always within 10% of the mean.

Characterization of PP2Acα Gene Promoter—To investigate the mechanism of the transcriptional regulation of PP2Acα expression, we used the 1.6-kb genomic fragment containing the promoter region of PP2Acα inserted into a luciferase vector, pGL3 Basic. The transcriptional initiation site (nucleotide +1) was denoted in accordance with the report of Kitagawa *et al.* (23) (Fig. 6, *arrows*). The promoter region contains neither a TATA box nor a consensus CAAT sequence. The promoter region contains various transcription factor binding sites such as, CRE at position −26, GC box for Sp1 (31) at positions −155 and −10, and binding sites for receptor-related orphan receptor α (RORα) (32) at positions −778 and −553.

Both CRE and Sp1 Contribute to the Basal Expression of the PP2Acα Gene—In the report by Kitagawa *et al.* (23), a fragment of 118 bp (−162 to −44) was defined as the essential region for the gene expression of *PP2Acα*. As shown in Fig. 6, the deletion fragments of the gene promoter were made from the HindIII-digested fragment (−1209 to +258, full-length) (*Construct 1*), and subcloned into a pGL3 Basic vector. Then, the activity of luciferase was assayed with the cells transfected with each deletion mutant vector. The activity was fully maintained in the 537-bp fragment (−279 to +258) (*Construct 2*), but it decreased to ~65% of the full activity in the 403-bp fragment (−145 to +258) (*Construct 3*) containing the CRE site. In the upstream fragment of 1064-bp (−1209 to −145) (*Construct 6*) containing the GC box, the activity decreased to ~40% of the full activity. However, the activity was almost lost in the upstream fragment of 1044 bp (−1209 to −165) (*Construct 5*) and the downstream fragment of 259 bp (−1 to +258) (*Construct 4*). The results indicated that the full promoter activity was located in the sequence between −279 and −1. To examine whether the CRE and GC box contribute to the full promoter activity, disabled mutants were generated for the consensus sequences of CRE at −26 and GC box at −155 in the luciferase vector containing the 537-bp Construct 2. In Construct 2-Mt/G containing a CRE but no GC box, the activity decreased to ~70% of the full activity, and the level was similar to that of Construct 3. In Construct 2-Mt/G&C, the activity was significantly suppressed to less than 5% of full activity by both mutations. Furthermore, by the mutation with CRE, the activity was completely lost in Construct 3-Mt/C. Taken together, these results indicate that both the CRE and GC box contribute to the basal expression of the *PP2Acα* gene in H9c2 cells.

Thapsigargin Enhanced Protein-CRE Complex Formation—To examine if a DNA-binding protein like Sp1 or CREB could interact with the *PP2Acα* promoter, EMSA was performed with nuclear extracts from the cells treated with 5 μM thapsigargin or 10 μM BAPTA-AM using ^{32}P-labeled oligonucleotides designed for the GC box at −155 and CRE at −26. In the case of the CRE, a major band appeared but it disappeared in the presence of an excess of unlabeled probe (not shown) or ^{32}P-labeled probe with the disabled-mutant for CRE (Fig. 7, *A* and *C*). The intensity of shifted band for CRE significantly increased with the nuclear extracts treated with thapsigargin for 1–2 h (Fig. 7*A*, *left*). In contrast, the intensity decreased with the nuclear extracts treated with BAPTA-AM for 1–2 h (Fig. 7*A*, *right*). In the case of the GC box, a major band appeared but it too disappeared in the presence of an excess of unlabeled probe (not shown) or ^{32}P-labeled probe with the disabled mutant for GC box (Fig. 7, *B* and *C*). However, the band intensity was not influenced by the treatment with thapsigargin or BAPTA-AM (Fig. 7). In the case of the GC box-like site at −10, no major band was observed in EMSA, suggesting that the site is nonfunctional (data not shown). In the case of the RORα site at −553, a gel shift was observed but was not changed by the treatment with Ca^{2+} modulators such as thapsigargin and BAPTA-AM (data not shown). Furthermore, there was no gel-shift observed with the probe for the RORα site at −778 in EMSA, suggesting that the site is nonfunctional (data not shown). Together, these results indicate that the DNA binding activity involves both the CRE at −26 and GC box at −155, but it is specifically influenced by Ca^{2+} modulators, such as thapsigargin and BAPTA-AM, especially in the CRE at −26.

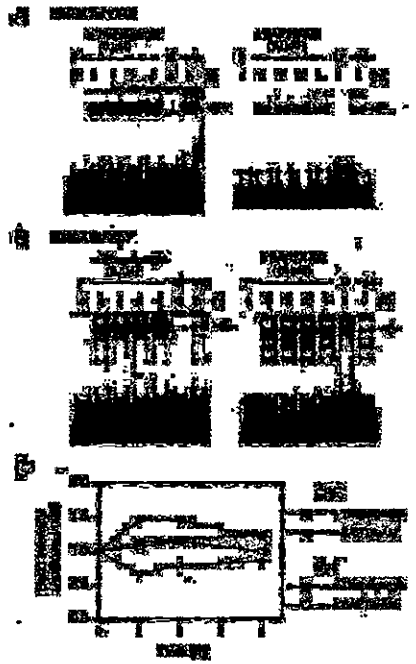

FIG. 7. **Ca^{2+} modulators influence DNA binding to CRE in EMSA.** H9c2 cells were incubated with thapsigargin (5 μM) or BAPTA-AM (10 μM) for the periods indicated, then the nuclear extracts were prepared as described under "Materials and Methods." ^{32}P-labeled oligonucleotides specific to CRE (−26) (*A*) and GC box (−155) (*B*) of the *PP2Acα* gene promoter were prepared and incubated with each nuclear extract, and then subjected to a 6% nondenatured PAGE. In *line F*, nuclear extract is free. In *line M*, ^{32}P-labeled mutant oligonucleotides were used for the original ones. In *line +Ab*, specific antibodies were added to the reaction mixture during the binding reaction for the supershift assay. *C*, quantitative data for the DNA binding to CRE and GC box shown in *A* and *B*. The intensity of gel-shift bands (*arrows*) was estimated densitometrically. Each value represents the mean of three experiments, and the S.D. was always within 10% of the mean.

The Gene Promoter Activity of PP2Acα Is Regulated by Ca^{2+} Modulators via CRE—To confirm the CRE-dependent regulation of the gene expression of PP2Acα by Ca^{2+} modulators, the gene promoter activity was examined by assaying the luciferase activity as describe above. The cells were transfected with luciferase vector construct 3 (−145 to +258) (Fig. 6), which contains a CRE but no GC box. After 24 h of transfection, the cells were treated with 5 μM thapsigargin or 10 μM BAPTA-AM for predetermined periods, and the cell lysates were prepared and subjected to the assay for luciferase activity. As shown in Fig. 8*A*, the activity increased with thapsigargin to ~130% of the initial activity. The increase was not observed in the case of the disabled mutant for CRE with thapsigargin (data not shown). In contrast, with BAPTA-AM, the gene promoter activity decreased to ~85% of the initial level after 3 h of treatment, but returned to the initial level after 4 h (Fig. 8*B*). In the disabled mutant of CRE (*Construct 3-Mut/C* in Fig. 6), the promoter activity was lost and no activity was observed even on treatment with thapsigargin or BAPTA-AM (data not shown). Collectively, the results indicate that the gene expression of PP2Acα is transcriptionally regulated by the change of intracellular Ca^{2+} via CRE, and are consistent with the results of the EMSA for CREB binding (Fig. 6).

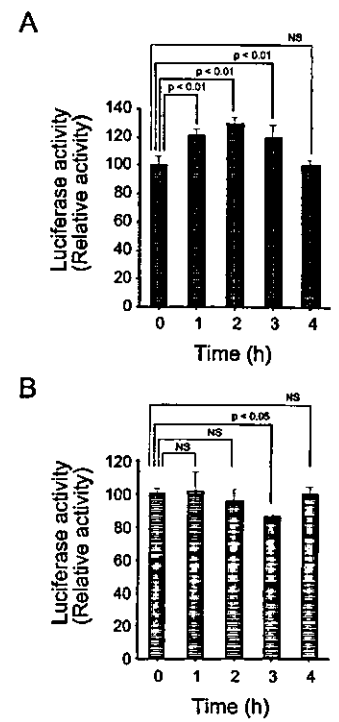

FIG. 8. **Ca^{2+} modulators influence the promoter activity of the rat *PP2Acα* gene in H9c2 cells.** H9c2 cells were transiently transfected with the *PP2Acα* promoter luciferase gene fusion plasmid (−145 to +258) (*Construct 3* in Fig. 6) containing CRE but no GC box. After 24 h of transfection, cells were treated with 5 μM thapsigargin (*A*) or 10 μM BAPTA-AM (*B*) for the periods indicated. Then luciferase activity was assayed with cell nuclear extracts as described under "Materials and Methods." Each value represents the mean ± S.D. of at least three experiments. The statistical analysis was performed with a factorial analysis of variance test.

The Phosphorylation and Intracellular Localization of CREB Is Regulated by Ca^{2+} Modulators in H9c2 Cells—CREB is a pivotal transcription factor for the regulation of cellular survival, and its activation is mediated by phosphorylation at a specific residue, Ser-133 (33). To investigate the effect of Ca^{2+} modulators on the phosphorylation status of CREB, the level of CREB phosphorylated at Ser-133 was examined by immunoblot analysis using specific antibodies in the cells treated with thapsigargin or BAPTA-AM. As shown in Fig. 9*A*, the phosphorylation level of CREB increased on treatment with thapsigar-

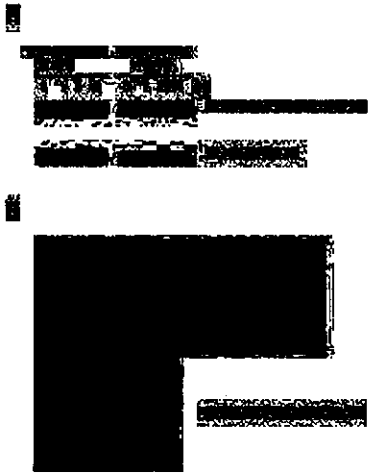

FIG. 9. **The phosphorylation of CREB is regulated by Ca^{2+} modulators in H9c2 cells.** *A*, H9c2 cells were incubated with thapsigargin (5 μM) or BAPTA-AM (10 μM) for the periods indicated, then cell extracts were prepared. The phosphorylation level of CREB was estimated by immunoblot analysis using specific antibodies. *B*, after the treatment with thapsigargin (5 μM) or BAPTA-AM (10 μM) for 2 h, the intracellular localization of phosphorylated CREB was examined by indirect immunofluorescence (*IF*) microscopy using specific antibodies as described under "Materials and Methods." The data represent two independent experiments.

gin, but decreased with BAPTA-AM, indicating that the phosphorylation status is regulated by the change of $[Ca^{2+}]_i$. In Fig. 9*B*, the intracellular localization of phosphorylated CREB (Ser-133) was investigated by indirect immunofluorescence microscopy in cells treated with or without the Ca^{2+} modulators. The fluorescent signals for phosphorylated CREB increased especially in the nucleus of the cells treated with thapsigargin, but it was significantly decreased by BAPTA-AM compared with untreated cells. These results are consistent with the results that the gene of *PP2Ac*α is regulated via CRE in a Ca^{2+}-dependent manner.

DISCUSSION

Akt is a Ser/Thr protein kinase with antiapoptotic and oncogenic activities (8–10). With regard to the Ca^{2+}-dependent regulation of Akt, Conus *et al.* (15) reported that the activation of Akt was independent of Ca^{2+} in mouse fibroblasts treated with thapsigargin. However, Yano *et al.* (13) identified a Ca^{2+}-triggered signaling cascade in which CaMK kinase activates Akt in a PI 3-kinase-independent manner. Then Huber *et al.* (14) found that Akt was rapidly activated by treatment with thapsigargin through the activation of PI 3-kinase. In the present study, we found that Akt was suppressed by a long term elevation of $[Ca^{2+}]_i$ induced by thapsigargin, but was enhanced by a long term lowering of $[Ca^{2+}]_i$ caused by BAPTA-AM in H9c2 cells. The inactivation of Akt is highly correlated with susceptibility to apoptosis. Recently, Luo *et al.* (34) reported that inactivation of Akt is a causal mediator of cell death, and this is consistent with our present results. Although the underlying mechanism for these differences in the Ca^{2+}-dependent regulation of Akt was not clear, we showed that transcriptional regulation of PP2Acα was important to control the activation status of Akt in H9c2 cells under conditions where there is a long term change in $[Ca^{2+}]_i$ levels.

PP2A is a multifunctional protein Ser/Thr phosphatase that regulates a variety of signaling pathways in eukaryotic cells (35, 36, 37). The core structure is a dimer, consisting of a 36-kDa catalytic subunit (PP2Acα, β) and a 65-kDa constant regulatory (structural) subunit (PR65/Aα, β). A third, variable regulatory subunit (B, PR55/Bα, β, γ, δ; B′, PR56/61α, β, γ, δ, ϵ; B″, PR48/58/72/130; B‴, PR99/110) can associate with this core enzyme. There are various reports of PP2A as a positive regulator of apoptosis (38), although a specific subunit of PP2A containing B′/PR61 is reported to be inhibitory for apoptosis in *Drosophila* (39, 40). Bad is a pro-apoptotic member of the Bcl-2 family, whose function is highly regulated by reversible phosphorylation (41). PP2A was responsible for the dephosphorylation of Bad (42), and dephosphorylated Bad bound antiapoptotic Bcl-2 members at the mitochondrial membrane leading to apoptotic cell death (43). PP2A was also found to co-localize at the mitochondrial membrane with Bcl-2, and the proapoptotic sphingolipid ceramide has been shown to activate the PP2A involved (44, 45). In anti-Fas-induced apoptosis, activation of caspase-3 caused cleavage of the regulatory Aα subunit of PP2A, and this in turn increased PP2A activity (46). On the other hand, Liu *et al.* (47) reported that 4-hydroxynonenal induced dephosphorylation of Akt through activation of PP2A in a caspase-dependent apoptosis of Jurkat cells. In the study, the authors described that PP2A was activated by an altered intracellular localization of tyrosine-dephosphorylated PP2A, but not by the caspase-dependent cleavage of the regulatory Aα subunit of PP2A. Furthermore, C2-ceramide induced dephosphorylation of both GSK3β and Akt by activating PP2A, resulting in apoptosis in rat cerebellar granule cells, and the apoptosis was blocked with lithium by inhibiting PP2A activity (48). Together, these findings indicate that PP2A plays a critical role in the positive regulation of apoptosis by dephosphorylating various apoptotic regulators including Akt, but the molecular mechanism for the activation of PP2A in apoptosis is not clearly understood.

PP2A is considered a phosphatase responsible for the dephosphorylation and inactivation of Akt (47, 48, 49, 50, 51, 52, 53). Previously, we also showed that Akt was dephosphorylated by PP2A in H9c2 cells (16), and PP2A interacted transiently with Akt in H9c2 cells under oxidative stress with H_2O_2 (27). In the present study, the treatment with okadaic acid decreased PP2A activity to −70% of the untreated control value, and it reversed the thapsigargin-dependent suppression of the phosphorylation of Akt in H9c2 cells (Fig. 4). Furthermore, treatment with okadaic acid could suppress the thapsigargin-induced enhancement of apoptosis in cells exposed to H_2O_2, although the effective okadaic acid concentration was limited to a range around 100–200 nM (Fig. 5). These findings strongly suggest that PP2A plays an up-regulating role in the thapsigargin-induced enhancement of apoptosis by inhibiting Akt signaling. Collectively, the finding that up-regulation of *PP2Ac*α gene expression led to an increase of PP2A activity is consistent with the enhanced dephosphorylation and inactivation of Akt in H9c2 cells following the long term elevation of $[Ca^{2+}]_i$ due to thapsigargin exposure (Fig. 2). Although the expression of PP2Ac is tightly controlled by an autoregulatory translational mechanism (54), there are reports describing changes in PP2Ac levels, for instance, during all-*trans* retinoic acid-induced differentiation of HL-60 cells (55, 56), during adipocyte differentiation induced by peroxisome proliferator-activated receptor-γ (57), during stimulation by colony-stimulating factor in macrophages (58), and during a response to the disruption of cellular attachment in mouse C3 10T1/2 cells (59).

We also observed transcriptional activation of PP2Acα in H9c2 cells transfected with the expression vector for calreticulin, a molecular chaperone in the endoplasmic reticulum (16). However, the underlying mechanism for these differences in the regulation of PP2A expression was not clarified.

To investigate the molecular mechanism behind the Ca^{2+}-dependent transcriptional regulation of PP2Ac expression in H9c2 cells, the promoter function of the *PP2Ac*α gene was characterized using a luciferase-based reporter assay in cells treated with Ca^{2+} modulators such as thapsigargin and BAPTA-AM. The results showed that the expression of PP2Acα was transcriptionally regulated by the change of $[Ca^{2+}]_i$. The *PP2Ac* gene has been isolated and characterized in humans (60) and rats (23). In both species, the promoter region of PP2Acα has a high GC content and does not contain either a TATA box or a CAAT box, which suggests that the gene is a typical housekeeping gene. Among various transcription factors including CREB, Sp1, and RORα within the promoter region, CREB was revealed to be responsible for Ca^{2+}-dependent regulation of the *PP2Ac*α gene in H9c2 cells treated with thapsigargin and BAPTA-AM. CREB is a bZIP transcription factor that forms homo- or heterodimers with itself or other members of the CREB family including ATF1 and CREM, and is a pivotal transcription factor that regulates cell proliferation, differentiation, and survival in a variety of cell types in vertebrates (33). The CREB dimers interact with a specific DNA sequence having the consensus motif TGACGTCA in the regulatory region of CREB target genes. CREB is inactive as a transcription factor until a cell is exposed to any extracellular stimuli that trigger its phosphorylation at a specific site, Ser-133, within its kinase-inducible domain (33). CREB was originally identified as a target of the cAMP signaling pathway, and regulated in response to diverse signals, including peptide hormones, growth factors, and Ca^{2+}. CREB is known to be activated at high $[Ca^{2+}]_i$ and this is consistent with our findings that the level of CREB phosphorylated at Ser-133 increased with thapsigargin but decreased with BAPTA-AM in the nucleus of H9c2 cells. Though minute changes in $[Ca^{2+}]_i$ are quickly transformed into changes in the activity of several kinases including cAMP-dependent kinase, protein kinase C, MAPKs, Ca^{2+}/CaMK and CaMK kinase, it is not clear whether these kinases are influenced in the case of long term change of $[Ca^{2+}]_i$ in H9c2 cells treated with Ca^{2+} modulators. Among these kinases, CaMKII and CaMKIV were reported to be able to phosphorylate CREB directly (61, 62). In addition, Rsk protein kinase phosphorylates CREB at Ser-133 through activation of the Ras/MAPK signaling pathway by Ca^{2+} (63, 64). Although CaMKIV mediates the early phase in the phosphorylation of Ser-133 in membrane-depolarized neurons, the MAPK pathway is responsible for prolonging the phosphorylation (65). In the present study, an increase in both the phosphorylation of CREB at Ser-133 and activity of CREB to bind the CRE site was observed after 2 h treatment with thapsigargin, suggesting a late activation of CREB caused by the long term elevation of $[Ca^{2+}]_i$. The treatment with BAPTA-AM influenced the phosphorylation of CREB and suppressed the binding of CREB to the CRE after 1 h, and this also suggests a late inactivation of CREB on the long term suppression of $[Ca^{2+}]_i$. Taken together, the long term change of $[Ca^{2+}]_i$ may regulate CREB function through the MAPK pathway rather than CaMK pathway in H9c2 cells treated with Ca^{2+} modulators.

Thapsigargin causes an increase of $[Ca^{2+}]_i$, and is also known as an inducer of endoplasmic reticulum (ER) stress (unfolding protein stress in the ER) (66). Recently, Song *et al.* (67) reported that thapsigargin-induced ER stress induced dephosphorylation of both GSK3β and Akt by activating PP2A, resulting in apoptosis in neuroblastoma cells, and this was consistent with our results. In the study, the authors described that dephosphorylation of GSK3β by activated PP2A was critical for the activation of caspase-3 in ER stress-induced apoptosis, but the mechanism for the PP2A activation by ER stress was not clarified. In ER stress, a transcriptional up-regulation is seen in the ER stress responsive genes that code a variety of ER proteins related to molecular chaperone functions, such as BiP/Grp78, Grp94, Grp58/ERp57, ERp72, and calreticulin (66). In mammalian cells, the 19-nucleotide motif CCAAT-N9-CCACG was identified as an ER responsive element (ERSE) of various ER chaperone genes, and was recognized by the human bZIP transcriptional factor ATF6 for ER stress response (68). However, we could not find a consensus sequence within the 1.6-kbp promoter region of the *PP2Ac*α gene, and this suggests that the *PP2Ac*α gene is not a direct target for the ER stress response in thapsigargin-treated cells.

In this study, CREB was linked to the enhanced susceptibility to apoptosis through the induction of the *PP2Ac* gene in cells exposed to the long term elevation of $[Ca^{2+}]_i$. To further investigate whether CREB is specifically responsible for the up-regulation of apoptosis in cells treated with thapsigargin and H_2O_2, CREB expression was suppressed by the short interfering RNA (siRNA) for the CREB gene. Using mammalian CREB siRNA expression plasmid (pKD-CREB-v2, Upstate Biotechnology), the CREB expression level was suppressed in H9c2 cells to ~30% of non-transfected cells. Using the transfected cells, thapsigargin-dependent enhancement of cell damage and apoptosis were examined in cells treated with thapsigargin (5 μM) and H_2O_2 (75 μM). However, despite the suppression of CREB protein, cell damage and apoptosis were not inhibited in cells treated with thapsigargin and H_2O_2, but rather were more enhanced (data not shown). This indicates that CREB is not specifically responsible for the mechanism enhancing apoptosis in cells showing long term elevation of $[Ca^{2+}]_i$. However, this may be reasonable because CREB is well known as a transcription factor for cell survival and antiapoptotic genes such as Bcl-2 (69), and the suppression of CREB may firstly decrease the expression of such cell survival genes resulting in enhanced susceptibility to apoptosis. The effect of the suppressed expression of CREB on cell survival was also consistent with previous findings using dominant-negative CREB polypeptides (69). Although the suppressed expression of CREB itself did not specifically reduce the thapsigargin-dependent enhancement of apoptotic cell damage in H9c2 cells, it still may be possible that CREB works for apoptosis on some negative feedback-like loop of cell survival signaling in cells demonstrating long term elevation of $[Ca^{2+}]_i$.

In conclusion, we found that the Akt kinase pathway was regulated by a long term change of $[Ca^{2+}]_i$ through transcriptional regulation of PP2Acα. With an elevation of $[Ca^{2+}]_i$ induced by thapsigargin, *PP2Ac*α gene expression is up-regulated through the activation of CRE at a late phase of the response. As a consequence, Akt is dephosphorylated and inactivated by PP2A, and this leads to an increase in susceptibility to apoptosis under the conditions with thapsigargin. Although the activation of CRE has been considered to function as a cell survival signaling, Ca^{2+}-induced late activation of CRE leads to an enhancement of apoptotic signaling, and this suggests some feedback mechanism of CRE-mediating cell survival signaling. Luo *et al.* (34) reported that NMDA-induced deactivation of Akt was causative of neural cell death, and this suggests some Ca^{2+}-dependent mechanism is involved in the inactivation of Akt. Although the Ca^{2+}-dependent regulation of cell survival and death has been extensively studied (1, 3, 4), the

present findings may indicate another novel regulatory pathway of Akt through PP2A in the cell survival signaling controlled by calcium homeostasis.

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Original Contribution

NUCLEAR GLUTATHIONE *S*-TRANSFERASE π PREVENTS APOPTOSIS BY REDUCING THE OXIDATIVE STRESS-INDUCED FORMATION OF EXOCYCLIC DNA PRODUCTS

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Abstract—We previously found that nuclear glutathione *S*-transferase π (GSTπ) accumulates in cancer cells resistant to anticancer drugs, suggesting that it has a role in the acquisition of resistance to anticancer drugs. In the present study, the effect of oxidative stress on the nuclear translocation of GSTπ and its role in the protection of DNA from damage were investigated. In human colonic cancer HCT8 cells, the hydrogen peroxide (H_2O_2)-induced increase in nuclear condensation, the population of sub-G_1 peak, and the number of TUNEL-positive cells were observed in cells pretreated with edible mushroom lectin, an inhibitor of the nuclear transport of GSTπ. The DNA damage and the formation of lipid peroxide were dependent on the dose of H_2O_2 and the incubation time. Immunological analysis showed that H_2O_2 induced the nuclear accumulation of GSTπ but not of glutathione peroxidase. Formation of the 7-(2-oxo-hepyl)-substituted $1,N^2$-etheno-2′-deoxyguanosine adduct by the reaction of 13-hydroperoxyoctadecadienoic acid (13-HPODE) with 2′-deoxyguanosine was inhibited by GSTπ in the presence of glutathione. The conjugation product of 4-oxo-2-nonenal, a lipid aldehyde of 13-HPODE, with GSH in the presence of GSTπ, was identified by LS/MS. These results suggested that nuclear GSTπ prevents H_2O_2-induced DNA damage by scavenging the formation of lipid-peroxide-modified DNA.

Keywords—Oxidative stress, DNA damage, Glutathione *S*-transferase π, 7-(2-Oxo-hepyl)-substituted $1,N^2$-etheno-2′-deoxyguanosine adduct, Free radical

INTRODUCTION

The role of oxidative stress as a mediator of apoptosis has been extensively studied. In particular, hydrogen peroxide (H_2O_2), a by-product of oxidative stress and a major reactive oxygen species (ROS), has been implicated in triggering apoptosis in various cells. H_2O_2 induces peroxidation of cellular components such as proteins, lipids, and nucleic acids [1]. H_2O_2 also stimulates intracellular signal cascades, such as mitogen-activated protein kinases, and activates transcription factors, such as AP-1 and nuclear factor kappa-B [2].

Glutathione *S*-transferase (GST, EC 2.5.1.18) is mainly expressed in the cytoplasm and is ubiquitous in nature. GST functions in xenobiotic biotransformation [3], drug metabolism [4], protection against peroxidative stress of lipids and the nucleus [5–7], and isomeration of prostaglandins [8]. Human GSTπ is one of a family of

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GSTs; it has been reported to accumulate in various human cancer tissues or precancer tissues and is employed in cancer research as a tumor marker [9–13]. An increase in GSTπ was also found in cancer cell lines resistant to doxorubicin hydrochloride (DOX), *cis*-diamminedichloroplatinum(II) (cisplatin: CDDP) [14–16], and alkylating agents [17].

In addition to its main location in the cytoplasm, GSTπ has been found in the nucleus in uterine cancer cells [18] and glioma cells [19]. These findings suggest a negative correlation between the existence of GSTπ in the nucleus of cancer cells and the survival of the patient. However, there has been no report on the mechanisms responsible for the nuclear survival of GSTπ or on the physiological role of nuclear GSTπ.

Edible mushroom lectin (*Agaricus bisporus* lectin; ABL) efficiently internalizes into the cytoplasm of cultured cells, localizes around the nucleus, and inhibits the nuclear transfer of proteins [20]. Previous reports presented evidence that ABL inhibits the nuclear transport of GSTπ and increases the sensitivity of cancer cells to anticancer drugs [21,22].

Endogenous lipid peroxidation products react with DNA and exocyclic DNA adducts to cause the covalent modification of nuclear bases [23,24]. During the lipid peroxidation process, lipid hydroperoxides are formed as the initial products, and the decomposition of the lipid hydroperoxides leads to the formation of aldehydes as the end products. Several aldehydes possess high reactivity against DNA bases, especially guanine [25–27]. Lipid-peroxide-induced DNA adduct formation and site-specific cleavage of double-stranded DNA have been reported [28,29]. Previously, Kawai et al. [30] studied the reaction of lipid hydroperoxides with DNA components and established a method to detect the formation of 7-(2 oxo-hepyl)-substituted $1,N^2$-etheno-2′-deoxyguanosine adducts (oxo-heptyl-εdG) by the reaction of 13-hydroperoxyoctadecadienoic acid (13-HPODE) with 2′-deoxyguanosine (dG).

Recently, it was reported that 4-hydroxy-2-nonenal (4-HNE) and 4-oxo-2-nonenal (4-ONE), the end products of lipid peroxides, are nonenzymatically transformed to conjugate with GSH [31]. Moreover, 4-ONE, a major end product of 13-HPODE, had a higher affinity for the nucleus than 4-HNE. Even though it has been found that GSTs catalyzes the formation of a conjugate of 4-HNE with GSH [32], its role in the formation of 4-ONE-GSH adducts was not known. In this study, we examined whether the nuclear GSTπ plays a role in the cellular sensitivity to oxidative stress caused by H_2O_2 and found that GSTπ prevents DNA damage by scavenging the oxo-heptyl-εdG formed from 13-HPODE and forming a conjugate of 4-ONE with GSH.

MATERIALS AND METHODS

Materials

ABL was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Invitrogen Corp. (Carlsbad, CA). Sheep polyclonal antibodies against human glutathione peroxidase (GPX) were purchased from The Binding Site Ltd. (Birmingham, UK). Horseradish peroxidase (HRP)-labeled anti-rabbit IgG, HRP-labeled anti-mouse IgG, and HRP-labeled anti-sheep IgG were from DAKO A/S (Glostrup, Denmark). The Enhanced Chemiluminescence (ECL) kit was obtained from Amersham Biosciences (Buckinghamshire, UK). All other chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO).

Preparation of cells

We used the human cancer cell lines HCT8 (colonic carcinoma) kindly donated by Dr. K. J. Scanlon. HCT8 cells were supplemented with 10% FBS at 37°C in 5% CO2 with 100% humidity. Six hours before treatment with ABL, the cells were maintained in medium with 1% FBS. About 2×10^6 cells were harvested with trypsin and washed with phosphate-buffered saline (0.137 M NaCl, 2.68 mM KCl, and 10 mM NAH2PO4/Na2HPO4, pH 7.4: PBS) twice at 4°C. The pellets were stored at −80°C before use.

TUNEL assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using an Apop Tag Plus Fluorescein in situ Apoptosis Detection Kit (Intergen Co., Purchase, NY). Briefly, approximately 2×10^6 cells were harvested, fixed in 70% ethanol, treated with terminal deoxynucleotidyl transferase for 1 h and then flourescein isothiocyanate (FITC) conjugate anti-digoxigenin for 1 h at room temperature, washed with 0.1% Triton X-100/PBS, and resuspended in propidium iodide containing RNase A. Fluorescence intensity was estimated simultaneously using a FACScan flow cytometer (Becton–Dickinson, San Jose, CA).

Nuclear condensation

For the histochemical analysis, HCT8 cells were maintained with RPMI 1640 medium containing 10% FBS in a four-well Lab Tec Chamber (Nalge Nunc International, Naperville, IL). After treatment with H_2O_2, cells were treated with 10 μM Hoechst 33342 for 30 min to estimate the extent of nuclear condensation. They were then washed again with PBS. Fluorescence intensity was examined using an Axioskop2 fluorescence microscope (Carl Zeiss, Jena, Germany), and the findings were

analyzed using a charge-coupled device camera (Axio-Cam) and AxioVison software.

Analysis of double-stranded breaks of DNA

DNA damage was determined by flow cytometry, based on the formation of sub-G1 peaks of DNA as described by Gong et al. [33]. HCT8 cells were washed with PBS, fixed with 70% ethanol for 12 h at -20°C, and then centrifuged and further incubated with citrate–phosphate buffer (1 v of 0.1 M citric acid and 24 v of 0.2 M Na_2HPO_4) for 15 min at 25°C. The DNA content per nucleus was evaluated in a FACScan flow cytometer after the nuclei were stained with propidium iodide.

Preparation of proteins

The cytoplasmic and nuclear proteins were prepared as described by Digram et al. [34]. Proteins in the whole cells were prepared as described previously [35].

Preparation of antibodies

GSTπ was purified from human placenta, and polyclonal antibody against human GSTπ was obtained by immunizing rabbits as described previously [21]. The monoclonal antibody to Oxo-heptyl-edG was prepared as described previously [30].

Immunological estimation

Immunological levels of GSTπ in the cytoplasm and nucleus were estimated by Western blotting. Lysate from the extract of cells was separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in a 12.5% gel, transferred to a nitrocellulose membrane, and immunologically stained using rabbit IgG against human GSTπ or sheep IgG against human GPX as the primary antibody and HRP-labeled anti-rabbit IgG or HRP-labeled anti-sheep IgG as the secondary antibody. Blots were developed by enhanced chemiluminescence using the ECL kit and the relative immunological activity was analyzed by NIH Image. The protein concentration was determined according to Redinbaugh and Turley [36], with bovine serum albumin as the standard.

Estimation of lipid peroxide in the nucleus

Nuclei extracts were prepared as described by Abmayr and Warkman [37]. Nuclear thiobarbituric acid reactive substance (TBARS) levels were determined according to the method of Ohkawa et al. [38] using tetramethoxyprepane (Wako Pure Chemical Industries).

Estimation of oxo-heptyl-edG

Cells incubated in various conditions were harvested with trypsin and washed with PBS two times at 4°C. The cells were then suspended in 10 mM citrate buffer (pH 6.0) and incubated for 10 min at 95°C. After a wash with PBS two times, the cells were suspended in 2 M HCl for 30 min at room temperature and rewashed with PBS two times. The levels of oxo-heptyl-edG in the cells were estimated by flow cytometry using anti-oxo-heptyl-edG mouse monoclonal antibody (mAb6A3) and FITC-conjugated anti-mouse IgG antibody.

Effect of GSTπ on the formation of oxo-heptyl-edG

13-HPODE (20 mM) was mixed with 1 mM $FeCl_2$ and stood for 12 h at 37°C. The solution (13-HPODE, 5 mM and $FeCl_2$, 0.2 mM, as a final concentration) was incubated with or without GSH (1 or 5 mM) and GSTπ (0.2 U) in the presence of 5 μg of calf thymus DNA for 1 h at 37°C. Then 1 and 5 μg of DNA extracted from the solution by ethanol precipitation

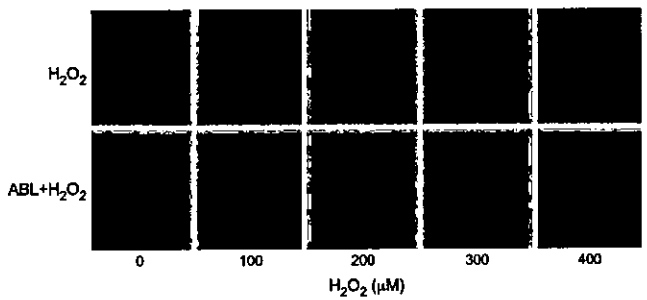

Fig. 1. Nuclear condensation. For the estimation of nuclear condensation, cells were incubated in a four-well Lab Tec Chamber. After treatment with various concentrations of H_2O_2 for 24 h, cells were treated with 10 μM Hoechst 33342 for 30 min for the estimation of nuclear condensation (top). The observation of fluorescence intensity was done using an Axioskop fluorescence microscope, and the findings were analyzed using a charge-coupled device camera and Axio Vision software. Cells were pretreated with ABL (40 μg/ml) for 10 h and then treated with H_2O_2 (bottom).

were spotted on a nitrocellulose membrane and immunologically stained using MAb6A3 as the primary antibody and HRP-conjugated anti-mouse IgG antibody as the secondary antibody. Blots were developed by enhanced chemiluminescence using the ECL kit and the relative immunological activity was analyzed by NIH Image.

Liquid chromatography/mass spectrometry

The chemical structure of the product of the incubation of 4-ONE and GSH in the presence of GSTπ was characterized by liquid chromatography/mass spectrometry (LC/MS). The LC/MS was conducted using a Platform II (VG Biotech) in an electrospray ionization positive (ESP+) mode. The gradient condition (solvent A, 0.01% trifluoroacetate; solvent B, acetonitrile containing 0.01% trifluoroacetate) was as follows: 100% A (0 min), 50% B (20 min), 100% B (30 min), 100% B hold (30–35 min), 100% A (40 min).

Statistical analysis

Data are presented as the mean ± SD. Differences were examined using a Student *t* test. A value of $p < 0.05$ was considered significant.

RESULTS

Nuclear condensation

Nuclear condensation is a characteristic of apoptosis. The nuclear condensation caused by H_2O_2 was estimated morphologically using Hoechst 33342 (Fig. 1). Human colonic cancer HCT8 cells were incubated with various concentrations of H_2O_2 for 24 h. No DNA condensation was observed (100–400 μM H_2O_2). ABL, a mushroom lectin, inhibits the nuclear transfer of GSTπ [1]. The cells were previously treated with 40 μg/ml of ABL for 10 h and further incubated with H_2O_2 for 24 h. Nuclear condensation was observed in

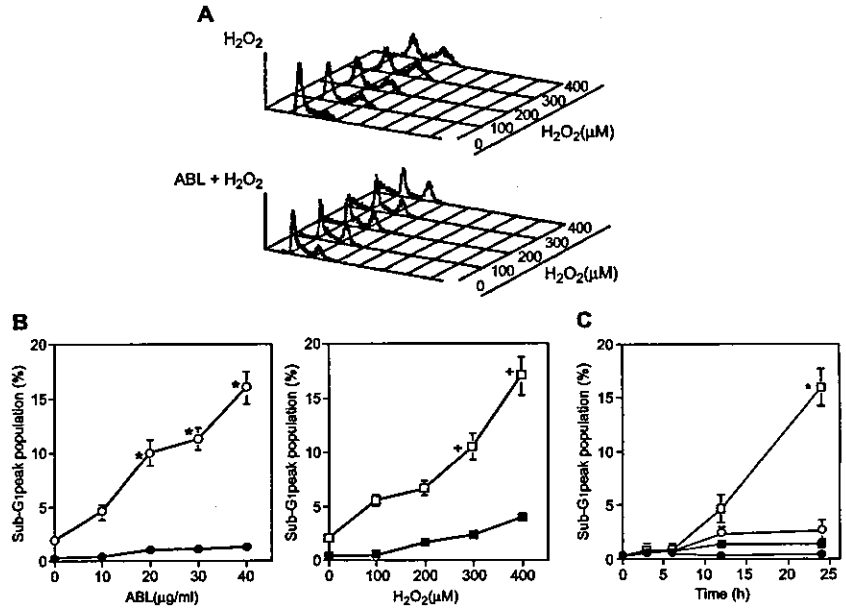

Fig. 2. Flow cytometric analysis of the DNA damage. (A) Effect of H_2O_2 on the DNA damage was analyzed using a FACScan flow cytometer. The sub-G_1 peak was estimated as a marker of the double-strand break of DNA. Treatment of cells with H_2O_2 or pretreatment with ABL was performed as described in Fig. 1 legend. (B) Effect of various concentrations of ABL (left) and H_2O_2 (right) on the formation of the sub-G_1 peak (%). ○400 μM H_2O_2 (+); ●H_2O_2 (−); □40 μg/ml ABL (+); ■ABL (−). *$p < 0.05$ compared with cells without H_2O_2 treatment. †$p < 0.05$ compared with cells without ABL pretreatment. (C) Effect of incubation time on the formation of the sub-G_1 peak (%). ○400 μM H_2O_2 (+); ●control; □40 μg/ml ABL (+); □400 μM H_2O_2 with ABL pretreatment. *$p < 0.05$ compared with H_2O_2-treated cells. Data are the means of three independent analyses. Bars show the SD.

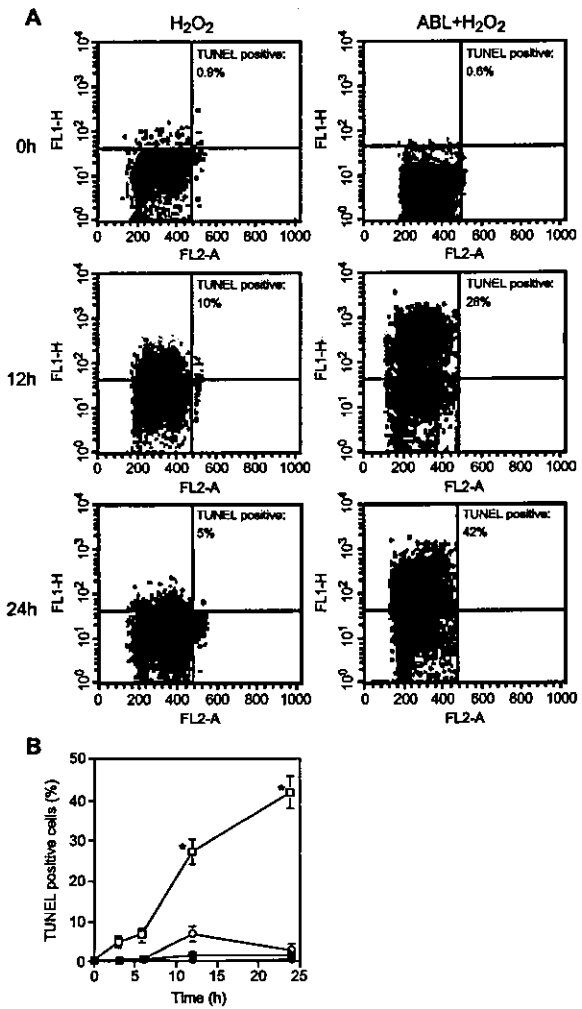

Fig. 3. TUNEL assay. The effect of ABL on the cytotoxicity of H_2O_2 was examined by TUNEL assay using an Apop Tag Plus Fluorescein In Situ Apoptosis Detection Kit as described under Materials and methods. (A) Cells (2×10^5) treated with 400 μM H_2O_2 for 12 and 24 h (left) or pretreated with ABL (40 μg/ml) for 10 h (right). (B) Effect of incubation time on the TUNEL-positive cells (%). ○400 μM H_2O_2 (+); ●control; □40 μg/ml ABL (+); ■400 μM H_2O_2 with ABL pretreatment. Data are the means of three independent analyses. Bars show the SD. *$p < 0.05$ compared with H_2O_2-treated cells.

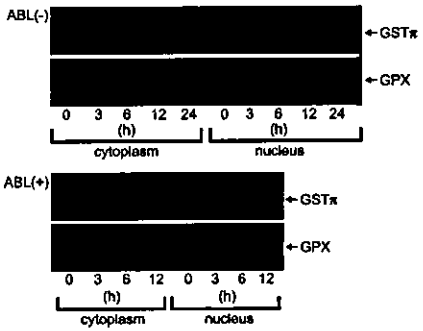

Fig. 4. Immunological estimation of the amount of GSTπ in the cytoplasm and nucleus. Proteins prepared from cellular cytoplasm and nucleus (1×10^5 cells) were separated by SDS–PAGE in a 12.5% gel, transferred to a nitrocellulose membrane, and immunologically stained using rabbit IgG or sheep IgG antibody against human GSTπ or GPX. Cells were treated with 400 μM H_2O_2 for the period indicated with (bottom) or without (top) pretreatment with ABL for 10 h.

a manner dependent on the dose of H_2O_2 when the cells were pretreated with ABL. These results suggest that inhibition of the nuclear transfer of GSTπ by ABL enhanced the H_2O_2-induced DNA damage.

Effect of H_2O_2 on DNA damage

To understand the extent of the DNA damage by H_2O_2, the sub-G_1 peak was estimated flow cytometrically as a marker of the double-stranded breaks of DNA (Fig. 2A). The DNA damage induced by pretreatment with ABL was not apparent (Fig. 2B, left) and damage was slightly induced by H_2O_2 alone (Fig. 2B, right). ABL increased the population of the sub-G_1 peak induced by 400 μM H_2O_2 in a dose-dependent (10–40 μg/ml) manner (Fig. 2B, left). Pretreatment with 40 μg/ml of ABL for 10 h enhanced the population of the sub-G_1 peak induced by H_2O_2 (100–400 μM) for 24 h dose dependently (Fig. 2B, right). The effect of ABL on the H_2O_2-induced DNA damage was dependent on the incubation time with H_2O_2 (Fig. 2C). These results suggest that ABL increases the sensitivity of cells to H_2O_2.

TUNEL assay

The H_2O_2-induced DNA damage was also estimated by TUNEL assay (Fig. 3). At 400 μM H_2O_2 increased the proportion of TUNEL-positive cells in 12 h (10%) with a subsequent decrease at 24 h (5%). Pretreatment with ABL (40 μg/ml) caused an increase in TUNEL-positive cells induced by 400 μM H_2O_2, 28% in 12 h and 42% in 24 h (Figs. 3A and 3B). These results also suggest that ABL increases the sensitivity of cells to H_2O_2, leading to DNA damage and apoptosis.

Immunological estimation of nuclear GSTπ

The amount of GSTπ induced by H_2O_2 was estimated. Fig. 4 shows results of Western blotting for GSTπ. H_2O_2 increased the levels of cytoplasmic GSTπ in a time-dependent manner (top). A concomitant increase in the level of GSTπ was observed in the nucleus. The effect of ABL on the nuclear transfer of GSTπ was studied (bottom). The H_2O_2-induced transfer of GSTπ to the nucleus was inhibited by ABL. The results were consistent with previous data [21].

In the cytoplasm, H_2O_2 is detoxified to H_2O by GPX. Then, the immunological activity of GPX was estimated (Fig. 4). Treatment with H_2O_2 increased the levels of GPX in the cytoplasm. During the experiment, no GPX was transferred to the nucleus. Similarly, the immunological activity of glutathione reductase was not found in the nucleus in the presence or absence of H_2O_2 (data not shown).

Estimation of lipid peroxide in the nucleus

The formation of lipid peroxides was determined as the TBARS levels (Fig. 5). H_2O_2 increased the levels of nuclear TBARS when the cells were pretreated with ABL.

Role of nuclear GSTπ

Kawai et al. [30] reported that DNA bases are modified with lipid peroxide of linoleic acids leading to DNA damage. 4-ONE is nonenzymatically formed from 13-HPODE, which reacts with dG to form oxo-heptyl-edG. Doorn and Petersen [31] reported that 4-ONE has a higher affinity for nucleotides than 4-HNE and, on the other hand, spontaneously reacts with GSH to form its GSH conjugate [31]. We then speculated that

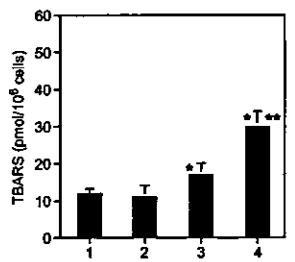

Fig. 5. Estimation of lipid peroxide in the nucleus. The formation of the nuclear TBARS was determined using tetramethoxyprepane. Data are the means of three independent analyses. Bars show the SD. *$p < 0.05$ compared with control cells. **$p < 0.05$ compared with H_2O_2-treated cells.

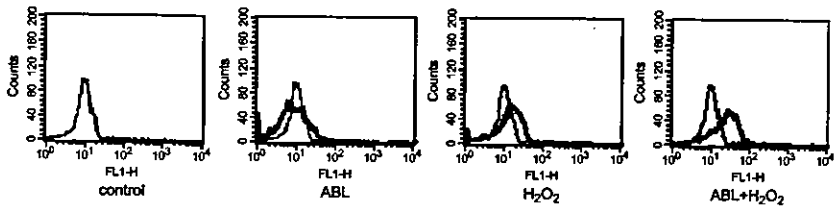

Fig. 6. Immunological estimation of oxo-heptyl-εdG. Effects of ABL (panel 2), H_2O_2 (panel 3), and H_2O_2 with ABL pretreatment (panel 4) on the levels of oxo-heptyl-εdG in the cells were estimated by flow cytometer using anti-oxo-heptyl-εdG mouse monoclonal antibody (mAb6A3) and FITC-conjugated anti-mouse IgG antibody.

human GSTπ catalyzed the formation of 4-ONE conjugated with GSH, which can then prevent the DNA from being modified with lipid peroxide. The immunological activity of lipid-peroxide-modified DNA was estimated flow cytometrically using anti-oxo-heptyl-εdG. The formation of oxo-heptyl-εdG was observed following treatment with 400 μM H_2O_2 for 12 h (Fig. 6, panel 3) and increased on pretreatment with ABL (Fig. 6, panel 4). The possible role of GSTπ in preventing the formation of lipid peroxide-DNA was affirmed in vitro. A mixture of 13-HPODE and $FeCl_2$ stood for 12 h at 37°C. The mixture was incubated with calf thymus DNA for 1 h at 37°C in the presence or absence of 5 mM GSH and 0.2 U of GSTπ. The formation of oxo-heptyl-εdG was estimated from immuno blots (Fig. 7). The formation of oxo-heptyl-εdG was inhibited by 20% in the presence of GSH (Fig. 7, lane 2) and by 60% in the presence of GSTπ and GSH (Fig. 7, lane 4). The results suggest that GSTπ inhibits the formation of oxo-heptyl-εdG in the nucleus. Fig. 8 shows the results of LC/MS measurements of the adduct formation of 4-ONE and GSH in the presence or absence of GSTπ. In the absence of GSTπ, the LC/MS analysis of the product gave a pseudomolecular ion peak [M + H]+ at *m/z* 462 (Fig. 8B). In the presence of GSTπ, this value apparently increased (Fig. 8C). Since the possible molecular weight of the ONE-GSH adduct is 641.18 (Fig. 9), the data obtained by LC/MS support the idea that GSTπ catalyzes the formation of the product.

DISCUSSION

In this study, we found for the first time that nuclear GSTπ functions to scavenge lipid-peroxide-induced DNA damage. We showed that (1) hydrogen peroxide increased the modification of nuclear DNA induced by lipid peroxide to cause DNA damage followed by the induction of apoptosis, (2) the nuclear GSTπ prevented DNA damage from lipid peroxide by scavenging the oxo-heptyl-εdG formed by the reaction of 13-HPODE with dG (the product of the conjugation of 4-ONE, one of the major breakdown products of 13-HPODE, with GSH catalyzed by GSTπ was identified), and (3) ABL inhibited the nuclear transfer of GSTπ to increase the sensitivity of the nucleus to oxidative stress. These findings suggest that nuclear GSTπ prevents H_2O_2-induced DNA damage by scavenging lipid-peroxide-modified DNA.

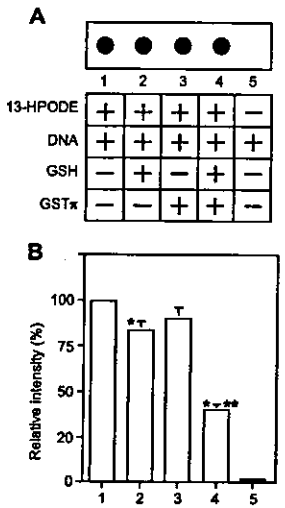

Fig. 7. Effect of GSTπ on the formation of oxo-heptyl-εdG in vitro. (A) 13-HPODE was mixed with $FeCl_2$ and stood for 12 h at 37°C. The solution was incubated with or without GSH and GSTπ in the presence of calf thymus DNA for 1 h at 37°C. DNA extract was spotted on a nitrocellulose membrane and immunologically stained using MAb6A3 as the primary antibody and HRP-conjugated anti-mouse IgG antibody as the secondary antibody. Blots were developed by enhanced chemiluminescence using the ECL kit and the relative immunological activity was analyzed by NIH Image. (B) Relative intensity (%) of the levels of oxo-heptyl-εdG in each lane corresponds to A. Data are the means of three independent analyses. Bars show the SD. *$p < 0.05$ compared with control cells; **$p < 0.05$ compared with H_2O_2-treated cells.

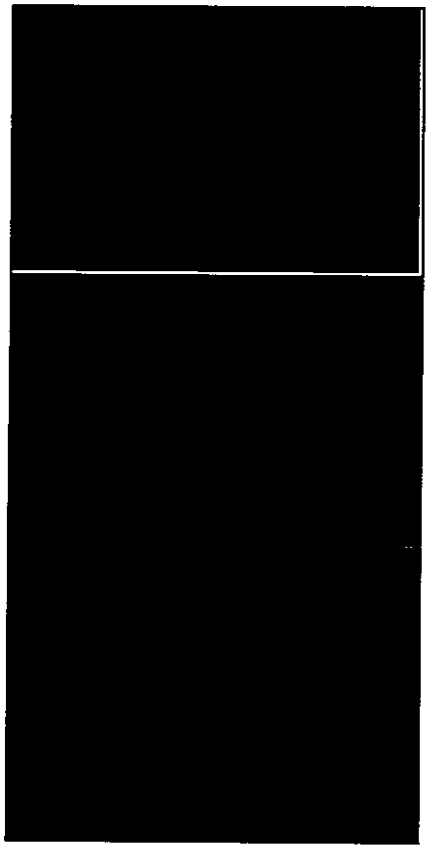

Fig. 8. LC/MS analysis. The chemical structure of the product of the incubation of 13-HPODE and GSH in the presence or absence of GSTπ was characterized by LC/MS. Materials were prepared as described in Fig. 6 legend. (A) 13-HPODE; (B) 13-HPODE with GSH; (C) 13-HPODE with GSH and GSTπ. Arrow indicates 4-ONE-GSH adduct.

Previously, we found that the nuclear GSTπ is an important factor in the acquisition of drug resistance in cancer cells [21,22]. Cancer cells which expressed the nuclear GSTπ in response to anticancer drugs such as DOX and CDDP showed resistance to these drugs, whereas the cells that did not express nuclear GSTπ were more sensitive to the drugs. The conjugation of the drugs with GSH was found in the resistant cells and correlated with decreased drug-induced DNA damage. In the present study, H_2O_2-induced DNA damage was observed when the cells were previously treated with ABL, an inhibitor of the nuclear transfer of GSTπ (Figs. 1–3). Strikingly, HCT8 cells were not sensitive to H_2O_2 (-400 μM). Treatment of the cells with H_2O_2 increased the nuclear transfer of GSTπ in a dose- (data not shown) and time-dependent manner (Fig. 4). The resistance of HCT8 cells to oxidative stress was abolished by pretreatment with ABL. The results strongly suggest an important role for the nuclear GSTπ in the sensitivity of the cells to oxidative stress.

There are many antioxidants in cells. Most of them are localized in the cytoplasm. In addition, each microorgan possesses its own defense system against oxidative stress. A nuclear superoxide dismutase, GPX, and GSTπ have been reported [21,39,40].

Adler et al. [41] reported that GSTp associates with Jun N-terminal kinase (JNK) to regulate its activity in mouse fibroblast NIH3T3 cells. Moreover, Yin et al. [42] demonstrated that GSTp coordinates the activation of extracellular signal-regulated kinases/p38 mitogen-activated protein kinase/inhibitor of κ kinase and suppression of JNK as part of the mechanism underlying its ability to elicit protection against H_2O_2-induced cell death. These findings indicate that GSTp plays an important role in the defense system against oxidative stress through its function as a regulator of stress kinases. It is interesting that GSTp has at least two different functions, to scavenge lipid peroxide and to regulate stress kinases as an antioxidant.

Lipid hydroperoxides are known to be relatively short lived. They are enzymatically and/or nonenzymatically metabolized to stable alcohols in vivo. They also react with metal to form reactive end products

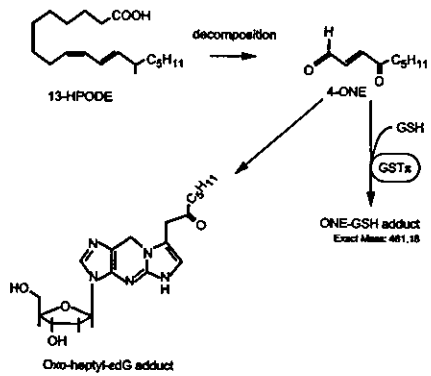

Fig. 9. Schema of the metabolism of 13-HPODE in the nucleus.

such as aldehydes. However, the importance of lipid hydroperoxides to the covalent modifications of biological components has not been thoroughly investigated. oxo-heptyl-εdG is formed by the reaction of 13-HPODE with dG [43]. During this reaction, 4-ONE directly mediates the formation of oxo-heptyl-εdG [30,43], suggesting that the lipid-hydroperoxide-derived production of 4-ONE contributes to DNA damage. 4-ONE and 4-HNE also form adducts with proteins. These adductions of proteins and DNA are thought to be involved in the pathogenesis of several diseases such as atherosclerosis [44], diabetes mellitus [45], and carcinogenesis [30].

With regard to the reduction of lipid peroxide, reduction of linoleic acid hydroperoxide by GPX was reported [46]. Lipid peroxide once formed is reduced to alcohol by GPX. With regard to the role of GST in the reduction of lipid peroxide, Cao et al. [32] reported that GSH and GST function in protecting against the cytotoxicity of 4-HNE in vascular smooth muscle cells. Depletion of GSH by buthionine sulfoximine and inhibition of GST activity by sufasalazine potentiated the 4-HNE-mediated cytotoxicity. The results suggested that GST functions to form a conjugate of 4-HNE with GSH.

Zimniak et al. [47] reported that mouse GSTA4-4 belongs to the alpha subclass of GST and functions to form a conjugate of 4-HNE with GSH. Additionally, Singhal et al. [48] reported that the human GST corresponding to mouse GSTA4-4 catalyzes the conjugation of 4-HNE with GSH. These reports indicate that GSTA4-4 plays an important role with GSH in the removal of 4-HNE. It is possible that GSTA4-4 functions to form a conjugate of 4-ONE with GSH. On the other hand, the colon cancer cell line employed in the present study possessed mainly GSTπ, which may detoxify 4-HNE and 4-ONE. It has been reported that aldose reductase prevents the formation of 4-HNE [49]. However, there has been no report on the role of GST in the reduction of another lipid peroxidation product, 4-ONE. As shown in Fig. 9, this is the first report to show that GSTπ reduces the formation of DNA adducts with 13-HPODE, characterized as oxo-heptyl-εdG. GSTπ catalyzes the conjugation of 4-ONE, a lipid-peroxide-derived product, with GSH, the adduct of which is thought to contribute to age-related diseases or carcinogenesis.

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ABBREVIATIONS

ABL—edible mushroom (*Agaricus bispourus*) lectin
GPX—glutathione peroxidase
GSH—reduced form of glutathione
GST—glutathione *S*-transferase
LC/MS—liquid chromatography/mass spectrometry
Oxo-heptyl-εdG—7-(2 oxo-hepyl)-substituted 1,N^2-etheno-2′-deoxyguanosine adduct
TBARS—thiobarbituric acid reactive substance
TUNEL—terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
4-HNE—4-hydroxy-2-nonenal
4-ONE—4-oxo-2-nonenal
13-HPODE—13-hydroperoxyoctadecadienoic acid

Functional variation in *LGALS2* confers risk of myocardial infarction and regulates lymphotoxin-α secretion *in vitro*

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Myocardial infarction (MI) has become one of the leading causes of death in the world. Its pathogenesis includes chronic formation of plaque inside the vessel wall of the coronary artery and acute rupture of the artery, implicating a number of inflammation-mediating molecules, such as the cytokine lymphotoxin-α (LTA)[1]. Functional variations in *LTA* are associated with susceptibility to MI[2]. Here we show that LTA protein binds to galectin-2, a member of the galactose-binding lectin family[3]. Our case–control association study in a Japanese population showed that a single nucleotide polymorphism in *LGALS2* encoding galectin-2 is significantly associated with susceptibility to MI. This genetic substitution affects the transcriptional level of galectin-2 *in vitro*, potentially leading to altered secretion of LTA, which would then affect the degree of inflammation; however, its relevance to other populations remains to be clarified. Smooth muscle cells and macrophages in the human atherosclerotic lesions expressed both galectin-2 and LTA. Our findings thus suggest a link between the LTA cascade and the pathogenesis of MI.

To understand better the role of LTA in the pathogenesis of this disease, we searched for proteins that interact with LTA. Using the *Escherichia coli* two-hybrid system and the phage display method, we identified galectin-2 as a binding partner of LTA. We purified the two recombinant proteins using a bacterial expression system, and confirmed direct binding of galectin-2 to LTA using an *in vitro* binding assay (Fig. 1a). We further examined their interaction in mammalian cells using constructs designed to express Flag-tagged LTA or S-tagged galectin-2 and western blot analysis; co-immunoprecipitation of galectin-2 and LTA confirmed their interaction (Fig. 1b). Using antibodies specific to each protein, we also investigated subcellular localization of native galectin-2 and LTA in U937 cells, and found that these proteins co-localized in the cytoplasm (Fig. 1c).

We then examined whether any genetic variation in *LGALS2* was associated with susceptibility to MI. Re-sequencing the *LGALS2* genomic region using DNA from 32 MI samples revealed 17 additional single nucleotide polymorphisms (SNPs) (Fig. 2a). Next, we compared the genotype frequencies of approximately 600 individuals with MI, and 600 controls at these SNP loci, and found that one SNP (3279C → T) in intron-1 of *LGALS2* was significantly associated with MI (designated SNP9 in Supplementary Table 1). This association was confirmed by increasing the number of samples to 2,302 for patients with MI and 2,038 for controls, and by including another set of samples (Table 1 and Supplementary Table 2).

We also analysed linkage disequilibrium using a subset of markers with minor allele frequency of >0.20, and investigated haplotype structure within the *LGALS2* region (Supplementary Table 3). This allowed us to identify a haplotype block containing three SNPs (SNP9, SNP10 and SNP11); no particular haplotype showed a higher statistical significance for association with MI than the single SNP alone (Supplementary Table 4). Because the minor allele frequency of the associated SNP was lower in the patients' group (Table 1), we concluded from these genetic studies that the minor variant protects against the risk of MI, although our study included only Japanese subjects and so its relevance to other populations remains to be clarified.

To investigate the biological significance of this genetic variation in intron-1 of *LGALS2*, we constructed reporter plasmids with a genomic fragment containing the SNP downstream of a luciferase gene and the SV40 enhancer sequence, and examined the effect of the intron-1 SNP on gene expression. The clone containing the 3279T allele showed nearly 50% less transcriptional activity than those containing the 3279C allele or the vector alone (Fig. 2b, c). These observations indicated that the nucleotide substitution could potentially reduce the level of the galectin-2 transcript.

We then hypothesized that the amount of intracellular galectin-2 might regulate the extracellular secretion level of LTA, thereby influencing the degree of inflammation. To test this hypothesis, we examined the effect of the level of galectin-2 on the secreted level of LTA in the medium by repressing galectin-2 expression using a small interference (si)RNA technique, and by over-expressing

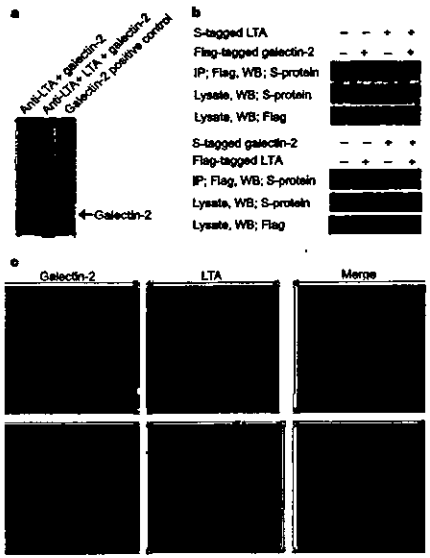

Figure 1 LTA binds to galectin-2. a, *In vitro* binding assay. b, Co-immunoprecipitation of tagged LTA and galectin-2 in COS7 cells. c, Co-localization of endogenous LTA with galectin-2 in U937 cells (top row) with enlarged images of representative cells in the upper panels (bottom row). IP, immunoprecipitation; WB, western blot.

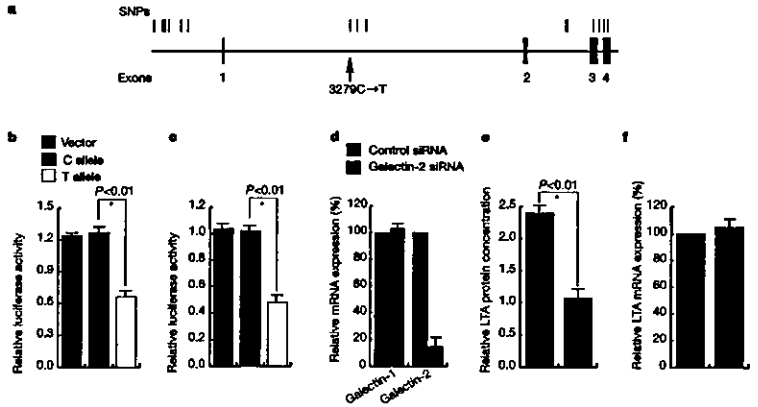

Figure 2 Association of a SNP in *LGALS2* with myocardial infarction and its functionality. a, Map of SNPs in *LGALS2* locus. b, c, Transcriptional regulatory activity of intron-1 SNP of *LGALS2* in HeLa (b) and HepG2 (c) cells. d–f, Inhibition of galectin-2 expression. Levels of galectin-1 or galectin-2 mRNA (d), supernatant LTA protein (e), and LTA mRNA (f) after 48 h transfection with siRNA vectors. *Student's *t*-test. Each experiment was repeated three times and each sample was studied in triplicate.

galectin-2. One siRNA for galectin-2 repressed galectin-2 messenger RNA to nearly one-fifth of its original level (Fig. 2d) and resulted in inhibition of LTA secretion into the medium (Fig. 2e). Over-expression of galectin-2 enhanced LTA secretion (Supplementary Fig. 1a). As shown in Fig. 2f, the LTA mRNA level was unchanged by knockdown or over-expression of galectin-2 (see also Supplementary Fig. 1b).

To further investigate the regulatory mechanism of LTA secretion by galectin-2, we searched for intracellular molecules that associate with galectin-2, using a tandem affinity purification (TAP) system[4]. We identified two unique bands that could be observed only when the galectin-2-TAP tag was expressed (Fig. 3a). On the basis of a MALDI/TOF (matrix-assisted laser desorption/ionization–time of flight) mass spectrometry analysis, these two bands were shown to correspond to α- and β-tubulins—important components of microtubules. Using HeLa cells transfected with a plasmid to express Flag-tagged galectin-2, we confirmed co-immunoprecipitation of endogenous tubulins and galactin-2 (Fig. 3b and data not shown). Interestingly, the tubulins were also co-immunoprecipitated with LTA (Fig. 3b and data not shown). Images from serial confocal sections of double-immunostained U937 cells revealed that galectin-2 and α-tubulin co-localized as reticular filamentous networks developed in the cytoplasm (Fig. 3c). Recently, microtubule cytoskeleton networks have been implicated in the subcellular transport of some proteins, including glucose transporter isoform (GLUT4) or thiamine transporter (THTR1)[5,6]. It is likely that LTA is another molecule that uses the microtubule cytoskeleton network for translocation. It is also conceivable that galectin-2 has a role in intracellular trafficking, although the precise role of galectin-2 in this trafficking machinery complex has yet to be elucidated.

To examine whether these proteins are expressed in the lesion of MI—that is, in atherosclerotic lesions of the coronary artery—and if so, to investigate in which part of the lesion they are expressed, we performed immunohistochemical staining of human coronary atherectomy specimens with anti-LTA or anti-galectin-2 antibody. As shown in Fig. 4a, b, immunoreactivities for both LTA and galectin-2 were detected in intimal cells in atherosclerotic plaques, some of which were spindle-shaped or contained vacuolated, round cytoplasms. Immunostaining of adjacent sections with anti-smooth muscle cell (SMC) α-actin or anti-CD68 showed that the majority of these cells were either SMCs or SMC-derived foam cells, with

Table 1 Association between MI and intron-1 SNP in *LGALS2*

Genotype	Number of patients with MI	Number of patients with MI (%)	Number of controls	Number of controls (%)
LGALS2 Intron-1 3279C → T				
CC	1,077	46.8	856	42.0
CT	1,014	44.0	903	44.3
TT	211	9.2	279	13.7
Total	2,302	100.0	2,038	100.0

	χ^2	*P* value	Odds ratio	95% CI
Genotype frequency	25.2	0.0000034		
Allele frequency	21.1	0.0000045	1.23	1.13–1.35
CC versus others	10.0	0.0016	1.21	1.08–1.37
TT versus others	22.1	0.0000026	1.57	1.30–1.90

Nucleotide numbering is according to the mutation nomenclature[16]. CI, confidence interval.

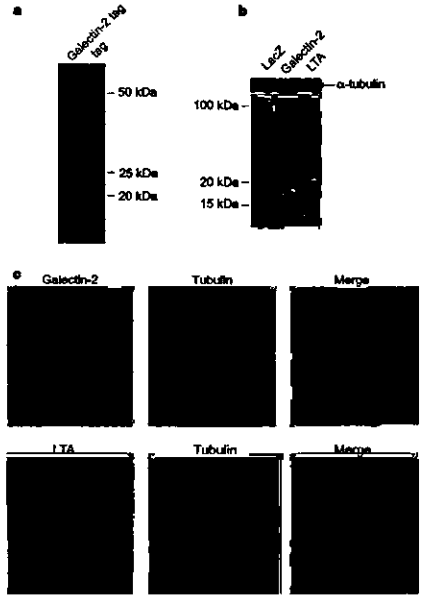

Figure 3 Galectin-2 interacts with microtubules. **a**, Isolation of TAP-tagged galectin-2 and interacting proteins. Arrowheads indicate α- and β-tubulins, revealed by MALDI/TOF mass spectrometry analyses. **b**, Co-immunoprecipitation of endogenous α-tubulin with Flag-tagged galectin-2 or LTA. Immunoprecipitations were done using anti-Flag M2 agarose, and immunoprecipitates were detected using anti-α-tubulin antibody (upper panel) or anti-Flag antibody (lower panel). Flag-tagged LacZ encoding β-galactosidase was used as a negative control. **c**, Co-localization of endogenous α-tubulin with endogenous galectin-2 or LTA in U937 cells.

occasional macrophages being observed (Fig. 4c, d and Supplementary Fig. 2). Co-expression of LTA and galectin-2 was also observed in the majority of polymorphic SMCs by double-labelled immunohistochemistry (Fig. 4e). In contrast, expression of either protein was not detectable in atrophic SMCs of fibrous plaques with scanty cellularity or in normal medial SMCs (Fig. 4f for LTA; data not shown for galectin-2).

These results showed that LTA and galectin-2 were co-expressed in SMCs and macrophages in the intima of human atherosclerotic plaques, but were absent in quiescent or normal medial SMCs. We also confirmed their co-expression by counting immunohistochemically double-labelled cells (Supplementary Table 5). A recent report also indicated that LTA was expressed in atherosclerotic lesions in mice and that loss of LTA reduced the size of the lesions[7]. Together, these results indicate that LTA, as one of the mediators of inflammation, along with galectin-2, as a regulator of LTA secretion, might have roles in the pathogenesis of MI, although the functional correlation of galectin-2 and LTA with the development, progression or rupture of atherosclerotic plaques has yet to be clarified.

We have identified galectin-2 as another risk factor for MI. We believe that the results of this study provide an anchoring point for better understanding of the pathogenesis of MI. □

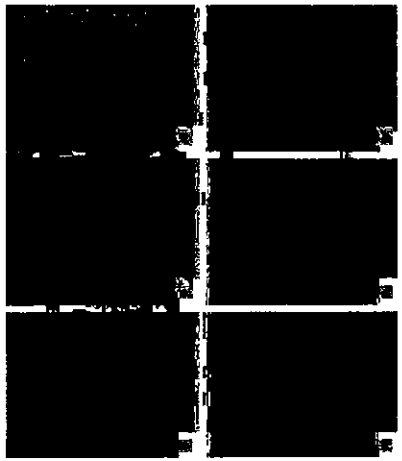

Figure 4 Expression and co-localization of galectin-2 and LTA in the coronary atherectomy specimen. Single-labelled immunohistochemistry of serial sections of primary atherosclerotic lesions from human coronary arteries obtained by directional coronary atherectomy, stained with anti-human LTA (**a**), anti-human galectin-2 (**b**), monoclonal anti-SMC α-actin (**c**), and monoclonal anti-CD68 (**d**). Magnification, ×100. **e**, Double-labelled immunohistochemical staining with anti-LTA (brown) and galectin-2 (blue) antibodies. Magnification, ×119. **f**, Single-labelled immunohistochemical staining with anti-human LTA for atrophic SMCs of fibrous plaques with scanty cellularity and normal medial SMCs. Magnification, ×100.

Methods

DNA samples

The study included 2,638 Japanese patients with MI, referred to as the Osaka Acute Coronary Insufficiency Study (OACIS) group. The diagnosis of definite MI has been described previously[8]. The control subjects consisted of 2,499 general populations recruited through several medical institutes in Japan. All subjects were Japanese and gave written informed consent to participation in the study, or if they were under 20 years old, their parents gave consent according to the process approved by the Ethical Committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama.

SNP analysis

Design for polymerase chain reaction (PCR) primers, PCR experiments, DNA extraction, DNA sequencing, SNP discovery, genotyping of SNPs and statistical analysis has been described previously[9,10].

E. coli two-hybrid and phage display screening

We constructed a two-hybrid complementary DNA (cDNA) library using mRNA isolated from Jurkat cells (RIKEN Cell Bank; RCB0806) and BacterioMatch two-hybrid system library construction kit (Clontech), and screened the library using pBT-human LTA as the 'bait'. For the phage display system, we constructed a phage display cDNA library using mRNA isolated from Jurkat cells and T7 select cDNA cloning system (Novagen). We screened the library using the immobilized LTA as the 'bait'.

Tandem affinity purification

The tandem affinity purification procedure was carried out essentially as described in ref. 4, with some modifications. We constructed a fusion cassette encoding a His tag, a TEV cleavage site, and an S tag as a TAP tag sequence in pCMV–Myc vector (Sigma). This TAP vector expresses amino-terminal Myc-tagged target protein with carboxy-terminal TAP tags in mammalian cells under the control of cytomegalovirus promoter. The TAP vector was transiently transfected into HeLa cells (Health Science Research Resources Bank (HSRRB); JCRB9004). Target protein bands were analysed by MALDI/TOF mass spectrometry at APRO Life Science.

In vitro binding assay and co-immunoprecipitation experiment

We prepared purified S-tagged recombinant LTA and T7-tagged galectin-2 derived from *E. coli* using the pET system (Novagen), and combined them. The co-immunoprecipitation experiments were performed using a monoclonal antibody against LTA (R&D Systems) coupled to HiTrapTM NHS-activated Sepharose HP (Amersham). We visualized the immune complex using T7 tag antibody (Stratagene) and horseradish peroxidase (HRP) conjugated with anti-mouse IgG antibody. For co-immunoprecipitation in mammalian cells, we transfected expression plasmids of Flag or S-tagged LTA, galectin-2 and LacZ (as a negative control) into COS7 cells (HSRRB; JCRB9127) or HeLa cells using Fugene. Immunoprecipitations were done in lysis buffer (20 mM Tris pH 7.5, with 150 mM NaCl, 0.1 % Nonident P-40). Twenty-four hours after transfection, cells were lysed, and immunoprecipitations were performed using anti-Flag tag M2 agarose (Sigma). We visualized the immune complex using HRP-conjugated S-protein (Novagen), anti-Flag M2 peroxidase conjugate (Sigma) or mouse monoclonal antibody against human α-tubulin (Molecular Probes) and HRP-conjugated anti-mouse IgG antibody.

Confocal microscopy

Polyclonal anti-human galectin-2 antisera were raised in rabbits using recombinant protein synthesized in *E. coli*. The antisera showed no cross-reactivity to structurally related molecules galectin-1 and galectin-3, analysed by western blot. Polyclonal anti-galectin-2 antisera and either goat anti-human LTA (gG (R&D Systems) or mouse anti-human α-tubulin monoclonal IgM antibodies were used with Alexa secondary antibodies (Molecular Probes). U937 cells (HSRRB; JCRB9021) were stimulated for 30 min with phorbol myristate acetate (PMA) (20 ng ml^{-1}) and fixed. They were subsequently incubated with the corresponding primary antibodies in phosphate-buffered saline containing 3% bovine serum albumin, and the corresponding Alexa secondary antibodies.

siRNA and over-expression experiments

The target sequences for galectin-2 (5′-AATCCACCATTGTCTGCAACT-3′) were cloned into pSilencer 2.0-U6 siRNA vector (Ambion). For the over-expression experiment, the galectin-2 was cloned into pFlag–CMV5a vector. After transfection, Jurkat cells were stimulated with PMA (20 ng ml^{-1}) for 24 h, and cells and supernatants were collected separately. LTA concentration was measured using an LTA-specific ELISA system (R&D Systems), and normalized by comparison with total protein concentration. The mRNA quantification procedure has been described previously[3].

Luciferase assay

A DNA fragment, corresponding to nucleotides 3,188 to 3,404 of intron-1 of *LGALS2*, was cloned into pGL3–enhancer vector (Promega) in the downstream of SV40 enhancer in the 5′ to 3′ orientation. After 24 h transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Immunohistochemistry

Tissue samples were obtained from 16 patients with MI by elective directional coronary atherectomy. Immunohistochemical protocols were carried out as described previously[3,4,8] using goat anti-human LTA IgG (R&D Systems) and rabbit polyclonal anti-human galectin-2 antibody. Staining of adjacent sections was carried out using human-cell-type-specific monoclonal antibodies against SMC 2–actin and CD68 (DAKO). For double-labelled immunohistochemistry, sections were incubated with anti-LTA antibody, then with biotinylated swine anti-goat IgG, and then with avidin–biotin–peroxidase conjugate, followed by visualization with 3,3′-diaminobenzidine tetrahydrochloride (Vector Labs). The section was subsequently incubated with rabbit polyclonal anti-human galectin-2 antibody, followed by incubation with alkaline phosphatase-conjugated swine anti-rabbit IgG and visualized with the 5-bromo-4-chloro-3-indoxyl phosphate and nitro-blue tetrazolium chloride (BCIP/NBT) substrate system.

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Differential modulation of endotoxin responsiveness by human caspase-12 polymorphisms

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Caspases mediate essential key proteolytic events in inflammatory cascades and the apoptotic cell death pathway. Human caspases functionally segregate into two distinct subfamilies: those involved in cytokine maturation (caspase-1, -4 and -5) and those involved in cellular apoptosis (caspase-2, -3, -6, -7, -8, -9 and -10)[1–3]. Although caspase-12 is phylogenetically related to the cytokine maturation caspases, in mice it has been proposed as a mediator of apoptosis induced by endoplasmic reticulum stress including amyloid-β cytotoxicity, suggesting that it might contribute to the pathogenesis of Alzheimer's disease[3]. Here we show that a single nucleotide polymorphism in caspase-12 in humans results in the synthesis of either a truncated protein (Csp12-S) or a full-length caspase proenzyme (Csp12-L). The read-through single nucleotide polymorphism encoding Csp12-L is confined to populations of African descent and confers hypo-responsiveness to lipopolysaccharide-stimulated cytokine production in *ex vivo* whole blood, but has no significant effect on apoptotic sensitivity. In a preliminary study, we find that the frequency of the Csp12-L