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Augmented glutathione synthesis decreases acrolein toxicity

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ABSTRACT

We have shown recently that acrolein is more strongly involved in cell damage than reactive oxygen species during brain infarction. Thus, we tried to isolate cells with reduced susceptibility to acrolein toxicity to clarify how acrolein is detoxified under cell culture conditions. The IC_{50} of acrolein in mouse mammary carcinoma FM3A cells and in neuroblastoma Neuro2a cells was 2.6 and 4.2 μM, respectively, but in acrolein toxicity-decreasing FM3A (FM3A-ATD) cells and Neuro2a (Neuro2a-ATD) cells, it was 7.6 and 8.4 μM, respectively. In both FM3A-ATD and Neuro2a-ATD cells, the concentration of glutathione (GSH) was increased, so that detoxification occurred through acrolein conjugation with GSH. In FM3A-ATD cells, the level of a rate-limiting enzyme of GSH synthesis, γ-glutamylcysteine ligase catalytic unit (GCLC), was increased through the reactivation of one inactive allele of GCLC genes in FM3A cells. In Neuro2a-ATD cells, phosphorylation of transcription factors (c-Jun and NF-κB) necessary for expression of genes for GCLC and glutathione synthetase (GSHS) involved in GSH synthesis was stimulated, so that transcription of two genes increased in Neuro2a-ATD cells. Phosphorylation of JNK (c-Jun N-terminal kinase), which catalyzes phosphorylation of c-Jun and NF-κB p65, was also increased in Neuro2a-ATD cells, suggesting that activation of JNK kinase is responsible for the increase in GSH. These results support the idea that GSH plays important roles in detoxification of acrolein, because GSH is increased in both FM3A-ATD and Neuro2a-ATD cells.

1. Introduction

Oxidative stress, which can lead to various disorders, is thought to be caused by two classes of compounds—first, reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH), and second, unsaturated aldehydes such as acrolein (CH_2=CHCHO) and 4-hydroxynonenal [1–3]. There are many reports concerning the roles of ROS and unsaturated aldehydes in cell damage, and it is thought that ROS play a predominant role in cell damage.

Polyamines (putrescine, spermidine and spermine) are essential for normal cell growth and are present in cells at millimolar concentrations [4]. However, when cells are damaged, the toxic compounds acrolein and H_2O_2 are produced from polyamines, in particular from spermine, by polyamine oxidases (PAO, spermine oxidase and acetylpolyamine oxidase) [5–7]. When the toxicities of acrolein and H_2O_2 were compared in a cell culture system, the major toxic factor produced from polyamines was acrolein [8,9]; furthermore, acrolein was more effectively produced from polyamines rather than from phospholipids [7,10]. We examined whether the levels of PAO and protein-conjugated acrolein (PC-Acro) in plasma were correlated with pathologies that involved tissue damage, and found that the levels of PAO and PC-Acro in plasma were well correlated with the severity of chronic renal failure [11,12] and stroke [5]. The size of stroke was nearly paralleled with the multiplied value of PC-Acro by PAO [5]. It was also shown that the induction of brain infarction in mice was well correlated with the increase in PC-Acro rather than ROS at the locus of infarction and in plasma [13,14]. However, it is not clear how acrolein is detoxified in cells. In this study, we tried to isolate acrolein toxicity-decreasing cells to clarify how acrolein is detoxified under cell culture conditions.

2. Materials and methods

2.1. Culture of FM3A and Neuro2a cells and isolation of the acrolein toxicity-decreasing cells from these cells

Mouse mammary carcinoma FM3A cells (Japan Health Science Foundation) and mouse neuroblastoma Neuro2a cells (DS Pharma

Abbreviations: FM3A-ATD, acrolein toxicity-decreasing FM3A; GCLC, γ-glutamylcysteine ligase catalytic unit; GSH, glutathione; GSHS, glutathione synthetase; JNK, c-Jun N-terminal kinase; Neuro2a-ATD, acrolein toxicity-decreasing Neuro2a; PAO, polyamine oxidases; PC-Acro, protein-conjugated acrolein; ROS, reactive oxygen species.

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Biomedical Co., Ltd.) were used for isolation of the acrolein toxicity-decreasing cells. These FM3A cells (2×10^4 cells/ml) and Neuro2a cells (5×10^4 cells/ml) were cultured in D-MEM (Wako Pure Chemical Industries, Ltd.) supplemented with 2% and 10% heat inactivated fetal calf serum, respectively, at 37 °C in an atmosphere of 5% CO_2 in air. Cells at the logarithmic growth phase (5×10^5 cells/ml) were diluted to 2.5×10^5 cells by the addition of 1 ml medium in a 50 mm dish. They were mutagenized by treatment with 0.1% ethylmethanesulfonate (Sigma–Aldrich) for 3 h, washed three times with the medium, and cultured for 4 days. Cells were then cultured in the medium containing acrolein (Tokyo Chemical Industry Co., Ltd.). The concentrations of acrolein in the medium were gradually increased in a stepwise manner from 10 to 35 μM over 6 months, and acrolein toxicity-decreasing cells were isolated.

2.2. Measurement of GSH and acrolein

Cells were homogenized with 5% trichloroacetic acid (TCA), and centrifuged at 12,000*g* for 10 min. The supernatant was used for the measurement of GSH, which was measured using total glutathione assay kit (Northwest Life Science Specialities LLC, USA) according to the manufacturer's instructions. Acrolein was measured as protein-conjugated acrolein (PC-Acro) by Western blotting as described below, because free acrolein is variable. Protein content in the precipitate was determined by the method of Bradford [15].

2.3. Western blot analysis

Cells (2×10^6 cells) were suspended in 0.1 ml of a buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF and 50 μM FUT-175 (6-amino-2-naphthyl-4-guanidinobenzoate dihydrochloride), an inhibitor of protease [16], and lyzed by repeated (three times) freezing and thawing with intermittent mechanical mixing. The supernatant was obtained by centrifugation at 17,000*g* for 15 min and used as cell lysate. Western blot analysis of cell lysate (20 μg protein) was performed by the method of Nielsen et al. [17], using ECL Western blotting reagents (GE Healthcare). Antibodies against γ-glutamylcysteine ligase catalytic unit (GCLC), glutathione synthetase (GSHS), c-Jun, NF-κB p65 and JNK1 (c-Jun N-terminal kinase 1) were obtained from Santa Cruz Biotechnology, Inc., and those against phospho-c-Jun, phopho-NF-κB p65, JNK2, JNK3, phospho-JNK1 and phospho-JNK2/3 were from Cell Signaling Technology, Inc. PC-Acro was detected by Western blotting using an antibody against [N^e-(3-formyl-3,4-dehydropiperidino)-lysine (FDP-lysine)] (NOF Corporation) [18]. The level of these proteins was quantified with a LAS-3000 luminescent image analyzer (Fuji Film).

2.4. Northern blot analysis

Total RNA was isolated from 2.5×10^7 cells using TRIzol reagent (Invitrogen). Poly(A)$^+$ RNA was isolated using a Fast Track 2.0 Kit (Invitrogen). Northern blot analysis was performed [19] using the ECL direct nucleic acid labeling and detection system (GE Healthcare) with 3 μg of poly(A)$^+$ RNA. The cDNA used for template DNA was prepared using SuperScript™ II RNase H$^-$ Reverse Transcriptase (Life Technologies) according to the manufacturer's instructions. Genes for GCLC and GSHS were amplified by polymerase chain reaction (PCR) using primer sets, 5′-TACGTGTCAGACATT GATTGTCGCTGGGGA-3′ (GCLC F), 5′-TCCAGATAGGAGTTCAGAAT GGGGATGAGT-3′ (GCLC R), 5′-ACGCTTTTCCCCTCACCAGTACC-3′ (GSHS F), 5′-GTCGGTGCACAGCTGGAGTCC-3′ (GSHS R), and the cDNA as templates. PCR products thus obtained were used as probes for Northern blot analysis.

2.5. Determination of nucleotide sequence of GCLC mRNA and genomic DNA in FM3A cells and acrolein toxicity-decreasing cells

The cDNA fragment for GCLC was obtained from total RNA prepared as described above by the reverse transcriptase PCR method [20] using Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). GCLC genome DNA was also prepared by PCR using DNA isolated by the same method as total RNA preparation as described above. A list of the primers used is available from the authors upon request. The nucleotide sequence was determined by the dideoxy chain-termination method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

2.6. Statistics

Values are indicated as means ± S.E. Data were analyzed by Student's *t*-test.

3. Results

3.1. Isolation and characteristics of acrolein toxicity-decreasing FM3A (FM3A-ATD) cells

FM3A-ATD cells were isolated by treatment with 0.1% ethylmethanesulfonate and culturing the cells for 6 months with a gradual increase in acrolein in the medium. The IC_{50} value for acrolein was determined by incubating the cells for 72 h (Fig. 1A), and was 7.6 μM in FM3A-ATD compared with 2.6 μM in FM3A cells. Growth of FM3A-ATD cells was compared with FM3A cells in the absence and presence of 4 μM acrolein (Fig. 1B). In the absence of acrolein, cell growth of FM3A-ATD cells was slightly slower than that of normal FM3A cells. In the presence of 4 μM acrolein, cell growth of FM3A cells was greatly diminished, but that of FM3A-ATD cells was not decreased significantly.

Since acrolein interacts with –SH groups very effectively and with $-NH_2$ groups less effectively [9], it is thought that GSH is the major factor for detoxification of acrolein [2]. Thus, the level of GSH in FM3A and FM3A-ATD cells was measured. As shown in Fig. 1C, the level of GSH in FM3A-ATD cells was approximately 2-fold higher than that in FM3A cells: i.e. 98.9 and 47.2 nmol/mg protein, respectively. Decrease in GSH by acrolein was then measured by incubating cells with a relatively high concentration of acrolein for a short period, because the GSH level is gradually recovered. By incubating cells with 200 μM acrolein for 1 h, the level of GSH in FM3A cells decreased greatly. However, a significant amount of GSH still remained in FM3A-ATD cells (Fig. 1C). Under these conditions, the level of PC-Acro was significantly higher in FM3A cells than in FM3A-ATD cells, especially the protein polymerized through crosslinking by acrolein (shown by square bracket in Fig. 1C). The level of polymerized protein crosslinked by acrolein in FM3A cells was approximately 2-fold more than that in FM3A-ATD cells, which was inversely correlated with the level of GSH. It is thought that one of the toxic effects of acrolein is inactivation of proteins [2]. Thus, the results indicate that an increase in GSH reduces the interaction of acrolein with proteins, and thereby decreases acrolein toxicity.

The mechanism of the increase in GSH in FM3A-ATD cells was studied. GSH is synthesized in two steps [21,22]. The first step in GSH synthesis is catalyzed by glutamate cysteine ligase (GCL), a heterodimeric enzyme with a 73-kDa catalytic unit (GCLC) and a 31-kDa modifier subunit (GCLM); the second step is catalyzed by GSH synthetase (GSHS). The level of GSH synthetic enzymes was measured by Western and Northern blot analysis. As shown in Fig. 1D, the level of GCLC protein was approximately 2-fold higher in FM3A-ATD cells than in FM3A cells, although the levels of GCLC

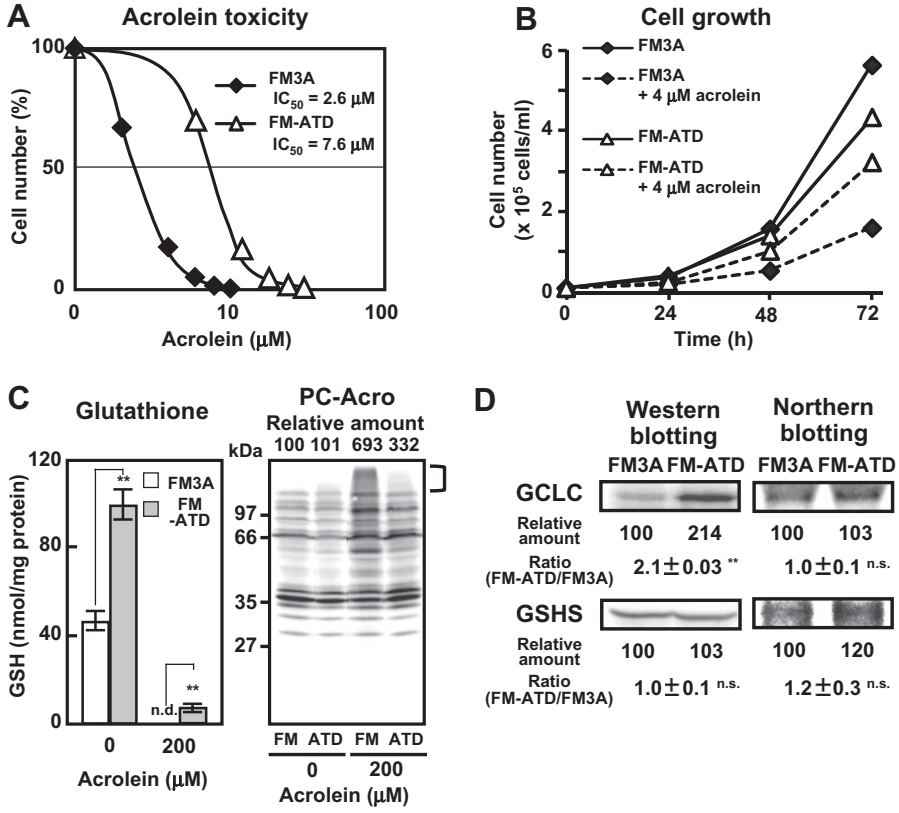

Fig. 1. Comparison of acrolein toxicity and characteristics of FM3A and FM3A-ATD cells. (A) Cell number was counted in the presence of 0.05% trypan blue after 72 h cell culture in the presence of various concentrations of acrolein. Degree of inhibition by acrolein was shown as percent cell number compared to that of non-treated cells. (B) Effect of 4 μM acrolein on cell growth of FM3A and FM3A-ATD cells was examined for 72 h. (C) GSH content and level of PC-Acro in FM3A and FM3A-ATD cells cultured for 1 h in the presence and absence of 200 μM of acrolein was measured as described in Section 2. Degree of protein polymerization through crosslinking by acrolein (shown by square bracket) is indicated as relative amount (%). (D) Levels of GCLC and GSHS proteins and mRNAs after 72 h cell culture in the absence of acrolein were measured by Western and Northern blot analyses as described in Section 2. Error bars in triplicate experiments were within symbols in (A and B). n.s., $p \geq 0.05$; $^{**}p < 0.01$. n.d., not detected; FM, FM3A; ATD, FM3A-ATD.

mRNA were very similar in both FM3A and FM3A-ATD cells. The level of GCLM was almost the same in these cells (data not shown). Levels of both protein and mRNA for the second enzyme, GSHS, were very similar in FM3A and FM3A-ATD cells (Fig. 1D). These results indicate that the increase in GSH in FM3A-ATD cells is due to an increase in GCLC. The nucleotide sequence of GCLC mRNA was then analyzed in FM3A and FM3A-ATD cells. As shown in Fig. 2, both full-length and exon-7 deficient GCLC mRNAs were present in FM3A cells. On the other hand, only full-length GCLC mRNA was present in FM3A-ATD cells. These results indicate that GSH in FM3A-ATD cells increased through the reactivation of one inactive allele of the GCLC genes. Thus, the nucleotide sequence of genomic GCLC DNA was determined. The reactivation of genomic GCLC DNA was due to the insertion of 11 nucleotides in intron-7 of the GCLC gene (data not shown).

3.2. Isolation and characteristics of acrolein toxicity-decreasing Neuro2a (Neuro2a-ATD) cells

In FM3A cells, the GCLC gene was unusual, because one of the allelic genes was inactive. Thus, isolation of mutants with reduced sensitivity to acrolein was also carried out using a different type of cell line (Neuro2a) to yield Neuro2a-ATD cells, to determine whether an increase in GSH is a common factor in decreasing acrolein toxicity. The IC_{50} value for acrolein was 8.4 μM in Neuro2a-ATD cells compared to 4.2 μM in Neuro2a cells (Fig. 3A). Growth of Neuro2a-ATD cells was slightly slower than that of normal Neuro2a cells in the absence of acrolein (Fig. 3B), and growth of Neuro2a-ATD cells was not inhibited significantly by 6 μM acrolein, although that of Neuro2a cells was strongly inhibited (Fig. 3B).

The level of GSH in Neuro2a cells was 20.4 nmol/mg protein, while that of Neuro2a-ATD cells was 33.9 nmol/mg protein (Fig. 3C). In contrast, the level of GSH in Neuro2a cells incubated with 200 μM acrolein for 1 h became negligible, while a significant amount of GSH remained in Neuro2a-ATD cells under the same conditions (Fig. 3C). In the presence of 200 μM acrolein, the level of PC-Acro, especially acrolein-conjugated polymerized protein, increased in Neuro2a cells approximately 1.5-fold compared with Neuro2a-ATD cells (Fig. 3C). These results support the idea that the increase in GSH plays important roles for resistance against acrolein toxicity, and is a common mechanism in different cell types.

The mechanism of the increase in GSH in Neuro2a-ATD cells was studied. Levels of GCLC and GSHS proteins and mRNAs were significantly higher in Neuro2a-ATD cells than in Neuro2a cells (Fig. 3D), suggesting that GSH synthetic enzymes are enhanced at the level of transcription in Neuro2a-ATD cells. It has been reported that transcription of both GCLC and GSHS mRNAs is stimulated by phosphorylation of c-Jun, a component of AP-1, and NF-κB p65, a subunit of NF-κB together with p50 (Fig. 4A) [23,24]. Thus, the level of phosphorylation of c-Jun and NF-κB p65 was evaluated, and it was found that phosphorylation of c-Jun and NF-κB p65 was increased significantly in Neuro2a-ATD cells, although the level of these two proteins was not altered (Fig. 4B). It has been also reported that phosphorylation of c-Jun and NF-κB p65 is catalyzed by JNK (c-Jun N-terminal kinase) [25]. Thus, the level of JNKs 1–3 and the phosphorylation of JNK1 and JNK2/3 were measured. As shown in Fig. 4C, phosphorylation of JNK1 and JNK2/3

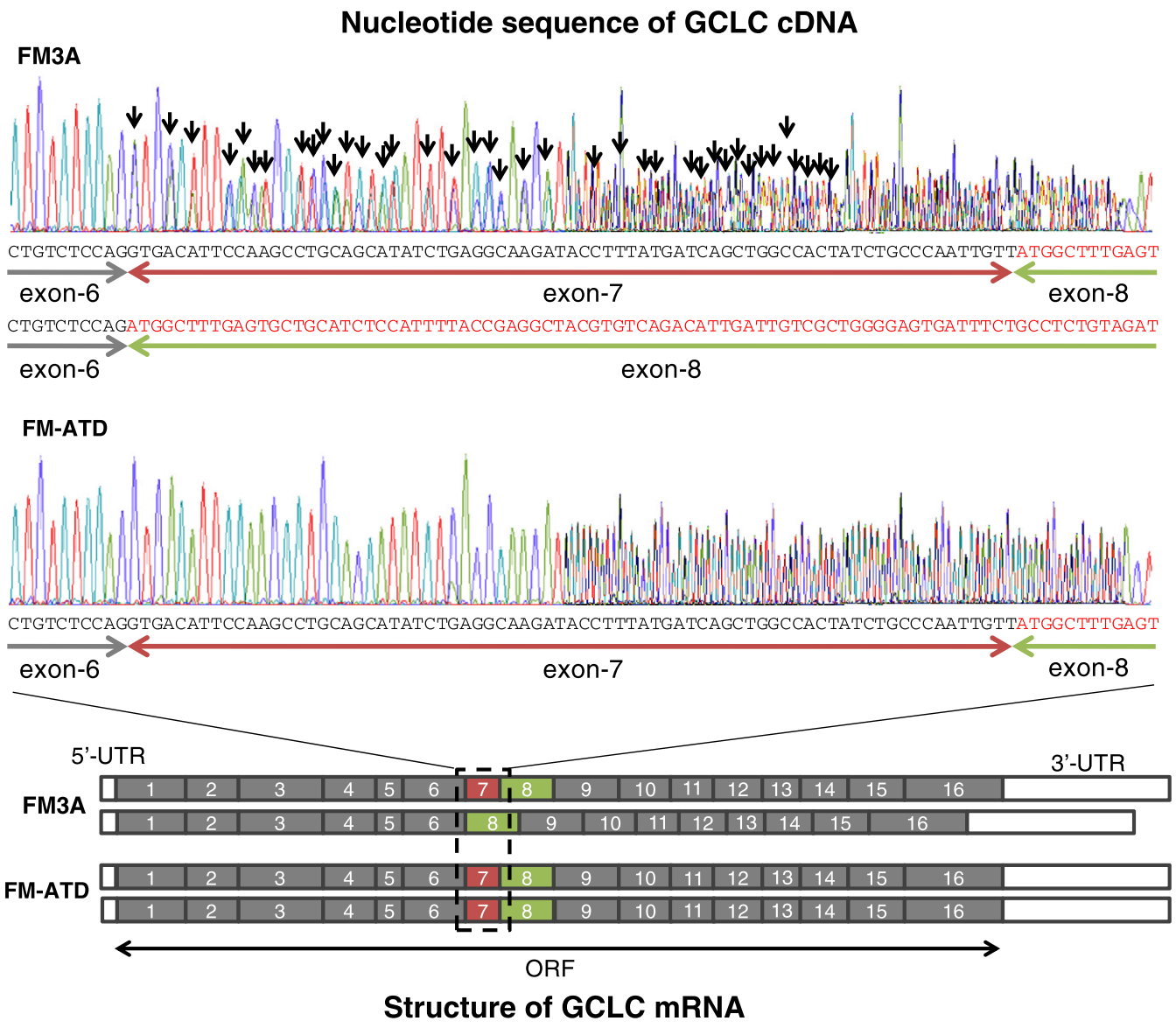

Fig. 2. Nucleotide sequences of GCLC mRNA in FM3A and FM3A-ATD cells. Nucleotide sequence at the region of exon-6 to exon-8 of GCLC mRNA (cDNA) and structure of GCLC mRNA consisting of exon-1 to -16 of GCLC genomic DNA in FM3A and FM3A-ATD cells were shown in the figure. The sequence was determined as described in Section 2.

was increased significantly in Neuro2a-ATD cells, suggesting that activation of JNK kinase is responsible for the increase in the level of GSH. The results indicate that the increase in GSH in Neuro2a-ATD cells is due to an increase in the activities of two GSH synthetic enzymes, through activation of JNK by phosphorylation.

4. Discussion

In this study, we isolated acrolein toxicity-decreasing cells to clarify how acrolein is detoxified in cells. In both mouse mammary carcinoma FM3A cells and mouse neuroblastoma Neuro2a cells, acrolein toxicity was decreased due to an increase in levels of GSH. These results clearly indicate that GSH is a key factor in detoxification of acrolein. The level of GSH in FM3A-ATD and Neuro2a-ATD cells was estimated as approximately 8–20 mM. Thus, it may be difficult to obtain more intense acrolein toxicity-decreasing cells through an increase in GSH, because very high levels of glycine, cysteine and glutamic acid are necessary to synthesize GSH. Our results are also in accordance with the finding that many effects of acrolein on cell death are closely associated with changes in cellular GSH [26,27]. We reported that acrolein is mainly produced from polyamines, especially from spermine [10,13,14]. However, in FM3A-ATD and Neuro2a-ATD cells, polyamine content and activities of acrolein producing enzymes, i.e. spermine oxidase and acetylpolyamine oxidase, did not change significantly (data not shown).

The mechanism of increase in GSH was different in the two cell types. In FM3A cells, one allele of GCLC genes was inactive, however, in the mutant (FM3A-ATD cells) the inactive gene was reactivated. In Neuro2a cells, phosphorylation of transcription factors (c-Jun and NF-κB p65) necessary for the transcription of the GCLC and GSHS genes was increased. The latter mechanism is presumably the more common mechanism leading to increased GSH levels in cells.

We have reported that PC-Acro increased in plasma of patients with chronic renal failure [11,12], and stroke and silent brain infarction [5,28,29]. Furthermore, we reported that PC-Acro is

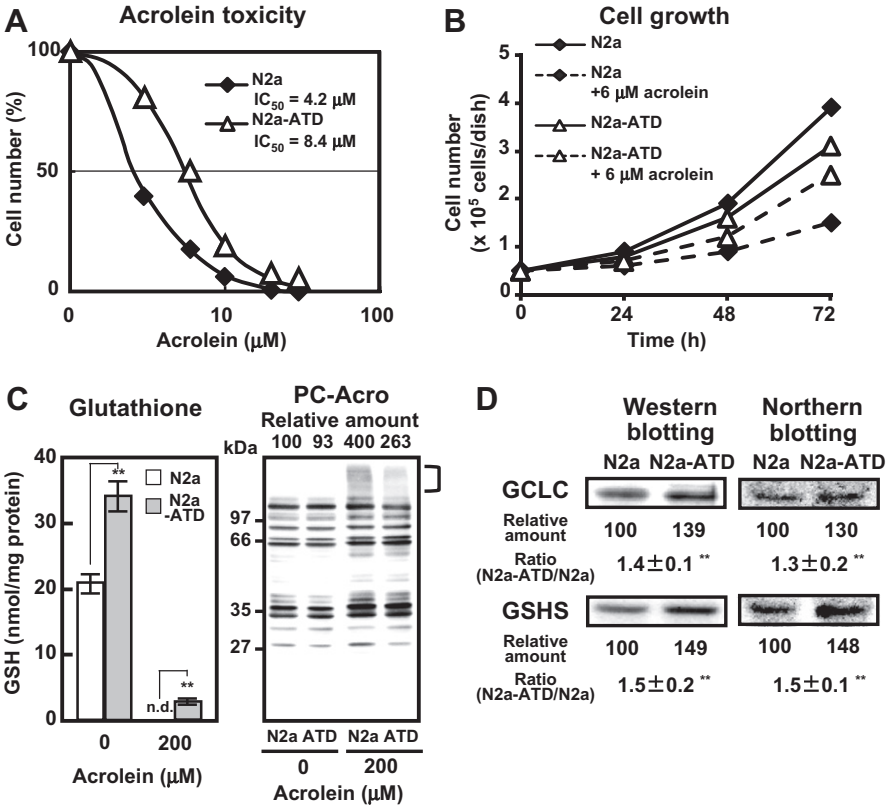

Fig. 3. Comparison of acrolein toxicity and characteristics of Neuro2a and Neuro2a-ATD cells. Experiments were performed as described in the legend of Fig. 1 except that acrolein concentration used was 6 μM instead of 4 μM in (B). $^{**}p < 0.01$. n.d., not detected. N2a, Neuro2a; ATD, Neuro2a-ATD.

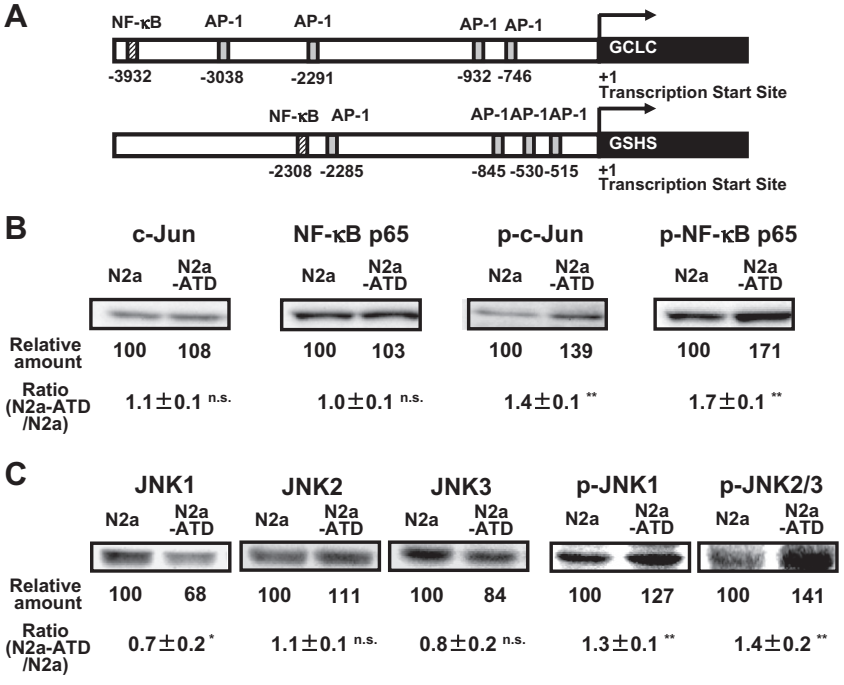

Fig. 4. Levels of c-Jun, NF-κB p65, JNK and their phosphorylated forms in Neuro2a and Neuro2a-ATD cells. (A) Locations of acting sites of transcription factors [AP-1 (c-Jun and c-Fos) and NF-κB] in GCLC and GSHS genes. (B and C) Levels of c-Jun, NF-κB p65, JNK 1–3 and their phosphorylated forms were measured by Western blotting using 20 μg protein as described in Section 2. n.s., $p \geq 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$. N2a, Neuro2a; N2a-ATD, Neuro2a-ATD.

elevated in saliva of primary Sjögren's syndrome [30]. It has been also reported that acrolein is involved in spinal cord injury and that GSH attenuates the injury [31]. Thus, it is clear that acrolein is well correlated with various pathologies caused by or involving cell damage, and the level of GSH is important to decrease acrolein toxicity.

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