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Inactivation of GAPDH as one mechanism of acrolein toxicity

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ABSTRACT

We have recently reported that acrolein is more toxic than reactive oxygen species. Thus, the mechanism of cell toxicity by acrolein was studied using mouse mammary carcinoma FM3A cells. Acrolein-conjugated proteins were separated by gel electrophoresis with subsequent determination of their amino acid sequence, and it was found that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was one of the major acrolein-conjugated proteins in cells. Acrolein interacted with cysteine-150 at the active site of GAPDH, and also with cysteine-282. When cells were treated with 8 μM acrolein, the activity of acrolein-conjugated GAPDH was greatly reduced, and the ATP content in cells was thus significantly reduced. In addition, it was shown that acrolein-conjugated GAPDH translocated to the nucleus, and the level of acetylated GAPDH and the number of TUNEL positive cells was increased, indicating that cell death is enhanced by acrolein-conjugated GAPDH. Inhibition of cell growth by acrolein was partially reversed when the cDNA encoding GAPDH was transformed into cells. These results indicate that inactivation of GAPDH is one mechanism that underlies cell toxicity caused by acrolein.

1. Introduction

It is thought that the major factor responsible for cell damage is reactive oxygen species (ROS) such as superoxide anion radical $O_2^{\cdot -}$, hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) [1]. However, when the toxicity of acrolein ($CH_2=CHCHO$) and ROS was compared, acrolein was more toxic than H_2O_2 [2] and slightly more toxic than $^{\cdot}OH$ [3] in cell culture systems. Furthermore, acrolein was thought to be produced from unsaturated fatty acids by ROS [4], but we found that it was more effectively produced from polyamines, especially from spermine [5–7], which are abundant and essential for eukaryotic cell growth [8]. Acrolein is spontaneously formed from 3-aminopropanal [$NH_2(CH_2)_2CHO$] produced from spermine by spermine oxidase [9], and less effectively from 3-acetamidopropanal [$CH_3CONH(CH_2)_2CHO$] produced from spermine and spermidine by spermidine/spermine N^1-acetyltransferase and acetylpolyamine oxidase [2,10].

An increase in protein-conjugated acrolein (PC-Acro) in plasma was well correlated with brain infarction and chronic renal failure [5,11]. Furthermore, silent brain infarction was identified with 84% sensitivity and specificity by combined measurement of PC-Acro, interleukin-6 and C-reactive protein in plasma [12].

The biological effects of acrolein are thought to be a consequence of its reactivity toward nucleophiles such as cysteine, lysine, histidine and arginine residues in critical regions of various proteins [13,14]. However, the mechanism of acrolein toxicity in cells has not been studied in detail. In the present work, the mechanism of acrolein toxicity was studied using mouse mammary carcinoma FM3A cells, and it was found that inactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by a relatively low concentration (8 μM) of acrolein contributes to cell toxicity. The mode of action of acrolein at GAPDH was similar to that of nitric oxide at GAPDH [15].

2. Materials and methods

2.1. Materials

Antibody against acetylated-lysine was obtained from Cell Signaling Technology. Antibodies against GAPDH (6C5), HA-probe (F-7) and β-actin (C4) were from Santa Cruz.

2.2. Cell culture

Mouse mammary carcinoma FM3A cells ($1–2 \times 10^4$ cells/ml) were cultured according to the method described previously [16]. Mouse neuroblastoma Neuro2a cells ($3–5 \times 10^4$ cells/ml) were

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cultured in D-MEM (Low-glucose) supplemented with 10% heat inactivated fetal bovine serum (FBS) and Non-Essential Amino Acids (Sigma) at 37 °C in an atmosphere of 5% CO_2 in air. The viable cell number was counted in the presence of 0.05% trypan blue.

2.3. Gel-electrophoresis of proteins

Cell lysate of FM3A cells was obtained by centrifugation at 20,000*g* for 15 min as described previously [17]. The S100 proteins were obtained by centrifugation of cell lysate at 100,000*g* for 2 h. Protein content was determined by the method of Bradford [18]. The S100 proteins (10 μg) were separated by SDS–PAGE on a 12% acrylamide gel and stained with Coommassie Brilliant Blue R-250. Two dimensional (2D) gel electrophoresis of S100 proteins was performed as described previously [19] using 100 μg proteins.

2.4. Plasmids

Total RNA was isolated from 2.5×10^7 FM3A cells using TRIzol reagent (Invitrogen), and cDNA was synthesized using Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). The cDNA for GAPDH, hemagglutinin (HA)-tagged GAPDH [GAPDH(HA)] or β-actin was amplified by *Pfu* DNA polymerase (Bioneer Corporation) with forward and reverse primer sets of GAPDH-F (5′-CGTAAGGATCCCAAAATGGTGAAGGTCGGT-3′) and GAPDH-R (5′-GGTGAATTCTTTCTTACTCCTTGGAGGCCA-3′), GAPDH-F and GAPDH(HA)-R (5′-GTGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTACTCCTTGGAGGC-3′), or β-actin-F (5′-CGCCACGGATCCGCCATGGATGACGATATC-3′) and β-actin-R (5′-TCAGTAGAATTCCGCCTAGAAGCACTTGCG-3′) using the first strand cDNA as templates. The amplified cDNA was digested with BamHI and EcoRI, and inserted into the same restriction sites of pcDNA3.1(+) (Invitrogen) to construct pcDNA-GAPDH, pcDNA-GAPDH(HA) or pcDNA-β-actin.

2.5. Purification of acrolein-conjugated HA-tagged GAPDH

Neuro2a (5×10^5/10 ml) cells were cultured for 48 h as described above, transfected with 4 μg of pcDNA-GAPDH(HA) by Lipofectamine™ Reagents (Invitrogen) and cultured further for 24 h. Then, cells were treated with or without 40 μM acrolein for 9 h. One ml of cell lysate (500 μg protein) was incubated with 10 μg of anti-HA antibody at 4 °C overnight, and incubated further with 30 μl of 50% Protein G Sepharose™ 4 Fast Flow (GE Healthcare) for 1 h. Bound proteins were extracted with 30 μl of SDS–PAGE sample buffer. Proteins were separated by SDS–PAGE, and GAPDH-HA protein was extracted from the gel.

2.6. Mass spectrometry

This was performed as described previously [20,21]. A protein band obtained with 2D gel electrophoresis or acrolein-conjugated GAPDH-HA protein was reduced with dithiothreitol and propionamidated by acrylamide. The protein was digested with either trypsin or endoproteinase Asp-N (Roche Applied Science, USA) at 37 °C overnight. An aliquot of digest was analyzed by nano LC-MS/MS using LCQ Deca XP (Finnigan, USA). Peptides were separated using nano spray column (100 μm i.d. × 375 μm o.d.) packed with a reversed-phase material (Inertsil ODS-3, 3 μm, GL Science, Japan) at a flow rate 400 nl/min. The mass spectrometer was operated in the positive-ion mode and the obtained spectra in a data-dependent MS/MS mode were searched against the NCBInr 20090606 database or in-house database with Mascot Version: 2.3 (Matrix Science) using the following parameters: Taxonomy: Mouse (144,768 sequences); Type of search: MS/MS Ion Search; Enzyme: Trypsin and/or Asp-N-ambic, Fixed Modification: none, Variable modifications: Gln- > pyro-Glu (N-term Q), Oxidation (M), Propionamide (C), and additional modifications (Acrolein adduct (C): C(3) H(4) O(1), Acrolein adduct (N-term): C(3) H(2), FDP-lysine (K): C(6) H(6) O(1), MP-lysine (K): C(6) H(4), Nim-propanalhistidine (H): C(3) H(4) O(1)); Mass values: monoisotopic; Peptide Mass Tolerance; ± 2 Da, Fragment Mass Tolerance; ± 0.8 Da, Peptide charge 1+, 2+ and 3+, Instrument ESI-TRAP and Allow up to 3–4 missed cleavages.

2.7. Measurement of GAPDH activity and ATP content in cells

FM3A cells (1×10^4/ml) were cultured with 0, 4 and 8 μM acrolein for 6, 12 and 24 h. GAPDH activity was measured using KDalert™ GAPDH Assay Kit (Applied Biosystems). ATP content was determined using the 2% trichloroacetic acid extract of FM3A cells by ENLITEN® ATP Assay System Bioluminescence Detection Kit for ATP measurement (Promega). Chemical luminescence was measured with a GloMax® 20/20n Luminometer (Promega).

2.8. Measurement of acrolein toxicity in GAPDH- or β-actin-overproducing Neuro2a cells

Neuro2a cells (5×10^5/10 ml) were cultured for 48 h, transfected with 4 μg of pcDNA-GAPDH, pcDNA-β-actin or vector pcDNA3.1(+) as described above and cultured further for 24 h. Then, cells (3×10^4/ml) were cultured in the presence and absence of various concentrations of acrolein for 3 days and the viable cell number was counted.

2.9. Cellular fractionation and detection of GAPDH and acetylated GAPDH

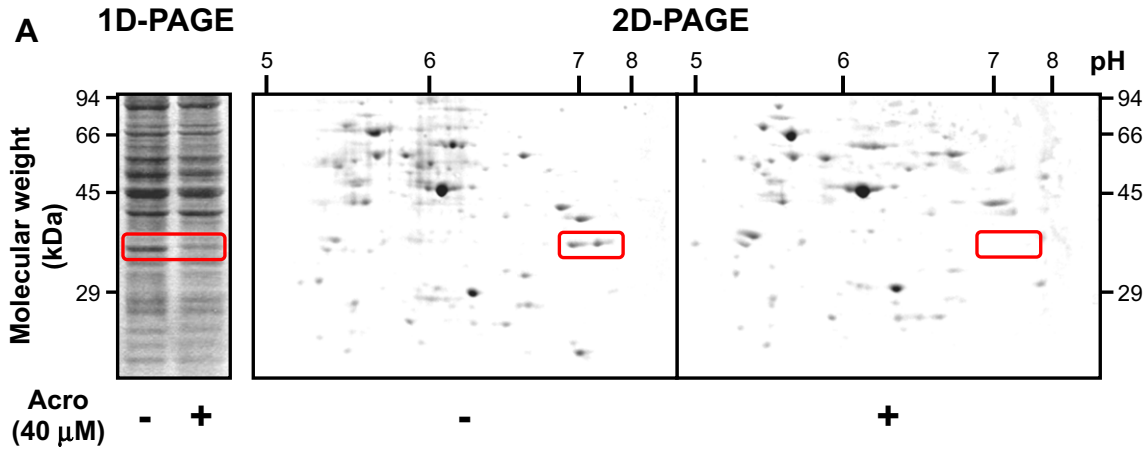
FM3A cells (1×10^4/ml) were treated with or without 8 μM acrolein for 6 h. Cytoplasm and nuclei were isolated according to the method of Park et al. [22]. SDS–PAGE and Western blot analysis were then performed using 10 μg protein of each fraction as described previously [17] using antibody against GAPDH. Immunoprecipitation of each fraction (100 μg protein) by anti-acetyl-Lys was performed as described in Section 2.5, and GAPDH protein was detected by Western blotting using anti-GAPDH antibody. The level of GAPDH in the cytoplasmic and nuclear fractions was quantified with a LAS-3000 luminescent image analyzer (Fuji Film).

2.10. Immunocytochemical detection of GAPDH

FM3A cells treated with 0, 4 and 8 μM acrolein for 6 h were fixed overnight in phosphate-buffered saline (PBS) containing 2% paraformaldehyde and 0.2% picric acid at 4 °C. Fixed cells were attached to poly-L-lysine coated glass, washed with methanol and dried by acetone. The membranes were permealized by exposing the fixed cells to PBS containing 0.3% Triton X-100 at room temperature for 30 min. Glasses were treated with PBS containing 5% FBS and 0.2 mg/ml RNase A at room temperature for 30 min. Detection of GAPDH was performed using antibody against GAPDH. Cellular immunofluorescence was obtained by treating cells with anti-mouse IgG Alexa Fluor 488 (Invitrogen), and images were acquired using a confocal microscope (LSM 510 META Laser Scanning Microscope, Carl Zeiss). DNA was stained with 50 μg/ml propidium iodide.

2.11. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

FM3A cells (1×10^4/ml) were cultured with 0, 4 and 8 μM acrolein for 24 h. TUNEL reaction was performed in TUNEL reaction



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  1 MVKVGVNGFG RIGRLVTRAA ICSGKVEIVA INDPFIDLNY MVYMFQYDST HGKFNGTVKA
 61 ENGKLVINGK PITIFQERDP TNIKWGEAGA EYVVESTGVF TTMEKAGAHL KGGAKRVIIS
121 APSADAPMFV MGVNHEKYDN SLKIVSNASC TTNCLAPLAK VIHDNFGIVE GLMTTVHAIT
181 ATQKTVDGPS GKLWRDGRGA AQNIIPASTG AAKAVGKVIP ELNGKLTGMA FRVPTPNVSV
241 VDLTCRLEKP AKYDDIKKVV KQASEGPLKG ILGYTEDQVV SCDFNSNSHS STFDAGAGIA
301 LNDNFVKLIS WYDNEYGYSN RVVDLMAYMA SKE
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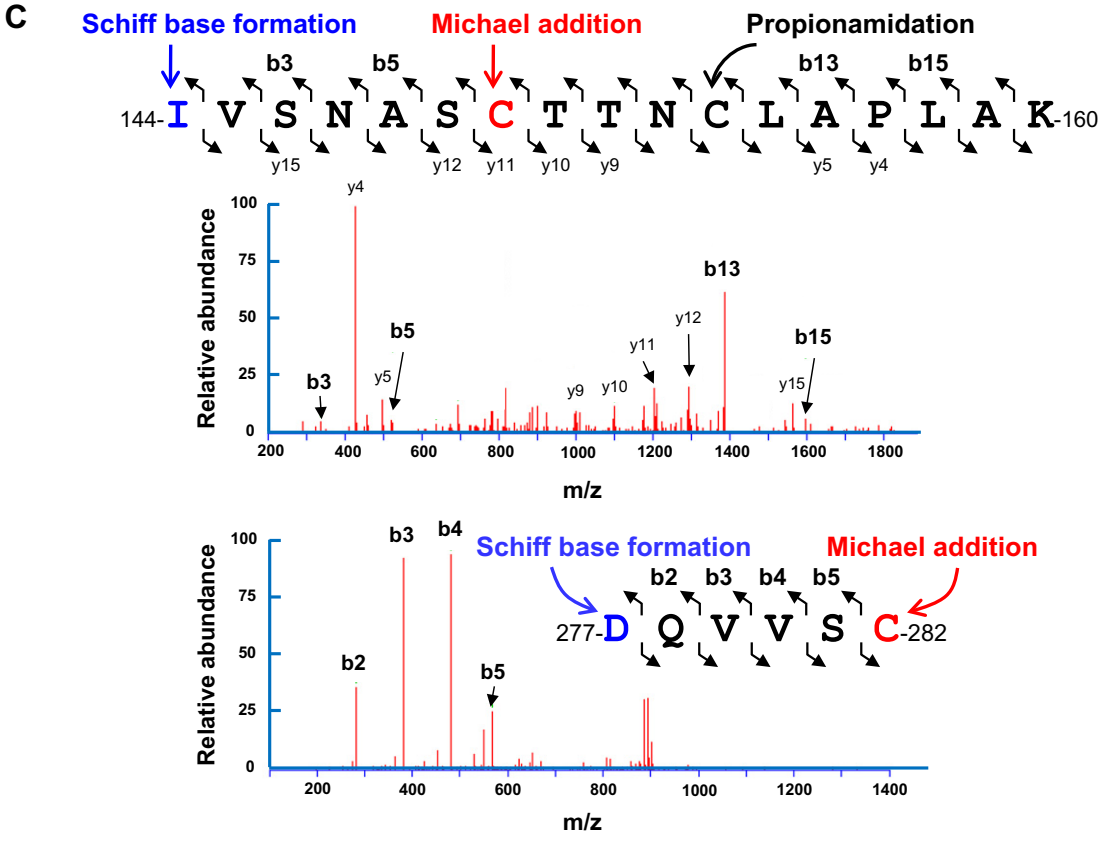

Fig. 1. Identification of acrolein-conjugated 37 kDa proteins as GAPDH by LC-MS/MS. (A) FM3A cells were treated with 0 or 40 μM acrolein for 9 h, and S100 proteins were analyzed by one-dimensional (left) or two-dimensional (right) polyacrylamide gel electrophoresis as described in Section 2. Disappearance of 37 kDa proteins by acrolein was observed. Acro, acrolein. (B) The gel containing 37 kDa proteins was analyzed by LC-MS/MS. Amino acid sequence of mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Swiss-Prot: P16858.2) was shown together with 11 identified peptides (underlined) by LC-MS/MS. (C) Neuro2a cells transfected with pcDNA-GAPDH(HA) were treated with 40 μM acrolein for 9 h. The amino acid residues of HA-tagged GAPDH conjugated with acrolein were determined by LC-MS/MS as described in Section 2. MS/MS spectra of the peptides 144–160 and 277–282 containing cysteine residues conjugated with acrolein were shown. Cys-150 and Cys-282 were conjugated with acrolein by Michael addition and Schiff base was formed intramolecularly at the N-terminus of the peptide.

mixture containing 0.5 μg/ml propidium iodide using In Situ Cell Death Detection Kit, Fluorescein (Roche). TUNEL positive cells were counted under a microscope.

3. Results

3.1. Identification of GAPDH as one of the acrolein-conjugated proteins

It has been reported that acrolein can conjugate with several different proteins [13,14]. Thus we tried to identify protein(s) conjugated with acrolein using 100,000*g* supernatant of FM3A cells treated with 40 μM acrolein for 9 h. As shown in Fig. 1A, in both 1D and 2D gel electrophoresis, the level of a protein of approximately 37 kDa strongly decreased in acrolein-treated FM3A cells compared with control cells. The results suggest that the acrolein conjugated 37 kDa protein became insoluble or shifted from the cytoplasm to mitochondria or nuclei.

Next, this 37 kDa protein obtained from control cells was identified by determining the peptide sequences by LC-MS/MS after protease digestion. As shown in Fig. 1B, amino acid sequences of 11 peptides were determined. These sequences were identical to the peptide sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Among 333 amino acid residues, 137 amino acid residues (41% of total amino acid residues) were identified. Although two spots were observed in proteins of control cells in 2D gel electrophoresis, both spots were identified as GAPDH. The results indicate that GAPDH is one of the major acrolein-conjugated proteins in cells.

3.2. Determination of amino acid residues of GAPDH conjugated with acrolein

Because GAPDH protein could not be immunoprecipitated by antibody against GAPDH after acrolein treatment in FM3A cells, acrolein-conjugated GAPDH was purified using HA-tagged GAPDH and acrolein-conjugated amino acid residues of GAPDH were determined. Neuro2a cells transfected with pcDNA-GAPDH(HA) were treated with 40 μM acrolein for 9 h. HA tagged-GAPDH protein was immunoprecipitated with anti-HA antibody, separated by SDS–PAGE and extracted from polyacrylamide gel. Acrolein-conjugated amino acid residues of GAPDH were then determined by LC-MS/MS using this protein after trypsin and Asp-N digestion. When a cysteine residue is conjugated with acrolein by Michael addition, intramolecular Schiff base formation with the N-terminal amino group of the digested peptide would occur [23]. Cysteine residues unreacted with acrolein are observed as the propionamidated forms. As shown in Fig. 1C, acrolein conjugated with two cysteine residues—Cys-150 and Cys-282. Since Cys-150 is at the active site of GAPDH [15], GAPDH is probably inactivated by acrolein through acrolein conjugation with Cys-150.

3.3. Activity and location of GAPDH in acrolein-treated cells

When FM3A cells were treated with 4 μM acrolein, cell growth slowed down, whereas it was arrested with 8 μM acrolein (Fig. 2A). GAPDH activity in cells treated with 4 μM acrolein was inhibited at 6 h after treatment of acrolein and partially recovered between 12 and 24 h. In contrast, GAPDH activity in cells treated with 8 μM acrolein was greatly inhibited at 6 h and no recovery was observed at 12 and 24 h (Fig. 2B). Although ATP content was not altered in cells treated with 4 μM acrolein, it was greatly diminished in cells treated with 8 μM acrolein in a time-dependent manner (Fig. 2C). To clarify whether the decrease in GAPDH activity correlates with the reduction of cell viability caused by acrolein, the effects of overproduction of GAPDH on cell toxicity caused by acrolein were

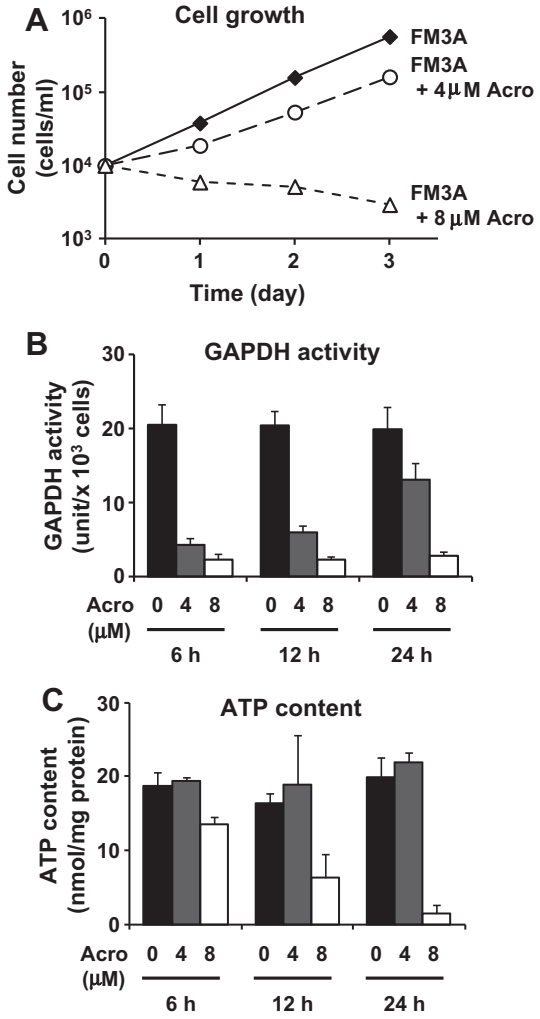

Fig. 2. Effect of low concentrations of acrolein on cell growth (A), GAPDH activity (B) and ATP content (C). FM3A cells were cultured in the absence and presence of 4 and 8 μM acrolein. (A) Cell growth was monitored by counting the viable cell number. Values are means ± S.E. of triplicate determinations. Error bars are within the symbols. (B and C) GAPDH activity and ATP content were measured at 6, 12 and 24 h after the onset of cell growth. Values are means ± S.E. of triplicate determinations. Acro, acrolein.

examined. Neuro2a cells were transfected with pcDNA-GAPDH, pcDNA-β-actin or the vector, and the effect of acrolein on cell growth was determined. The level of GAPDH or β-actin was about two fold higher in cells transfected with pcDNA-GAPDH or pcDNA-β-actin compared with control or mock-transfected cells (Fig. 3A). Under these conditions, inhibition of cell growth by acrolein was partially reversed by overproduction of GAPDH. The IC_{50} value of acrolein increased to 4.3 μM in GAPDH overproducing cells from 2.9 μM in mock-transfected cells (Fig. 3B). In contrast, overproduction of β-actin did not cause the reduction of acrolein toxicity (Fig. 3B), although actin has been reported to be conjugated with acrolein [24].

It is known that inactive GAPDH that is conjugated with nitric oxide (NO) at Cys-150 translocates to nuclei together with Siah, an E3 ubiquitin ligase, and activates p300/CBP acetylase resulting in apoptosis [25,26]. If acrolein-conjugated GAPDH also translocates to nuclei, the same phenomena may occur. Thus, the location of inactivated GAPDH by acrolein was compared with that of normal GAPDH. As determined by Western blotting, GAPDH in control cells was mainly located in the cytoplasm, whereas GAPDH in cells treated with 8 μM acrolein for 6 h was located in both cytoplasm

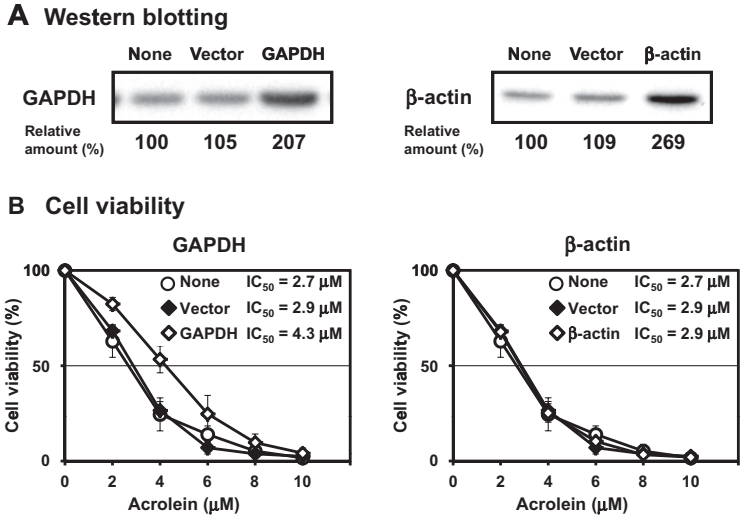

Fig. 3. Recovery of cell viability by overproduction of GAPDH. Neuro2a cells were transfected with vector pcDNA3.1(+), pcDNA-GAPDH or pcDNA-β-actin. (A) The content of GAPDH or β-actin in cells after 24 h of transfection was measured by Western blotting using 5 μg protein of cell lysate. (B) Cells were treated with various concentrations of acrolein and the viable cell number on day 3 was counted. IC_{50} values were indicated in the figure. Values are means ± S.E. of triplicate determinations.

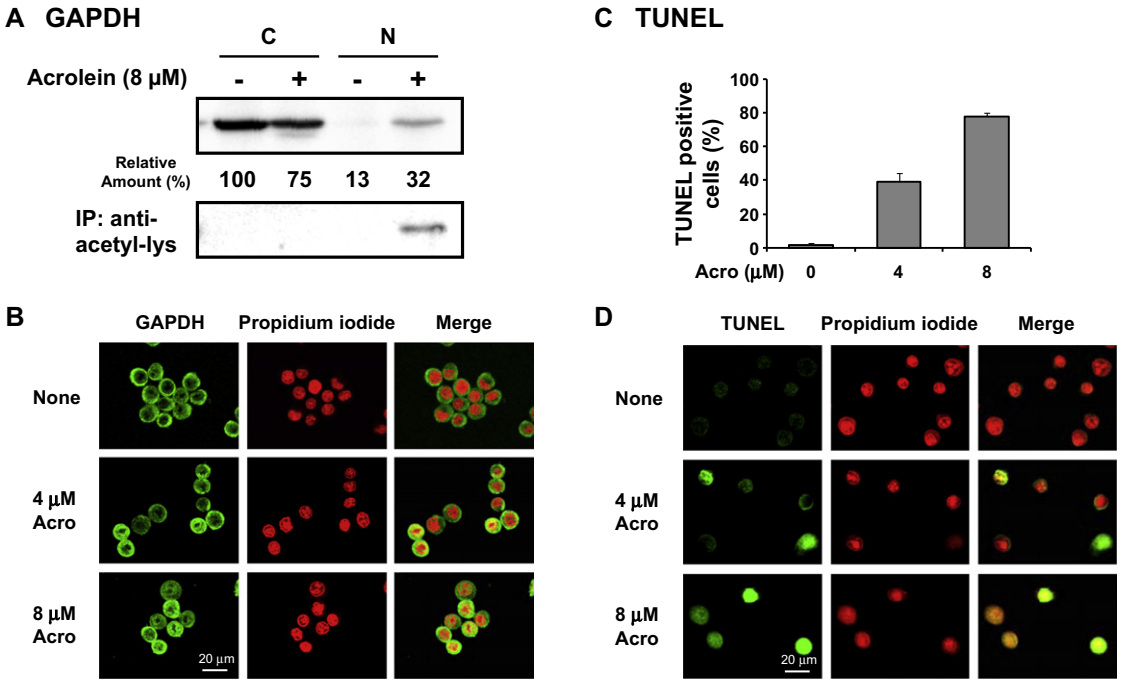

Fig. 4. Cellular distribution of GAPDH and increase in TUNEL positive cells after treatment with acrolein. (A) FM3A cells were treated with 0 or 8 μM acrolein for 6 h, and cytoplasm and nuclei were isolated. GAPDH in each fraction was identified by Western blotting. Immunoprecipitation by anti-acetyl-lysine and detection of GAPDH was performed as described in Section 2. C, cytoplasmic fraction; N, nuclear fraction. (B) FM3A cells were treated with 0, 4 or 8 μM acrolein for 6 h. Immunocytochemical detection using anti-GAPDH antibody and staining of DNA with propidium iodide were performed and the images were merged using a confocal microscope. (C) FM3A cells were treated with 0, 4 and 8 μM acrolein for 24 h and percentage of TUNEL positive cells was shown by counting approximately 500 cells. Values are means ± S. E. of triplicate determinations. (D) Images of TUNEL positive cells, staining of DNA with propidium iodide and the merge image were obtained with a confocal microscope. Bar indicates 20 μm. Acro, acrolein.

and nuclei (Fig. 4A). The location of GAPDH was confirmed by immunostaining of GAPDH in cells. In control cells there was little overlap in GAPDH staining (green) and DNA staining (propidium iodide, red), but in cells treated with 4 and 8 μM acrolein for 6 h, there was marked overlap (yellow) of GAPDH and nuclear (DNA) staining (Fig. 4B). It has been also reported that inactivated GAPDH is acetylated [25,26]. When FM3A cells were treated with 8 μM acrolein for 6 h, the level of acetyllysine in GAPDH localized in nuclei increased (Fig. 4A). These results confirmed that acrolein-treated GAPDH translocates to nuclei like NO-treated GAPDH.

The number of TUNEL positive cells increased after acrolein treatment (Fig. 4C and D). TUNEL positive cells treated with 0, 4 and 8 μM acrolein for 24 h were 1%, 39% and 78%, respectively. The results indicate that acrolein-conjugated GAPDH is acetylated by p300/CBP and causes apoptosis similar to nitrosylated GAPDH [26].

4. Discussion

We have recently reported that tissue damage such as brain infarction correlates more closely with acrolein than with ROS [3,7]. In this communication, the mechanism by which acrolein causes cell toxicity was examined. It was found that a decrease in GAPDH activity is well correlated with a decrease in cell proliferation and transformation of cells with pcDNA-GAPDH partially recovered cell growth.

It has been reported that several proteins conjugate with acrolein [13,14]. Among them, it seems that inactivation of ADP/ATP translocase 1 [27], actin [24], NF-κB1 [28] and protein tyrosine phosphatase 1B [29] are involved in cell toxicity, because these proteins are well associated with cell growth. We previously reported that acrolein interacts with SH-groups much more effectively than with NH_2-groups [3]. A cysteine residue is located at the active site of these proteins similar to GAPDH. However, the relationship between inactivation of these proteins by acrolein and the decrease in cell growth has not been studied in detail. When pcDNA-β-actin was transformed into Neuro2a cells, cytotoxicity of acrolein did not change significantly (Fig. 3B).

GAPDH is not only a classical metabolic enzyme involved in energy production, but also functions as a transcription factor of histone gene expression, nuclear membrane fusion protein, a recognition protein for inappropriately incorporated nucleotides in DNA, and a regulatory protein in the maintenance of telomere structure [15]. However, GAPDH modified by NO at the active site Cys-150 makes a complex with Siah, an E3 ubiquitin ligase, shifts to nuclei and causes cell toxicity [25,26]. GAPDH conjugated with acrolein at Cys-150 also caused cell toxicity by a similar mechanism. Cys-282 was also found to be conjugated with acrolein. It has been reported that Cys-149, at the active site in rabbit GAPDH is essential for oxidative stress-induced aggregation, and that Cys-281 assists the aggregation [30]. Accordingly, acrolein-conjugated Cys-282 is also involved in cell toxicity. Our experimental results indicate that inactivation of GAPDH by acrolein is at least partially involved in cell toxicity caused by acrolein.

We also reported that Lys-557 and Lys-560 are the critical sites on albumin for acrolein adduction in plasma of patients with brain infarction by using LC-MS/MS [21]. In this case, conjugation of acrolein with albumin contributed to a decrease in the toxicity of acrolein.

It has been reported that the function of mitochondria is inhibited by acrolein [31,32]. However, it is not clear which mitochondrial protein(s) are damaged at relatively low concentrations of acrolein. For elucidation of the mechanism of cell toxicity of acrolein, it is important to determine how many proteins are inactivated by acrolein at relatively low concentrations (<50 μM) in intact cells. In this study, it became clear that 8 μM acrolein causes not only a decrease in ATP content but also apoptosis through translocation of acrolein-conjugated GAPDH to nuclei.

Acknowledgments

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