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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