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Induction of Apoptotic Cell Death by Methylglyoxal and 3-Deoxyglucosone in Macrophage-Derived Cell Lines

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Production of 2-oxoaldehyde compounds increases during hyperglycemic conditions and is cytotoxic to susceptible cells. We have investigated the effects of methylglyoxal and 3-deoxyglucosone at physiological concentrations on monocytic leukemia U937 cells and other cell lines. Both ladder formation of DNA and nuclear fragmentation were observed in the cells treated with these agents, indicating that apoptotic cell death was induced. The fluorescent intensity of an oxidation sensitive dye (2′,7′-dichlorofluorescin) was increased in U937 cells but not in other cells in which apoptosis was not induced. The levels of intracellular glutathione, however, were only slightly changed. Apoptosis and intracellular oxidant levels were enhanced by buthionine sulfoximine, an inhibitor of glutathione biosynthesis, and partially blocked by *N*-acetylcysteine, an antioxidant. Thus, it is conceivable that elevation of intracellular oxidant stress is a cause of the apoptosis induced by cytotoxic 2-oxoaldehyde compounds.

The glycation reaction, which proceeds non-enzymatically between reducing sugars such as glucose and fructose and free amino groups in molecules, has been suggested to be involved in the pathogenesis of diabetic complications (1,2). Intermediate products in the glycation reaction are responsible for modification and cross-linking of long-lived proteins such as collagen and lens crystalline, and correlate with aging and diabetes. In hyperglycemic conditions, the levels of cytotoxic aldehydes such as methylglyoxal (MG) and 3-deoxyglucosone (3-DG) increase. 3-DG, a 2-oxoaldehyde, is produced through the degradation of Amadori compounds. MG, another reactive oxoaldehyde, is a physiological metabolite and serves as a substrate for the glyoxalase system. Both compounds are elevated during hyperglycemia and accelerate glycation reaction (3,4). Some oxide-reductases exist in cells to protect against their cytotoxicity (5). We have recently identified aldehyde reductase as one such enzyme (6). Aldose reductase, an enzyme constituting the polyol metabolic pathway, also belongs to this family of genes (7). Interestingly, the cytotoxic effects of 3-DG and glyceraldehyde are enhanced by treatment of cells with an aldose reductase inhibitor (8). Because diabetic complications develop at a slow rate, the long term effects of these compounds on the formation of advanced glycation end products (AGEs) have been well investigated (9). In addition, the inhibitory effects of 3-DG on progression during the S phase were reported in rat fibroblasts (10). However, the mechanism(s) by which these 2-oxoaldehydes exert their cytotoxicity remains virtually unknown.

Here we have investigated the cytotoxic effects of MG and 3-DG, and found that in certain

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Abbreviations: MG, methylglyoxal; 3-DG, 3-deoxyglucosone; GSH, reduced glutathione; GSSG, oxidized glutathione; PBS, phosphate-buffered saline; BSO, buthionine sulfoximine; NAC, *N*-acethyl cysteine; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescein.

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cells they trigger apoptotic cell death. This is seen as an acute effect acting through an oxidation-induced mechanism.

MATERIALS AND METHODS

Cell culture. Human monocytic leukemia cells U937 were cultured in RPMI1640 supplemented with 10% fetal bovine serum (Gibco/BRL, Gaithersburg, MD), containing 100 U/ml penicillin and 10 mg/l streptomycin sulfate (Nacalai Tesque, Tokyo, Japan). The cells were maintained under a humidified atmosphere of 5% CO_2 at 37°C and were treatment with varying concentrations of 3-DG (6) or MG. Since MG (Sigma) contained some impurities, we further purified MG by distillation (11), and used for some experiments. Experiments using these reagents gave essentially the same results.

Assay of DNA fragmentation. DNA fragmentation was assayed as described (12). Briefly, 10^6 cells were pelleted and lysed by the addition of ice-cold 20 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 0.2% Triton X-100. Following centrifugation to pellet high molecular weight DNA together with cell debris, the supernatant was treated sequentially with proteinase K and RNase A. The DNA remaining was extracted with 6 M Nal and 0.5% sodium *N*-lauroylsarcosine, and precipitated with isopropanol. The recovered DNA fragments were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining under UV irradiation.

Morphological analysis of cells by microscopy. After incubation with the reagents indicated, morphological changes of the cells were analyzed by either phase-contrast microscopy or fluorescent microscopy. U937 cells were washed twice with PBS and precipitated by centrifugation at 1,500 rpm for 10 min. They were fixed for 2 h with 4% paraformaldehyde buffered with PBS and stained with 1 mM Hoechst 33258 for 15 min at room temperature.

Assay of intracellular glutathione level. Intracellular glutathione levels were determined by the glutathione recycling method using glutathione reductase as a coupling enzyme as described (13).

Evaluation of intracellular oxidant levels by flow cytometry. Levels of intracellular oxidant were evaluated using an oxidation-sensitive fluorescent probe DCFH-DA as described (14). Cells treated with agents indicated were incubated with 5 mM DCFH-DA for 3 h. After washing with PBS, the fluorescence intensity of the cells was measured with a FACScan (Becton Dickinson, Jose, CA). For each analysis, 10,000 events were recorded.

RESULTS

DNA Ladder Formation by MG and 3-DG in U937 Cells

To investigate the mechanism by which 2-oxoaldehyde compounds exert acute cytotoxicity, we examined the size of nuclear DNAs extracted from cells after incubation with MG or 3-

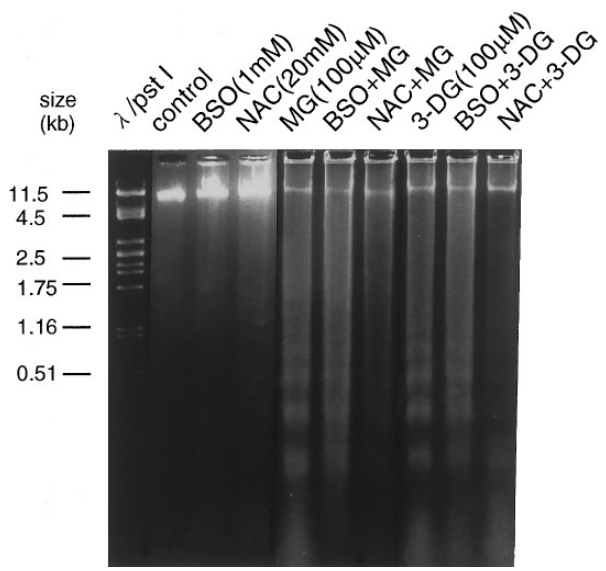

FIG. 1. Ladder formation of nuclear DNA isolated from U937 cells treated with MG and 3-DG. 10^6 cells were treated with 100 μM MG or 100 μM 3-DG in the presence or absence of 1 mM BSO or 20 mM NAC for 24 h. DNA was isolated and subjected to 1.5% agarose gel electrophoresis followed by staining with ethidium bromide.

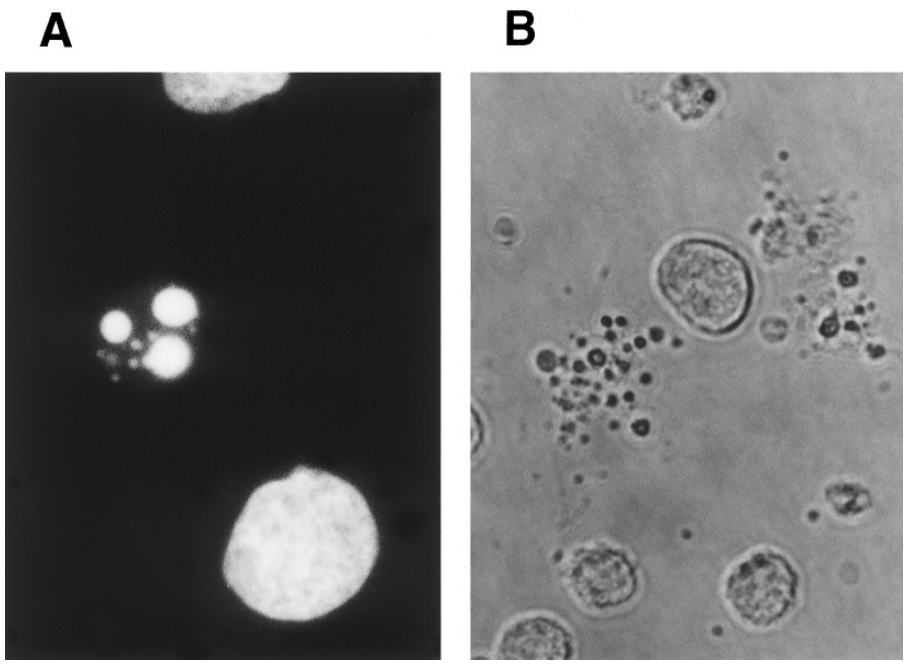

FIG. 2. Nuclear fragmentation and apoptotic body formation by MG. After treatment with 200 μM MG for 6 h, U937 cells were stained with 1 mM Hoechist 33258 for 15 min and examined by fluorescent microscopy (×1000) (A). The cells incubated for 24 h under the same conditions were examined by phase-contrast microscopy (×400) (B).

DG for 24 h on agarose gel (Fig. 1). Both MG and 3-DG induced the ladder formation of the DNA in U937 cells at 100 μM. BSO, an inhibitor for GSH biosynthesis, enhanced but NAC, an antioxidant, partially suppressed the effect of MG and 3-DG. Essentially the same effects were observed in RAW264.7 cells but not in other cell lines such as K562, HL60 and KATO III cells (data not shown). Thus, these compounds induced internucleosomal cleavage of the DNA and their cytotoxic effects were found to be cell-type specific.

Effects of MG and 3-DG on Morphology of Cells

Since internucleosomal cleavage of the DNA is one of the biochemical phenomena of apoptotic cell death, we investigated whether or not these compounds affect morphology of cells by fluorescent microscope using a fluorescent DNA binding dye Hoechst 33258 and by a phase-contrast microscopy (Fig. 2). Nuclear fragmentation was observed by treatment of the cells with 200 μM MG at 6 h (Fig. 2A) when cells were spherical and looked normal. Further incubation induced morphological change in the cells showing apoptotic cell bodies at 24 h with a phase-contrast microscopy (Fig. 2B). These morphological changes characteristic of apoptotic cell death were also observed with 1000 μM 3-DG (data not shown). These data together with Fig. 1 clearly show that MG and 3-DG triggered apoptosis in these cells.

Evaluation of Intracellular Oxidant Stress Using Flow Cytometry

We examined for changes in intracellular oxidant levels in cells after treatment with MG or 3-DG using an oxidation sensitive dye, DCFH-DA, (Fig. 3). Treatment of the U937 cells with MG or 3-DG shifted the peak in a dose-dependent manner (Figs. 3A and 3C), to one with a stronger fluorescent intensity, thereby indicating that the cells treated with these com-

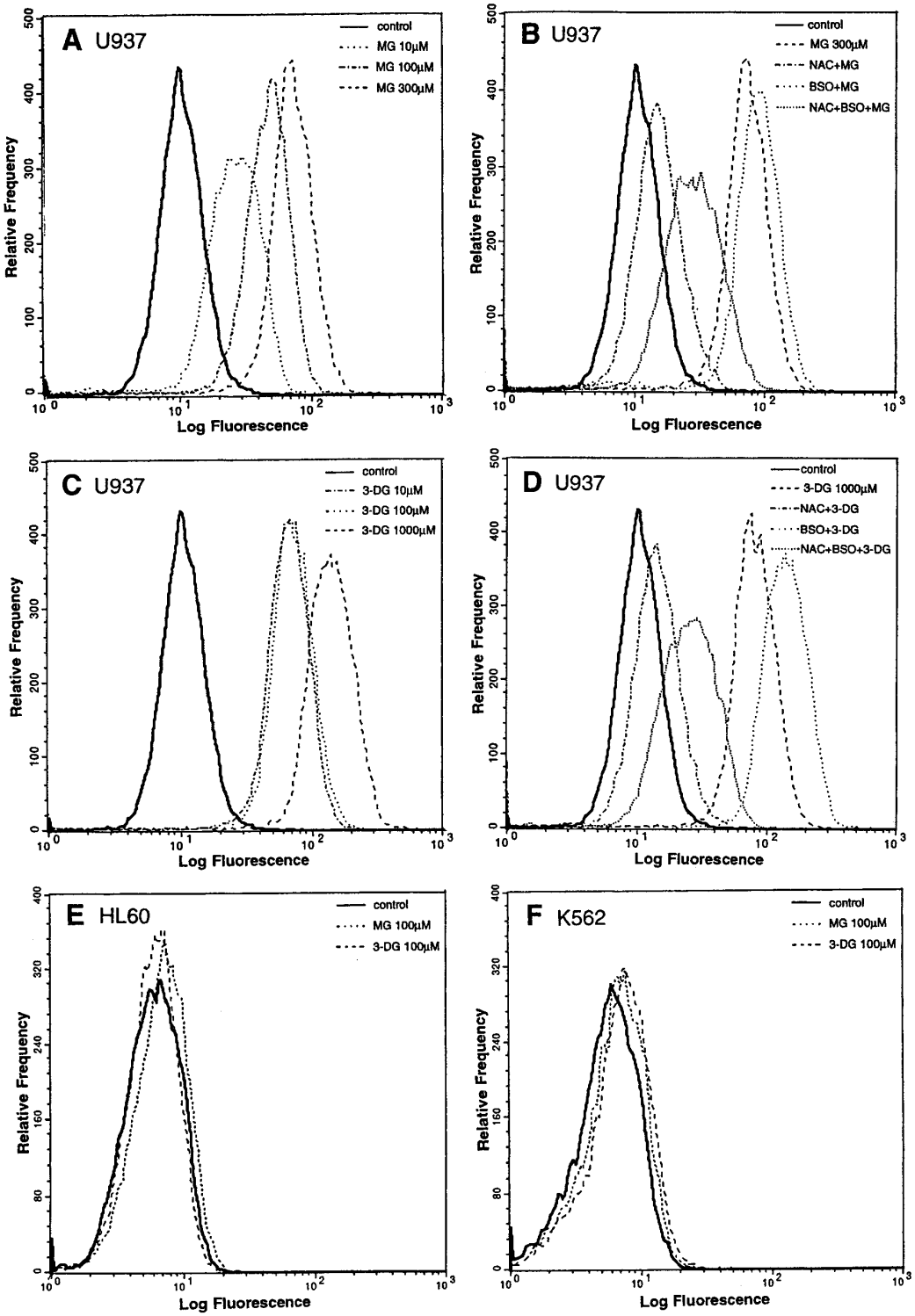
A U937
control
MG 10μM
MG 100μM
MG 300μM
B U937
control
MG 300μM
NAC+MG
BSO+MG
NAC+BSO+MG
C U937
control
3-DG 10μM
3-DG 100μM
3-DG 1000μM
D U937
control
3-DG 1000μM
NAC+3-DG
BSO+3-DG
NAC+BSO+3-DG
E HL60
control
MG 100μM
3-DG 100μM
F K562
control
MG 100μM
3-DG 100μM
Relative Frequency
Log Fluorescence

TABLE 1
Effects of MG and 3-DG on Intracellular Glutathione Levels in Several Cell Lines

	GSH + GSSG (μM/10^6 cells)		
Cell type	Control	MG (300 μM)	3-DG (1000 μM)
U937			
Vehicle	15.0 ± 0.3	14.8 ± 1.3	12.9 ± 0.3
BSO	5.0 ± 0.2	5.0 ± 0.5	3.3 ± 0.5
NAC	22.1 ± 0.8	21.4 ± 0.5	21.6 ± 0.5
BSO + NAC	6.2 ± 0.7	6.2 ± 0.3	6.8 ± 0.5
HL60	7.7 ± 0.5	7.8 ± 0.2	8.1 ± 0.3
K562	7.1 ± 0.5	7.7 ± 0.3	6.5 ± 0.2
KATO III	7.9 ± 0.3	8.2 ± 0.2	7.4 ± 0.3

Mean ± S.D. of triplicate experiments.

pounds were exposed to more oxidant stress than control cells. BSO enhanced, but NAC suppressed the increase in the fluorescent intensity caused by addition of MG or 3-DG (Figs. 3B and 3D). The fluorescent intensities of other cell lines including HL60 (Fig. 3E) and K562 (Fig. 3F), in which apoptosis were not induced by MG or 3-DG treatment (data not shown), were not affected. Thus, the apoptosis induced by MG and 3-DG correlated with the increase in DCFH fluorescent intensity.

Effects of MG and 3-DG on Levels of Intracellular Glutathione

Since GSH normally acts as an antioxidant to protect cells against various forms of oxidative stress, we examined the effect of MG and 3-DG on levels of GSH + GSSG in U937 and other cell lines (Table 1). Their effects on U937 cells pretreated with 1 mM BSO and/or 20 mM NAC for 24 h were also evaluated. 300 μM MG and 1000 μM 3-DG exerted only slight effects on both apoptosis-susceptible and -resistant cells under all conditions examined. BSO decreased the GSH level by about 70%, although it did not induce apoptotic cell death by itself, suggesting that GSH depletion due to conversion of GSH to hemithioacetol by MG (15) was unlikely to be a key mechanism.

DISCUSSION

Production of several 2-oxoaldehyde compounds are elevated during intermediary process of the glycation reaction, especially under diabetic conditions (1,2). While these compounds have reactive groups whereby they can accelerate the glycation process, they can also modify essential cellular components and induce acute damage to cells. Here, we demonstrated that two major carbonyl compounds MG and 3-DG, which reach 5 μM and 1 μM respectively under diabetic conditions (16,17), are capable of inducing apoptotic cell death in a macrophage-derived cell line U937. The involvement of reactive oxygen species in some types of apoptosis has been suggested (18,19), and here too the signal which triggers apoptosis by MG and 3-

FIG. 3. Examination of intracellular levels of oxidants by flow cytometry using DCFH-DA. U937 cells were incubated in varying concentrations of MG (A) or 3-DG (C) for 6 h. Effects of 300 μM MG (B) and 1000 μM 3-DG (D) on the cells pretreated with 1 mM BSO and/or 20 mM NAC for 24 h were also examined. After further incubation with DCFH-DA for 1 h, fluorescent intensity was measured by flow cytometry. Effects of 100 μM MG and 100 μM 3-DG on HL60 cells (E) and K562 cells (F) were also shown.

DG was also likely to be mediated by oxidation of an essential signal-transducing component. The levels of intracellular oxidants increased in the cells in which apoptotic cell death was induced by MG and 3-DG treatment, as judged by measuring DCF fluorescence, but not in other cells. The enhancing effect of BSO on the apoptosis and partial prevention by NAC also supported this hypothesis. However, the change in glutathione levels was not in itself a direct cause, since BSO alone was unable to induce apoptosis, and both MG and 3-DG only slightly affected the intracellular levels of GSH. It is not clear at the present time what signals the cells to die by apoptosis when subjected to these compounds.

It is well known that some types of apoptosis take place by a process of gene expression. Transcriptional factors such as AP-1 and NF-κB, which can be activated by oxidative stimulation of cells (20), may be involved in this type of apoptosis through activating a gene responsible for apoptotic cell death. Since aldehyde reductase is a major hepatic enzyme which detoxifies MG and 3-DG (6), inactivation of this enzyme by the glycation reaction under diabetic conditions may accelerate apoptotic death in certain cells (21).

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