Reactive oxygen species induce apoptosis of vascular smooth muscle cell

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Abstract Apoptosis of vascular smooth muscle cell (VSMC) plays an important role in the genesis of atherosclerosis and restenosis. In order to investigate the role of reactive oxygen species in the induction of VSMC apoptosis, rat VSMCs were treated with glucose oxidase/glucose (GO/G) or diethylmaleate (DEM). The results showed that GO/G and DEM led to VSMC death. Administration of catalase, superoxide dismutase and deferoxamine revealed that H_2O_2 was the major reactive oxygen species causing cell death, and H_2O_2O exerted its effect by formation of hydroxyl radical ($^{\cdot}OH$). GO/G- and DEM-induced VSMC death occurred by apoptosis characterized by "DNA ladders", condensation of nuclei, positive to in situ nick-end labeling and increases in histone-associated DNA fragmentation. This study suggests that H_2O_2 and its derived form $^{\cdot}OH$ might be related to apoptosis of VSMC in atherosclerosis and restenosis.

Key words: Apoptosis; Vascular smooth muscle cell; Reactive oxygen species

1. Introduction

There is an increasing body of evidence showing that VSMC apoptosis is involved in the pathogenesis of atherosclerosis and restenosis [1–4]. For example, apoptotic VSMCs are present in human atherosclerotic and restenotic lesions [2,4]. Balloon injury initiates a profound apoptotic response in neointimal VSMCs [4]. Recent investigations have demonstrated that simultaneous treatments with IFN-γ and TNF-α and/or IL-1β can trigger apoptosis in cultured human and rat VSMCs [5]. Oxidatively modified low density lipoprotein can also induce apoptosis in VSMCs [6]. Nevertheless, the mechanisms whereby apoptosis of VSMCs is triggered still remain largely unknown.

Previous experiments have shown that H_2O_2 is effective in stimulating the in vitro growth of several cell types such as fibroblasts of hamster and rat [7,8], and mouse osteoblastic cells [9]. Also, human and rat VSMCs have been reported to undergo DNA synthesis in response to H_2O_2 stimulation [10,11]. Recently, it has been found that VSMCs exposed to H_2O_2 undergo death rather than proliferation, although H_2O_2 can stimulate DNA synthesis in VSMCs [12]. Whether reactive oxygen species (ROS) can induce apoptosis of VSMC is as yet unclear.

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Abbreviations: VSMC, vascular smooth muscle cell; ROS, reactive oxygen species; DEM, diethylmaleate; GO/G, glucose oxidase/glucose; SOD, superoxide dismutase; CAT, catalase; DF, deferoxamine; MTT, 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide

In the present study, we examined the role of ROS in the induction of VSMC apoptosis. We employed GO/G, an enzymatic system which produces H_2O_2 [13], to treat cultured rat VSMCs. DEM, which leads to the accumulation of ROS in the cell by reducing intracellular glutathione [14], was also employed.

2. Materials and methods

2.1. Materials

Glucose oxidase, glucose, maleic acid diethyl ester, bovine erythrocyte superoxide dismutase (SOD), bovine liver catalase (CAT), deferoxamine (DF), propidium iodide, benzoic acid and mannitol were from Sigma Chemical Co. (St. Louis, MO). MTT kit and cell death detection ELISA kit were from Boehringer Mannheim. The in situ apoptosis detection kit was from Oncor.

2.2. Cell culture

VSMCs were obtained from the thoracic aortas of 200–250 g male Wistar rats using the collagenase and elastase digestion method [15]. Cells were seeded in Medium 199 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37°C. Cells at passage 8–13 were made quiescent by incubating in the above culture medium containing 0.2% FCS for 48 h before use.

2.3. Exposure of cells to GO/G or DEM

DEM was added to the culture medium. For the GO/G treatment, cells were washed twice with Hank's balanced salt solution (HBSS). Washed cells were exposed to indicated concentrations of GO/G in 10 ml of HBSS for 1 h in a humidified 5% CO_2 atmosphere at 37°C. The reaction was stopped by removing the HBSS containing GO/G. Cells were further cultured in freshly prepared culture medium for the indicated time. Control cells were incubated under the same conditions without GO/G or DEM. For the administration of antioxidants, SOD, CAT, benzoate and mannitol were added simultaneously with GO/G or DEM, whereas DF was preincubated for 2 h before treatment. 6 mM glucose alone and the antioxidants applied in the present study do not cause VSMC proliferation or death detected by MTT test (data not shown).

2.4. Cell viability assay

Cell viability was assessed with MTT test according to the kit instructions. Optical density was read at 570 nm.

2.5. Analysis of DNA fragmentation

Cells were digested with lysing buffer (10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS (w/v), 50 μg/ml proteinase K, 20 μg/ml RNase) at 37°C for 20 h. DNA was extracted with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1), subjected to electrophoresis on a 1.8% agarose gel and stained with ethidium bromide.

2.6. In situ nick-end labeling and propidium iodide staining

The terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay was used to detect DNA fragmentation in situ according to the kit instructions. After TUNEL labeling, samples were stained for 8 min with propidium iodide at 5 mg/ml in PBS, pH 7.4, containing 50 mg/ml RNase A (without DNase).

2.7. Cell death detection ELISA

Cell death detection ELISA was performed according to the man-

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ufacturer's instructions. Optical density was read using an ELISA reader at 405 nm.

2.8. Statistical analysis

The data are expressed as means ± S.E.M. of at least three independent experiments. Paired data were evaluated by Student's *t*-test. $p < 0.05$ was considered significant.

3. Results

As shown in Fig. 1A, GO in the presence of glucose (6 mM) produced dose-dependent decreases in VSMC viability. ROS include H_2O_2, ^1O, $^{\bullet}OH$, O_2^- and so on. To determine which species of reactive oxygen plays a critical role in causing cell death, several ROS scavengers were administrated. Simultaneous administration of CAT almost completely blocked GO/G-induced cell death (Fig. 1B). Pretreatment with DF, an iron chelator which prevents the formation of $^{\bullet}OH$ from H_2O_2 via the Fenton or Haber-Weiss reaction [16], partially prevented GO/G-induced cell death. The results suggest that H_2O_2 produced by GO/G is the major species in causing VSMC death, and H_2O_2 may exert its effect by formation of $^{\bullet}OH$. Administration of benzoate (1–500 μM) and mannitol (0.1–50 mM), two scavengers of $^{\bullet}OH$, failed to prevent GO/G-induced cell death. SOD also had no protective effect on GO/G-induced cell death.

Exposure to DEM resulted in VSMC death (Fig. 1C). This effect could be attenuated by pretreatment with DF or by simultaneous administration of CAT, but not by benzoate (1–500 μM), mannitol (0.1–50 mM) or SOD (Fig. 1D). These results confirm the role of H_2O_2 and $^{\bullet}OH$ in DEM-induced cell death.

To examine if GO/G- and DEM-induced VSMC death occurs by apoptosis, several criteria were applied. First, agarose gel electrophoresis was used to define apoptosis. Both GO/G (Fig. 2A) and DEM (Fig. 2B) exposure led to a "DNA ladder", a sign of fragmentation of nuclear DNA into oligonucleosomal subunits. Cell death by apoptosis was next confirmed by in situ nick-end labeling and propidium iodide staining. Untreated VSMCs had large and regularly shaped nuclei stained by propidium iodide (red), and they were concomitantly not positively labeled by TUNEL (Fig. 3A). However, condensed apoptotic nuclei were observed in VSMCs at 8 h after treatment with GO/G (Fig. 3B) or at 24 h after treatment with DEM (Fig. 3C), with the condensed apoptotic nuclei labeled by TUNEL (green or yellow). Cell death detection ELISA was further employed to characterize apoptosis. The data showed that exposure to GO/G or DEM led to increases in DNA fragments in the cytoplasmic fraction of cells (Fig. 4). Taken together, these criteria to characterize

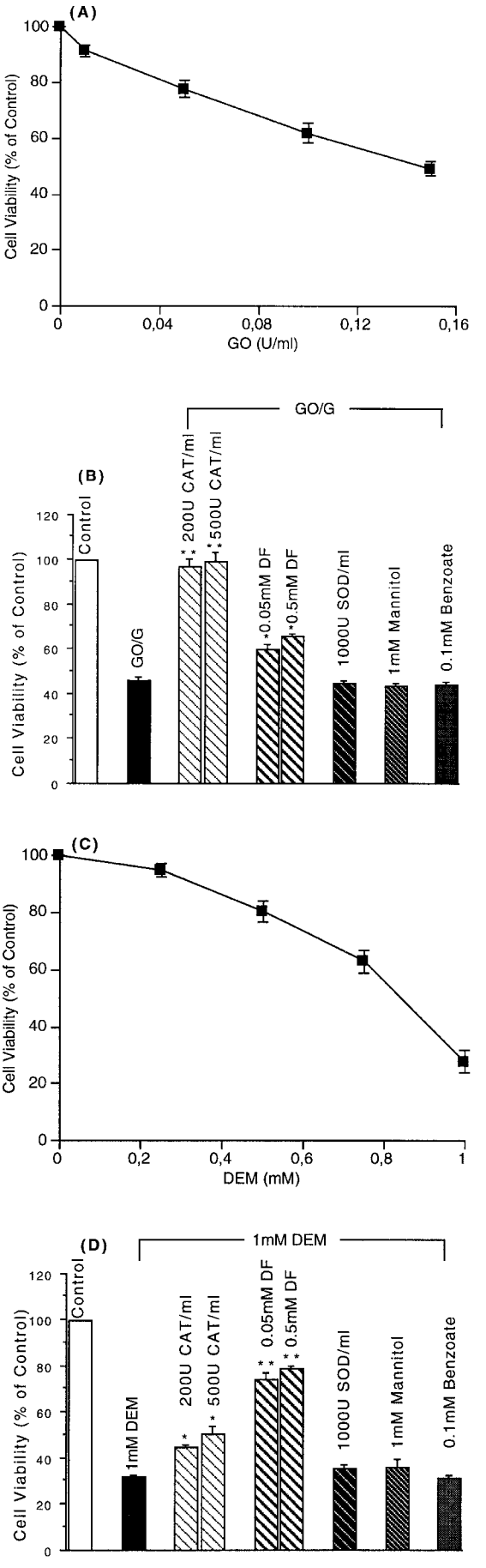

Fig. 1. Effect of GO/G and DEM on VSMC viability assessed by MTT. (A) VSMCs were exposed to increasing concentrations of GO plus glucose at 6 mM. (B) Cells were exposed to 0.15 U GO/ml plus 6 mM glucose with simultaneous administration of CAT, SOD, mannitol, benzoate, or pretreatment for 2 h with DF. Compared to GO/G alone (column 2), $^*p < 0.02$, $^{**}p < 0.005$. Cell viability was analyzed at 8 h after GO/G treatment. (C) VSMCs were exposed to increasing concentrations of DEM. (D) Cells were exposed to 1 mM DEM with simultaneous administration of CAT, SOD, mannitol, benzoate, or pretreatment for 2 h with DF. Compared to DEM alone (column 2), $^*p < 0.04$, $^{**}p < 0.005$. Cell viability was analyzed at 24 h after DEM treatment.

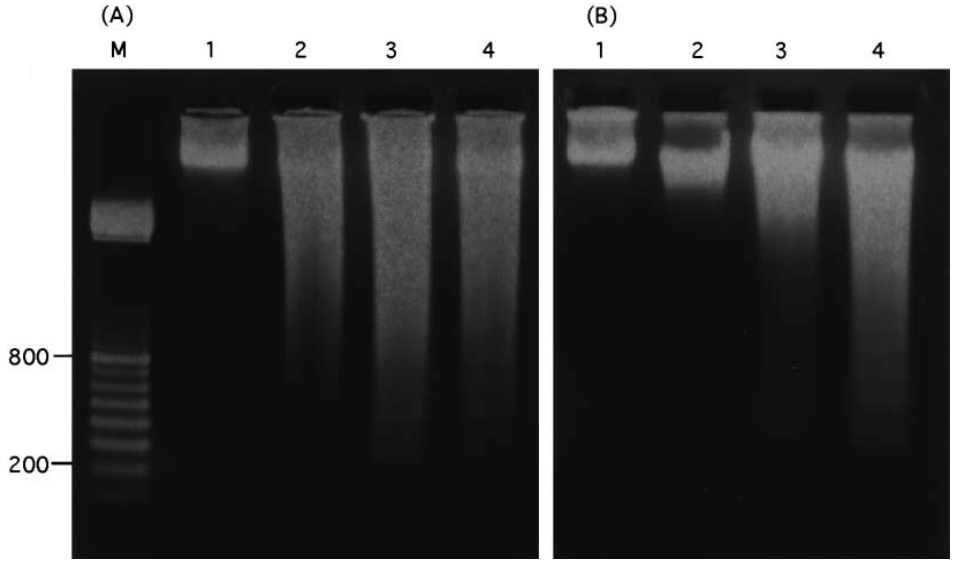

Fig. 2. DNA fragmentation detected by agarose gel electrophoresis. DNA was extracted at 8 h after treatment with GO/G or at 24 h after treatment with DEM. Lane M, 100 bp DNA marker. (A) Lane 1, control. Lanes 2–4, cells were treated with GO at 0.05, 0.1 and 0.15 U/ml, respectively; glucose was at 6 mM. (B) Lane 1, control. Lanes 2–4, cells were treated with DEM at 0.5, 0.75 and 1.0 mM, respectively.

apoptosis suggest that cell death caused by GO/G or DEM occurs by apoptosis.

4. Discussion

The present study employed GO/G as an enzymatic generator of extracellular H_2O_2. The data illustrate that H_2O_2 could lead to VSMC death but not proliferation. Such results are in accordance with the previous observation [12]. Thus, extracellular H_2O_2 can not function as a mitogen to stimulate VSMC proliferation, although it can stimulate DNA synthesis in VSMCs [10,11]. In contrast to exogenous H_2O_2, there is evidence showing that intracellular H_2O_2 may act as second messenger for a variety of growth factors. For example, stimulation of VSMCs by platelet-derived growth factor, epidermal growth factor, fibroblast growth factor or angiotensin II all led to a rise in intracellular H_2O_2 concentration, and the response of VSMCs to these growth factors was inhibited when H_2O_2 was blocked [17]. Nevertheless, the protective effect of CAT on DEM-induced VSMC apoptosis observed in the present study (Fig. 1D) reveals that intracellular H_2O_2 may also trigger apoptosis if it can alter the cellular redox state which has been shown to regulate cell death [18].

H_2O_2 itself has been shown to have little direct toxic effect. Its cellular injury is more likely related to $^{\bullet}OH$, which is formed in metal-catalyzed Fenton or Haber-Weiss reactions [19]. In our experimental model, we found that H_2O_2 participated in GO/G- and DEM-induced VSMC apoptosis, and it exerted its effect via formation of $^{\bullet}OH$. DF can prevent the formation of $^{\bullet}OH$, whereas benzoate and mannitol scavenge the formed $^{\bullet}OH$. Our data showed that DF rather than benzoate or mannitol protected VSMCs from death caused by GO/G or DEM, suggesting that blocking the formation of $^{\bullet}OH$ directly by DF is more effective than scavenging $^{\bullet}OH$ in preventing H_2O_2-induced VSMC apoptosis. Furthermore, the inconsistencies of $^{\bullet}OH$ scavengers in protecting GO/G-

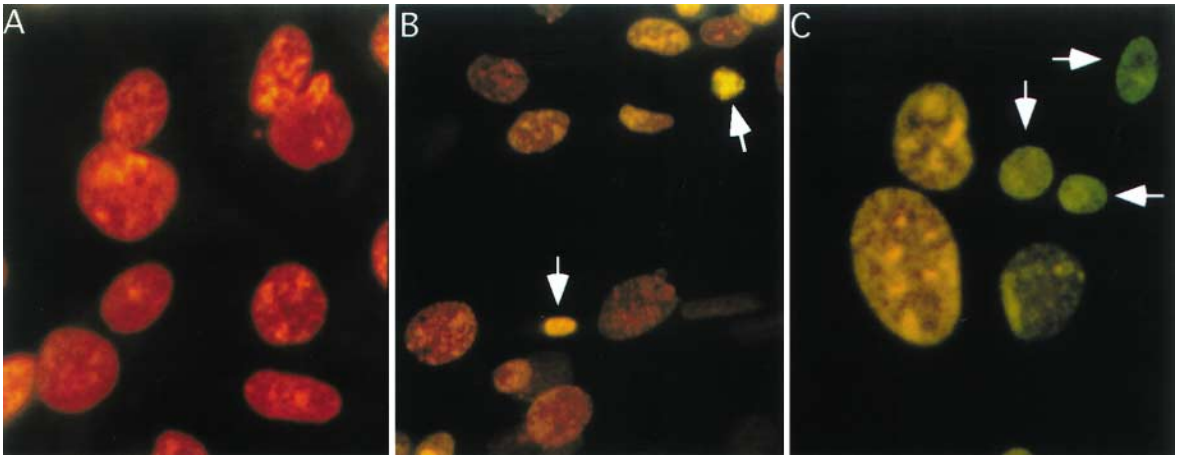

Fig. 3. TUNEL labeling and propidium iodide staining of VSMCs exposed to GO/G or DEM. (A) Control culture. (B) Cells treated with 0.15 U GO/ml plus 6 mM glucose. (C) Cells treated with 1 mM DEM. TUNEL labeling and propidium iodide staining were processed at 8 h after treatment with GO/G or at 24 h after treatment with DEM. Arrows indicate the apoptotic cells. Original magnification ×400.

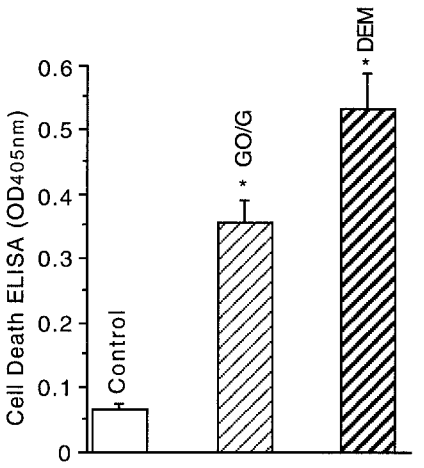

Fig. 4. Cell death determined by ELISA. The ELISA detection was processed at 8 h after treatment with 0.15 U GO/ml plus 6 mM glucose or at 24 h after treatment with 1 mM DEM. The histone-associated DNA fragments were presented as the optical density at 405 nm. Compared to control, $^{*}p < 0.0001$.

and DEM-induced VSMC apoptosis in our present study may be explained by site-specific action of $^{\cdot}OH$ which is derived from H_2O_2 in the presence of metals bound to critical cellular targets [20–22].

In atherosclerosis, apoptotic cells are prominent in the advanced atherosclerotic lesions and in the macrophage-enriched area [2,4]. It has been speculated that ROS might participate in triggering apoptosis of VSMC because excessive ROS can be generated by macrophages during phagocytosis [4,19]. ROS are overproduced after angioplasty [23]. Our present study for the first time reveals that ROS can trigger apoptosis of VSMC.

In conclusion, the present study provides evidence that extracellular H_2O_2 does not function as a mitogen for VSMC, instead, it is a stimulus to trigger VSMC apoptosis. ROS may participate in atherosclerosis and restenosis by triggering apoptosis of VSMC.

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