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Preventive effect of Ginkgo biloba extract on apoptosis in rat cerebellar neuronal cells induced by hydroxyl radicals

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Abstract

The ability of oxidative stress to induce apoptosis, and the effect of Ginkgo biloba extract (EGb761) on this induction were studied in primary cultured rat cerebellar neuronal cells. Cells were exposed to hydroxyl radicals by treating them with 20–50 μM hydrogen peroxide (H_2O_2) and 100 μM ferrous sulfate. Hydroxyl radical treatment fragmented the DNA in a manner typical of apoptosis cells, producing a ladder pattern of 200 base pair increments on 1% agarose gel electrophoresis. Pretreatment of cells with 100 μg/ml EGb reduced hydroxyl radical induced cells apoptosis (determined by flow cytometry) and DNA fragmentation. The results indicate that hydroxyl radicals induce apoptosis in rat cerebellar neuronal cells, and this induction can be prevented by EGb.

Keywords: Apoptosis; EGb761; Hydroxyl radicals; Primary cerebellar neurons culture; Flow cytometry

Recent evidence has focused attention on the role of oxidative stress in various acute and chronic neurodegenerative diseases. But the precise mechanism by which degenerative disorders of the human central nervous system associated with oxidative stress occurs is unclear [3]. Cell death can occur by either apoptosis or necrosis. Necrosis is characterized by a generalized breakdown of cellular structure and function followed by cell lysis and tissue inflammation. Apoptosis, on the other hand, is characterized by cell shrinkage, membrane blebbing, formation of apoptotic bodies, and fragmentation of nuclear DNA into oligonucleosomal subunits [2]. Apoptosis is thought to play a putatively central role in the pathogenesis of neurodegenerative disorders [17]. Apoptosis can be triggered by different conditions, such as tumor necrosis factorα [10]. Hydrogen peroxide (H_2O_2) has recently been implicated as a cytotoxic agent inducing apoptosis in thymocytes and cortical neurons, and antioxidant such as Torlox (water-soluble analog of vitamin E) or *N*-acetylcysteine inhibited cell apoptosis induced by oxidative stress [2,15,19]. Oxidative stress is a mediator of apoptosis, and antioxidants may provide an exciting preventive and therapeutic prospective for neurodegenerative disorders.

Ginkgo biloba extract (EGb761) has been reported to protect the brain against hypoxic damage, prolong the survival of rats and to inhibit the reactive oxygen species (ROS) formation in rat brain neurons [13]. But it was not reported whether EGb761 has a protective effect on apoptosis. Cerebellar neurons grown in primary culture provide a convenient in vitro preparation for examining the mechanisms by which neuronal degeneration is induced by oxidative stress. In this study, we investigated whether oxidative stress produced by treatment with hydroxyl radicals can induce apoptosis in rat cerebellar neurons, and we also studied the protective effects of Ginkgo biloba extract (EGb761) against apoptosis.

Primary cultures of cerebellar cells were prepared from 7-day-old rats (Wistar) as previously described [18] with minor modifications. Briefly, after mechanical dissociation of the cerebella, cells were plated (2×10^6 cells/ml, 2 ml/well) in 35 mm Costar culture dishes (6-well clusters; Cambridge, MA) previously coated with poly-L-lysine (40 μg/ml, MW 300 000; Sigma). Culture medium was Dulbecco's minimal essential medium (high glucose,

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GIBCO), supplemented with glutamine (2 mM), sodium bicarbonate (44 mM), HEPES (5 mM), fetal calf serum (FCS, 10%). The cells were grown at 37°C in 5% CO_2/95% air with 100% relative humidity. After 2 days in vitro, experiment culture medium was replaced with fresh medium containing low concentration serum (1%), and neurons were cultured for 8 h.

Neurons were exposed to 50 μM H_2O_2 supplemented with 100 μM ferrous sulfate and incubated for 8 h at 37 C under an atmosphere of 5% CO_2 in air. The control cells were incubated under the same conditions without H_2O_2 and ferrous sulfate. On the basis of MTT assay and quantitative analysis of DNA fragmentation (unpublished data), EGb761 (100 μg/ml) was chosen in our study. Neurons were treated with EGb761 (100 μg/ml) 15 min before exposure to 50 μM H_2O_2 and 100 μM ferrous sulfate. Hydrogen peroxide is known to produce hydroxyl radicals by the Fenton reaction in the presence of iron. The medium was supplemented with ferrous sulfate so that the concentration of iron would not be a limiting factor in the oxidative exposures. Unpublished results from our laboratory indicate that the addition of ferrous sulfate (100 μM) alone does not induce apoptosis.

Analysis of DNA fragmentation, which is a well-known marker of apoptosis, was performed as previously described [14] with minor modification. Briefly, cells were collected, rinsed with PBS, lysed in 100 μl volumes of lysis buffer (10 mM EDTA 50 mM Tris–HCl (pH 8.0), 0.5% sodium lauryl sarcosine, 0.5 mg/ml Proteinase K) and then incubated at 50°C for 1.5 h; RNase A (0.5 mg/ml) was added and lysates were incubated for an additional 1.5 h; phenol extractions (equal volume) were performed followed by one chloroform extraction. DNA was precipitated with 2 vols. of ice-cold ethanol and 0.1 vols. of 10 M ammonium acetate at −20°C overnight. DNA was pelleted by centrifugation at 13 000 × g for 20 min at 4°C, air dried, and resuspended in 60 μl TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). DNA was electrophoresed in a 1% agarose gel in 0.5 × TBE running buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). The gel was stained with 0.5 μg/ml ethidium bromide and photographed by UV transillumination.

Flow cytometric DNA analysis was performed in order to evaluate the percentage of apoptotic cells whose DNA content was lower than that of diploid cells. As is generally accepted, apoptotic cells can be recognized by their diminished stainability with DNA specific fluorochromes which is due to DNA degradation and its subsequent leakage from the cell [11]. The method for DNA labelling was performed as previously described [12] with minor modification. Briefly, culture cells were prepared as a single cell suspension in 200 μl PBS, fixed with 2 ml of ice cold 70% ethanol, maintained at 4°C overnight. The cells were harvested by 200 × g centrifugation for 10 min, resuspended in 500 μl PBS supplemented with RNase (100 μg/ml), incubated at 37°C for 30 min and stained with 50°μg/ml propidium iodide (PI) at 4°C for 30 min.. The red fluorescence of individual cell was measured with FACScan (Becton Dickenson, Mountain View, CA).

Hydroxyl radical induced DNA fragmentation and the protective effect of EGb761 were detected by electrophoresis. Electrophoresis of DNA extracted from cerebellar neurons incubated with 20 μM or 50 μM H_2O_2 and 100 μM Fe^{2+} for 8 h revealed oligonucleosomal fragmentation with the characteristic ladder pattern associated with apoptosis (Fig. 2, lanes 2, 3). DNA isolated from cerebellar cells, which were cultured in complete medium or in low concentration serum medium, and which had been pretreated by EGb761 or catalase for 15 min and exposed to 50 μM H_2O_2 + 100 μM Fe^{2+}, did not reveal this oligonucleosomal chromatin cleavage (Fig. 1, lanes 4–7). These results indicate that hydroxyl radicals can induce DNA fragmentation and EGb761 can inhibit the DNA fragmentation induced by hydroxyl radicals.

Hydroxyl radical induced apoptosis and the protective effect of EGb761 were analyzed quantitatively by flow cytometry. Fig. 2 shows DNA content histograms obtained after PI staining of cerebellar neurons which had been incubated for 8 h in complete medium (A), in low concentration serum medium (E3) in the presence of 50 μM H_2O_2 + 100 μM Fe^{2+} with or without pretreatment by EGb761 or catalase (C–E). The percentages of normal (diploid and supradiploid DNA content) and apoptotic

Fig. 1. Electrophoresis pattern of DNA fragmentation of cerebellar neurons exposed to exogenous hydroxyl radicals with or without EGb pretreatment for 8 h. Lane 1, DNA marker (λDNA/*Eco*RI + *Hin*dIII); lane 2, 20 μM H_2O_2 + 100 μM Fe^{2+} exposure; lane 3, 50 μM H_2O_2 + 100 μM Fe^{2+} exposure; lane 4, 50 μM H_2O_2 + 100 μM Fe^{2+} exposure with 100 U/ml catalase pretreatment; lane 5, 50 μM H_2O_2 + 100 μM Fe^{2+} exposure with 100 μg/ml EGb pretreatment; lane 6, low concentration serum (1%) medium; lane 7, complete (10% FCS) medium.

cells (subdiploid DNA content), calculated according to the DNA content are shown in Fig. 3. After 8 h incubation in complete medium, less than 7% cells underwent apoptosis, in low concentration serum medium about 21% cells underwent apoptosis, whereas in the presence of 50 μM H_2O_2 + 100 μM Fe^{2+} with low concentration serum medium over 41% cells underwent apoptosis. When the cells had been pretreated with EGb761 or catalase, and then exposed to 50 μM H_2O_2 + 100 μM Fe^{2+}, the apoptotic cells were 28%, or 19%, significantly lower than cells treated with hydroxyl radicals ($P < 0.01$). These results also indicate that hydroxyl radicals can induce cell apoptosis and EGb761 can protect the cell from apoptosis.

Several experimental results indicate the involvement of oxygen radicals in apoptosis. Hydrogen peroxide induces apoptosis, and Bcl-2 seems to inhibit apoptosis by decreasing the generation of reactive oxygen species [5,7]. Evidence has also been presented that oxidative stress induced apoptosis in rat cortical neurons and human neuroectodermal cell line [8,16,19]. In other experiments, stimuli that cause oxidative stress, including culture in high oxygen [1] and exposure to β-amyloid [9] have been reported to trigger the apoptotic process in neurons. Antioxidants, such as acetylcysteine and thioredoxin, protect against apoptosis induced by tumor necrosis factor-α [10]. In this paper, we demonstrate that cerebellar neuronal death in an in vitro model of oxidative stress is apoptotic. The data show hydroxyl radical exposure with low concentration serum medium can induce neuronal apoptosis. The low concentration medium alone or hydroxyl radical exposure with complete medium (50 μM H_2O_2 + 100 μM Fe^{2+}, data not shown) could only induce less neuronal apoptosis and did not produce DNA ladder in electrophoresis. The concentration of H_2O_2 (50 μM) that we used in this study is lower than others previously used (1–5 mM) in inducing apoptosis in neuronal cells [8,19]. Our present model may be closer to the physical condition in vivo. The possible interpretation may be the serum deprivation of cultured neurons which leads to increased endogenous free radical production [20].

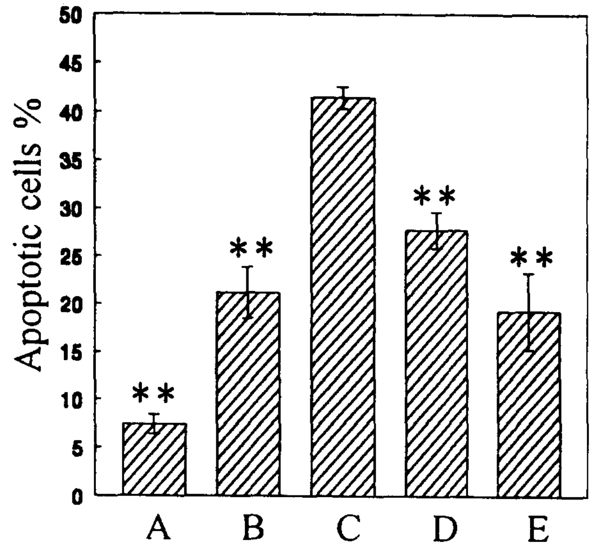

Fig. 3. Percentage of apoptotic cells after exposure to exogenous hydroxyl radicals with or without EGb treatment determined by flow cytometric analysis. The data were shown as means ± SEM by the cell incubated in complete medium (A), in low concentration serum medium (B), in medium with 50 μM H_2O_2 + 100 μM Fe^{2+}(C), in medium with EGb and H_2O_2 + Fe^{2+} (D), in medium with catalase and H_2O_2 + Fe^{2+} (E). Similar results were obtained in three experiments. $^{**}P < 0.01$ compared to (C).

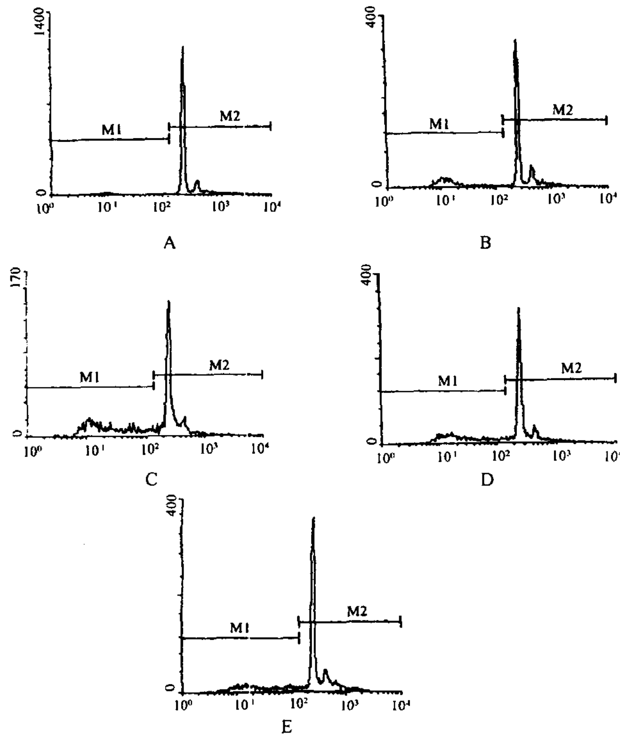

Fig. 2. Quantitative determination of apoptosis by flow cytometric DNA analysis following propidium iodide staining. Histogram of DNA content of cerebellar neurons after 8 h incubation under various experimental conditions. (A) Complete medium; (B) low concentration serum medium; (C) medium with 50 mM H_2O_2 + 100 μM Fe^{2+} (D) medium with H_2O_2 + Fe^{2+} + 100 μg/ml EGb; (E) medium with H_2O_2 + Fe^{2+} 100 U/ml catalase. Cells with subdiploid DNA content (indicated as m1) represent apoptotic cells.

The protective effect of EGb761 on apoptosis induced by hydroxyl radicals was studied and the results showed that EGb761 treatment before exposure to hydroxyl radicals blocked the DNA fragmentation that accompanied apoptosis in cerebellar neurons. EGb761 is a complex mixture which contains flavonoid and non-flavonoid fractions. The main chemical structures of flavonoids possess an aromatic ring and double bond, so flavonoids prefer to react with hydroxyl radicals and yield an addition product, thus directly scavenging the hydroxyl radicals. Flavonoids also possess phenolic hydroxyl groups which may chelate the Fe^{2+} and indirectly inhibit the formation of hydroxyl radicals [4]. Joyeux also reported only flavone presents an important effect against free radicals and lipid peroxidation, the presence of the 2–3 double bonds of the central

pyran ring and two free hydroxyl groups at the *ortho* position on the B ring are essential for the effect against free radicals and lipid peroxidation [6]. The protective effect of EGb761 on apoptosis may be via the direct scavenging of hydroxyl radicals and the indirect inhibiting of lipid peroxidation.

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