Enhancement of Apoptosis in Cerebral Endothelial Cells by Selected Inflammatory Signals[a]

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INTRODUCTION

Acute inflammation is characterized by (1) microvascular injury attracting acute inflammatory cells (mostly polymorphonuclear neutrophils (PMNs) and macrophages), (2) plasma extravasation leading to swelling, and (3) edema in the inflamed site and tissue destruction.[1] The accumulation of PMNs and macrophages in the acutely inflamed tissue results from activation and injury of endothelial cells (ECs). PMN-EC and/or macrophage-EC interaction perpetuates progressive vascular injury, edema formation, and tissue injury. In inflammatory reactions, the potential pathogenetic roles of ECs have been increasingly recognized.[2]

Interaction between ECs and PMNs/macrophages in acute inflammation is mediated by a number of inflammatory mediators.[3,4] The recruitment of circulating PMNs and macrophages into the inflammatory foci involves the expression of adhesive molecules on ECs, PMNs, and macrophages. Interaction between PMNs/macrophages and ECs engage such inflammatory mediators as cytokines, free radicals, eicosanoids, kinins, and others (*e.g.*, histamines and serotonin). Nitric oxide (NO) has emerged to be a key inflammatory mediator. Proinflammatory actions of NO are at least partly related to the formation of free radicals, such as peroxynitrite.[3,4]

In this communication, we summarize recent findings from our laboratory on the effects of inflammatory mediators, especially the free radicals and cytokines, on endothelial function *in vitro*.

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MATERIALS AND METHODS

All materials used in the present study were of molecular biology grades.

Endothelial Cultures and Treatment Protocols

BCEC Cultures

Bovine cerebral endothelial cells (BCECs) were prepared and characterized as previously described[5,6] with modifications.[7] Purity of BCECs was determined by immunocytochemical detection of the expression of factor VIII and vimentin, and in the absence of the expression of fibronectin, α-actinin,[8] and glial fibrillary acidic protein.[6] BCEC identity and culture purity were further established by light microscopic appearance, thrombomodulin activity, uptake of acetylated low-density lipoprotein,[9] and *Griffonia simplicifolia* agglutinin cytofluorimetry.[10] The cell cultures contained more than 95% endothelial cells, based on percent of cells exhibiting factor VIII and vimentin immunoreactivity. BCECs of passages 4-15, which still expressed functional bradykinin receptors based on agonist-induced calcium influx and phosphoinositide turnover,[7] were plated on 100 mm dishes or 24-well plates, maintained in Dulbecco's modified eagle medium (DMEM) with 10% fetal calf serum, and grown to confluence for study.

Treatment Protocols for Induction of DNA Fragmentation and Apoptosis

Cytokine/cycloheximide-induced BCEC Apoptosis. Confluent BCECs were incubated with lipopolysaccharide (LPS, 100 μg/mL) g/mL), tumor necrosis factor α (TNFα, 5 μg/mL), or cycloheximide (CHX, 5 μg/mL), an inhibitor of the synthesis of macromolecules, alone or sequentially in LPS or TNFα for 16 h followed by incubation in CHX for 6 hours. Control samples were treated with vehicles only.

Hyperoxia-induced BCEC Apoptosis. BCECs were exposed to 100% oxygen at room temperature for 2-8 hours. Control samples were exposed to room air for the same time intervals.

Quantification of Cytoplasmic Histone-associated DNA Fragments by ELISA

A prominent feature of apoptosis is DNA fragmentation. A Cell Death Detection ELISA kit (Boehringer Mannheim, Indianapolis, IN) was used to quantitatively determine levels of histone-associated DNA fragments, including mono- and oligonucleosomes after the induction of cell death. The assay was based on the sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed at DNA and histone, respectively. This assay allows the specific determination of mono- and oligonucleosome levels in the cell lysates.[11,12]

$$\text{enrichment factor} = \frac{\text{mU of the treated sample}}{\text{mU of the vehicle-treated sample}}$$

mU = absorbance (10^{-3})

Assessment of Apoptosis by Cell Morphology

BCECs were labeled with 0.1 μg/mL of Hoechst-3342 (Molecular Probes, Eugene, OR) at 37°C in the presence of 5% CO_2 for 10 minutes. All experiments were performed on a Nikon Diaphot inverted microscope equipped with a 75 W H lamp and a 20X or 40X objective. To monitor Hoechst-3342 fluorescence, images were acquired using an XFO3 filter (excitation, 330 ± 60 nm; emission, 450 ± 65) with a CCD camera (Quantex) and digitized by using Metamorph system software (Universal Image, NY, NY). In some experiments (hyperoxic exposure), apoptosis in BCECs were assessed using a TUNEL method, as previously described.[13]

Assessment of DNA Fragmentation by Agarose Gel Electrophoresis

A DNA isolation kit from Puregene (Research Triangle, NC) was employed for the extraction of DNA after the induction of cell death. The DNA samples (10 μg/lane) were electrophoresed at 75 V for 2 h in 1.5% agarose gel with 0.4 μg/mL ethidium bromide in a Tris-acetate buffer (0.4 M Tris, 0.25 M sodium acetate, 0.22 mM EDTA, pH 7.8). DNA was visualized through ultraviolet transillumination and photographed. The latter consists of DNA fragments that differ in size by 180-200 base pairs, the approximate molecular sizes of DNA fragments after internucleosomal cleavage by endonucleases during apoptosis.

Electrophoretic Mobility Shift Assay

Following appropriate treatments of BCECs, crude nuclear "miniextracts" were prepared according to the method described by Lee *et al.*[14] with modifications. NF-κβ consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was from Promega (Madison, WI). The oligonucleotide was end labeled with [γ-^{32}P]ATP according to Promega technical bulletin number 106. The binding reaction was performed in 20 μL of binding buffer (10 mM Tris-HCl, 20 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, pH 7.6) containing 15 μg nuclear protein, 0.0175 pmol of labeled probe (30,000 cpm), and 1 μg of poly-dIdC. After incubation for 20 min at room temperature, the mixtures were subjected to electrophoresis on a nondenaturing 6% polyacrylamide gel at 180 V for 2 hours under the low-ionic-strength condition described by Chodosh.[15] The gel was dried and autoradiographed. For competition assay, unlabeled NF-κβ oligonucleotide or nonspecific oligonucleotide was added at a 20-or 100-fold excess of the end-labeled probe.

Determination of Glutathione Levels in BCECS

The method for glutathione assay was described by Tietze.[16]

Statistical Analysis

Multiple samples were analyzed by one-way analysis of variance. Differences between the control and an experimental group or between two experimental groups were analyzed by a post hoc Tukey test. A p value of less than 0.05 was considered insignificant.

RESULTS

In BCECs, pretreatment with LPS, an endotoxin; TNFα; or CHX alone did not have an appreciable effect on the extent of DNA fragmentation. Sequential treatment of BCECs with LPS or TNFα followed by CHX led to DNA fragmentation as reflected by an increase in DNA fragment levels by ELISA. The same treatment also resulted in an increase in NF-κβ binding activity. Morphologically, these BCECs showed cell shrinkage with membrane blebbing and condensation of nuclear chromatin on Hoechst stain. In some cells, apoptotic bodies could also be detected. DNA ladders were also noted on gel electrophoresis of DNA extracted from BCECs sequentially treated with LPS or TNFα followed by CHX.

The extent of DNA fragmentation in BCECs caused by LPS/CHX could be reduced by pretreatment of BCECs with N-acetyl-cysteine (NAC) in a dose-dependent manner. NAC at concentrations that reduced LPS/CHX-induced DNA fragmentation also increased BCEC glutathione levels. By contrast, maleic acid diethyl ester (MADE), a glutathione depleter, and DL-buthionine-(S,R)-sulfoximine (BSO), a glutathione synthase inhibitor, which decreased glutathione levels in BCECs, enhanced DNA fragmentation.

LPS, TNFα, or CHX alone did not affect transcription factor NF-κβ binding activity. Sequential LPS and CHX treatment substantially increased NF-κβ binding activity. LPS/CHX-induced increased in NF-κβ binding activity was inhibited by NAC pretreatment.

Hyperoxia caused a time-dependent DNA fragmentation. Morphologically, BCECs underwent similar changes as noted following LPS/CHX treatment. BCEC death following hyperoxia was further confirmed by the appearance of clear DNA ladders on agarose gel electrophoresis of DNA extracted from BCECs following hyperoxia.

In contrast to LPS/CHX treatment, NAC or BSO have little effect on the extent of DNA fragmentation in BCECs exposed to 100% oxygen. Melatonin, an antioxidant, however, was partially effective in blocking DNA fragmentation triggered by hyperoxia. A novel antioxidant, carboxy-buckminsterfullerene, was most effective in inhibiting DNA fragmentation.

DISCUSSION

Recent advances in inflammation research have increased the pathogenetic roles of the endothelium in a number of disorders in which blood vessels were predominantly affected.[2] SLE is one of these diseases. Because CNS manifestation of SLE is also characterized by an inflammatory process engaging cerebral endothelial cells (CECs), understanding CEC dysfunction in response to inflammatory signals may aid in the future development of novel therapeutic agents for treating neuropyschiatric manifestation of SLE.

In a series of *in vitro* studies using BCECs as models, we noted that inflammatory signals (LPS or TNFα) may accelerate DNA fragmentation and enhance cell death. Morphological and biochemical studies suggest BCECs die by apoptosis after sequential exposure to LPS or TNFα followed by CHX treatment. DNA fragmentation in BCECs appeared to depend upon glutathione-related oxidative stress. This contention is supported by the observation that elevation of BCEC glutathione levels by NAC decreased and glutathione depletion by BSO or MADE accentuated DNA fragmentation. Another link between oxidative stress and LPS/CHX-induced apoptosis was the finding that NF-κβ binding activity was increased by LPS/CHX treatment, which was also inhibited by NAC.

Additional support for a role of oxidative stress in DNA fragmentation and apoptosis in BCECs was derived from experiments on hyperoxia. Oxygen-reactive species have been shown to be a major cause of DNA damage. Cells with damaged DNA may trigger a molecular cascade to enter growth arrest and DNA repair. The same cascade triggered by DNA damage (*e.g.*, p53) has also been implicated in initiating apoptosis. The regulatory mechanisms that may control a branching point of cell cycle and death remain to be elucidated.[17–20]

Recently, ceramide has been noted to be a stress signal and mediator of apoptosis in certain cell types. This novel cascade appears to be downstream of a cytokine-mediated signaling process.[21] In a preliminary study in BCECs, we also noted that ceramide content was increased following LPS/CHX treatment, which caused DNA fragmentation and apoptosis. BCEC exposure to ceramide led to increased DNA fragmentation and cell death. These preliminary findings raise the possibility of a cytokine-ceramide cascade in which cytokine signaling may activate a sphingomyelin cycle to accelerate DNA fragmentation and cell death by apoptosis. Further studies are needed to define the link between cytokine action and the sphingomyelin pathway in cerebral endothelial DNA damage and apoptosis.

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