LABORATORY INVESTIGATION

J Korean Neurosurg Soc 40 : 103-109, 2006

Endothelial Cell Products as a Key Player in Hypoxia-Induced Nerve Cell Injury after Stroke

Chul-Min Cho, M.D.,[1] Se-Un Ha,[2] Hae-Rahn Bae, M.D., Ph.D.,[2] Jae-Taeck Huh, M.D., Ph.D.[1]

Departments of Neurosurgery,[1] Physiology,[2] College of Medicine, Dong-A University, Busan, Korea

Objective : Activated endothelial cells mediate the cascade of reactions in response to hypoxia for adaptation to the stress. It has been suggested that hypoxia, by itself, without reperfusion, can activate the endothelial cells and initiate complex responses. In this study, we investigated whether hypoxia-induced endothelial products alter the endothelial permeability and have a direct cytotoxic effect on nerve cells.
Methods : Hypoxic condition of primary human umbilical vein endothelial cells(HUVEC) was induced by $CoCl_2$ treatment in culture medium. Cell growth was evaluated by 3,4,5-dimethyl thiazole-3,5-diphenyl tetrazolium bromide (MTT) assay. Hypoxia-induced products (IL-1β, TGF-β1, IFN-γ, TNF-α, IL-10, IL-6, IL-8, MCP-1 and VEGF) were assessed by enzyme-linked immunosorbent assay. Endothelial permeability was evaluated by Western blotting.
Results : Prolonged hypoxia caused endothelial cells to secrete IL-6, IL-8, MCP-1 and VEGF. However, the levels of IL-1, IL-10, TNF-α, TGF-β, IFN-γ and nitric oxide remained unchanged over 48 h hypoxia. Hypoxic exposure to endothelial cells induced the time-dependent down regulation of the expression of cadherin and catenin protein. The conditioned medium taken from hypoxic HUVECs had the cytotoxic effect selectively on neuroblastoma cells, but not on astroglioma cells.
Conclusion : These results suggest the possibility that endothelial cell derived cytokines or other secreted products with the increased endothelial permeability might directly contribute to nerve cell injury followed by hypoxia.

KEY WORDS : Hypoxia · Primary human umbilical vein endothelial cells · Cytokines · Endothelial permeability · Neurotoxicity.

Introduction

The endothelium plays a key role in maintaining vascular homeostasis coping with all changes within blood. Because of its location the endothelium is exposed directly to a diverse array of extracellular stresses that might disrupt the homeostatic function. Disrupted vascular homeostasis has been linked to a variety of pathological situations including ischemia and inflammation.

Hypoxia is one of the most important forms of stressors frequently exposed to the endothelial cells. In response to oxygen deficiency the endothelial cells are activated and mediate the cascade of reactions for adaptation to the stress. Researches of this subject have been focused on the injury of ischemia followed by reperfusion, but discrete works have implicated that hypoxia, by itself, without reperfusion, can activate the endothelial cells and initiate complex molecular responses as host defense mechanism[21].

The response of endothelial cells to hypoxia differs depending upon the degree and duration of oxygen deficiency. Acute hypoxia activates endothelial cells to release numerous inflammatory mediators and growth factors, promoting leukocyte adherence[4,12] and endothelial permeability[3,26]. Prolonged hypoxia results in the transcriptional induction of specific genes encoding blood vessel growth and remodeling as well as cytokines and growth factors[13,17], via activation of the transcriptional regulator, hypoxia-inducible factor 1(HIF-1)[33]. However, the exact list of cytokines and other products secreted by hypoxic endothelial cells remains uncertain.

The blood-brain barrier(BBB) plays a crucial role in protecting the central nervous system from any changes in homeostasis. The selective nature of the BBB is endowed with the continuous tight junctions between the endothelial cells as well as specific transport systems and enzymes in the endothelium[6].

• Received : February 15, 2006 • Accepted : April 18, 2006
• Address for reprints : Jae-Taeck Huh, M.D., Ph.D., Department of Neurosurgery, College of Medicine, Dong-A University, Dongdaesin-dong 3-ga, Seo-gu, Busan 602-715, Korea Tel : +82-51-240-5242, Fax : +82-51-242-6714, E-mail : jthuh@donga.ac.kr

Disruption of endothelial permeability of this barrier contributes to the pathogenesis of a wide range of diseases, including ischemic and inflammatory brain injury[11,28]. It has been reported that the endothelial permeability is increased by hypoxic insult, which results in a breakdown of BBB[3,29]. On the basis of these findings, it was hypothesized that the released cytokines from endothelial cells by hypoxic stress, if passed though BBB owing to up-regulated endothelial permeability, might contribute to the neuronal injury without any involvement of inflammation, and thus explain the mechanism of pathogenesis of ischemic brain damage. However, it is not demonstrated that the endothelial products secreted in response to hypoxia has the direct cytotoxic effect on the underling nerve cells.

Therefore, in this study, we investigated what kinds of cytokines and growth factors are released from hypoxic human endothelial cells, and whether hypoxia alters cadherin-catenin complexes to increase the endothelial permeability. Finally, we also examined if there is a direct cytotoxic effect of hypoxia-induced endothelial products on nerve cells.

Materials and Methods

Cell culture

Primary human umbilical vein endothelial cells(HUVEC) were harvested by use of 0.25% trypsin (Gibco-BRL, Grand Island, NY, U.S.A.) digestion according to the method of Jeff et al. They were maintained in endothelial cell growth medium M199 (Gibco-BRL, Grand Island, NY, U.S.A.), containing 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL, Grand Island, NY, U.S.A.), 2mM glutamine, 5U/ml heparin, 100 μg/ml streptomycin, 100U/ml penicillin, and 3ng/ml basic fibroblast growth factor (bFGF; Upstate Biotechnology, Lake Placid, NY, U.S.A.). HUVECs were serially passaged on gelatin-coated flasks. Cells were used within five passages and identified as endothelial cells by their characteristic cobblestone morphology and immunohistochemical staining of Factor VIII antigen using anti-human von Willebrand factor antibody (Sigma, Saint Louis, MO, U.S.A.). To exclude endotoxin contamination, all cell suspensions at the end of each experiment were evaluated by chromogenic limulus amoebocyte assay (Sigma, Saint Louis, MO, U.S.A.) and all powdered mediums were reconstituted with endotoxin-free water.

Induction of hypoxia

For in vitro mod el of hypoxia, intracellular chemical hypoxia was induced by $CoCl_2$ treatment in culture medium. Cobaltous ions are known to substitute for ferrous ions in O_2-binding heme protein, depriving O_2 availability to cells[14]. HUVECs grown to 100% confluence on gelatin-coated flasks or plates were further cultured in endothelial cell medium containing 10% FCS with no bFGF added. The following day, fresh medium was replaced and $CoCl_2$ was added.

Measurement of cell growth

Cell growth was evaluated by 3,4,5-dimethyl thiazole-3, 5-diphenyl tetrazolium bromide(MTT) assay test according to standard procedures[32]. The ability of viable cells to reduce MTT was determined. MTT stock solution (5mg/ml) was prepared in PBS, pH 7.4, filtered through 0.22μM filter to remove formazan crystals and stored at -20°C in the dark. Cells were plated in a density of 3,000 cells/well on pre-coated 96-well plates and incubated for indicated times. Subsequently, 10-fold diluted MTT with M199 medium was added to each well and incubated for 4 hours. Plates were centrifuged at 275 x g for 5 min and the supernatant was aspirated. The formazan crystals were completely dissolved in dimethyl sulfoxide by vigorously shaking on microplate shaker. OD was read on microplate ELISA reader (El 312e, Bio-Tek) at wavelength of 550nm and 630nm. Survival of each experimental group was compared with that of control cells.

Quantification of cytokines, chemokines and VEGF

HUVEC monolayers were exposed to hypoxia as described above. Culture medium was collected at various time intervals, centrifuged for 5min at 1,000g to prepare cell-free supernatants, and stored at -20°C before assay. Hypoxia-induced products (IL-1β, TGF-β1, IFN-γ, TNF-α, IL-10, IL-6, IL-8, MCP-1, VEGF) were assessed by enzyme-linked immunosorbent assay (ELISA), which employed quantitative "sandwich" techniques with antibodies specific for the cytokines. Briefly, in all assays, standards (PeproTech, Endogen, or R&D) and test samples were dispensed in duplicate into wells of 96-well microtiter plates, which had been pre-coated with monoclonal antibodies directed against the materials (Pharmingen, Endogen, or R&D). Then, biotinylated detection antibodies (Pharmingen, Endogen, or R&D) were added. After an hour of incubation, HRP-conjugated streptavidin (Endogen) was added to the wells. For color reaction 100$\mu\ell$ of 3,3',5,5'-tetramethylbenzidine (TMB; Endogen) substrate was added for 15 to 30 min. After stopping the reaction with 1 M H_2SO_4, the absorption at 450~550nm was determined using an automated ELISA microplate reader (Bio-Tek, EL312e).

Western blotting

Cells were rinsed rapidly in ice-cold PBS and were lysed directly in boiling 2 sodium dodecyl sulfate(SDS) sample buffer. The lysates were boiled for 5 min, and separated by 12% SDS-polyacrylamide gel electrophoresis(PAGE), and the proteins were transferred onto a nitrocellulose membrane as previously described. After blocking with Tris-buffered saline (TBST;

20mM Tris [pH 7.5], 145mM NaCl, 0.05% Tween-20) containing 5% skim milk for 4 h at room temperature, the membranes were incubated with antibodies to pan-cadherin (Sigma, Saint Louis, MO, U.S.A.), VE-cadherin, or β- and γ-catenin (BD Transduction Laboratories, Franklin Lakes, NJ, U.S.A.) for 1 h at room temperature. The membranes were washed three times for 10 min each in TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham, Little Chalfont, Buckinghamshire, England) for 30 min at room temperature. After washing three times with TBST, the membranes were developed with an enhanced chemiluminescence detection system (Amersham, Little Chalfont, Buckinghamshire, England). The membrane was subjected to luminescent image analyzer (LAS-1000plus, Fuji, Japan) and analyzed quantitatively.

Results

Induction of chemical hypoxia by $CoCl_2$

Hypoxic condition of endothelial cells was induced by $CoCl_2$. To determine the optimal concentration and duration of $CoCl_2$ treatment inducing hypoxia in HUVECs, cells were incubated with different concentrations up to 500μM and cell viability assay was done after various time course of incubation. Fig. 1 shows the dose- and time- dependent toxicity of $CoCl_2$. Almost ninety percent of cells were dead as early as 6 h when incubated with 500μM $CoCl_2$. About 50% cells survived after 48h at 250μM of $CoCl_2$. However, at 100μM or less concentrations, there was no significant effect of $CoCl_2$ on cell viability even after 48h. Therefore, for the following experiments, the concentration of 100μM $CoCl_2$ was regarded as the optimal concentration which induces hypoxia without affecting cell viability. In hypoxic conditions, reduced O_2 availability to the cells induces the expression of transcriptional regulator HIF-1, an essential mediator of O_2 homeostasis. To investigate whether 100μM $CoCl_2$ adequately induce intracellular hypoxia in HUVECs, the activation of HIF-1 protein expression was evaluated by western blot. Fig. 2 shows that HIF-1 protein was up-regulated by 100μM $CoCl_2$. Induction of HIF-1 activation was peaked at 6h of $CoCl_2$ treatment, and sustained until 48h. This result suggests that treatment of 100μM $CoCl_2$ in our HUVECs is enough to induce hypoxia and hypoxia-related gene expression.

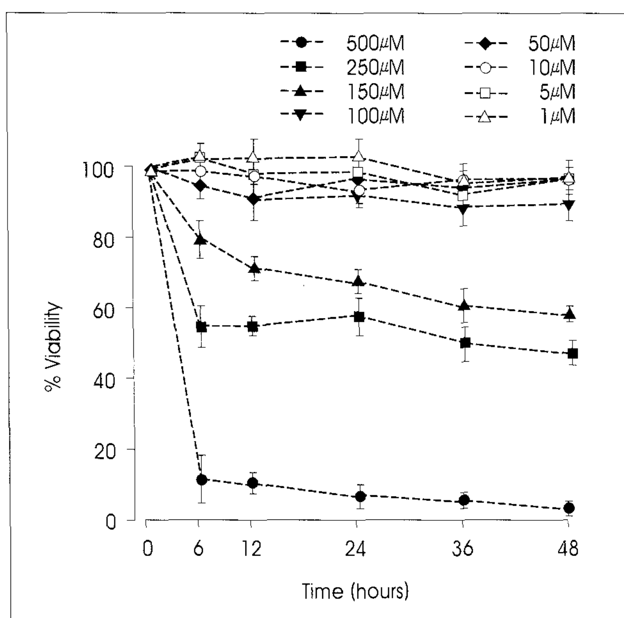

Fig. 1. The cytotoxic effect of $CoCl_2$ in HUVECs. Cells were incubated with various concentrations of $CoCl_2$ (0 to 500μM) and the cytotoxicity was assayed at different time interval using 3,4,5-dimethyl thiazole-3, 5-diphenyl tetrazolium bromide assay. Fifty percent cells were dead at 250μM of $CoCl_2$, but almost all the cells are viable even after 48 h at 100μM or less concentrations.

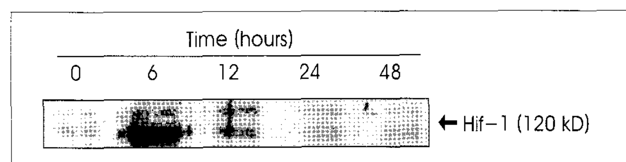

Fig. 2. HIF-1 activation by hypoxia induced by 100μM of $CoCl_2$. HUVECs were incubated with 100μM of $CoCl_2$ for 48 h and nuclear extracts were obtained at various intervals for western blot. Note that $CoCl_2$ markedly activates HIF-1 induction after 6h of treatment and the effect was sustained until 48 h.

Effect of hypoxia on the production of cytokines and other growth factors

Cytokine production in HUVECs exposed to hypoxia was investigated using ELISA. Prolonged hypoxia caused endothelial cells to secrete certain types of cytokines among those we tested. The levels of IL-6 in the supernatant started to increase significantly after 4h treatment of $CoCl_2$. It reached a concentration of 700pg/ml at 24 h and remained the level as a plateau (Fig. 3A). IL-8 levels also increased gradually over 48 h to reach the peak concentration of as high as 3600pg/ml (Fig. 3B). However, the levels of IL-1 remained unchanged over 48 h of hypoxia, as did the levels of IL-10, TNF-a , TGF-β, and IFN-v (data not shown).

We also investigated the release of chemokines and other hypoxia-related products in HUVECs subjected to hypoxia. Among the chemokines we tested, interestingly, monocyte chemoattractant protein-1 (MCP-1) levels increased dramatically in response to hypoxia (Fig. 3C). Control HUVECs were releasing considerable amount of MCP-1 without any treatment. However, hypoxic stress profoundly increased the level of MCP-1 secreted in the medium to reach the concentration of 3400pg/ml at 48 h.

Other than cytokines and chemokines, there are many hypoxia-regulated vasoactive substances in human endothelial

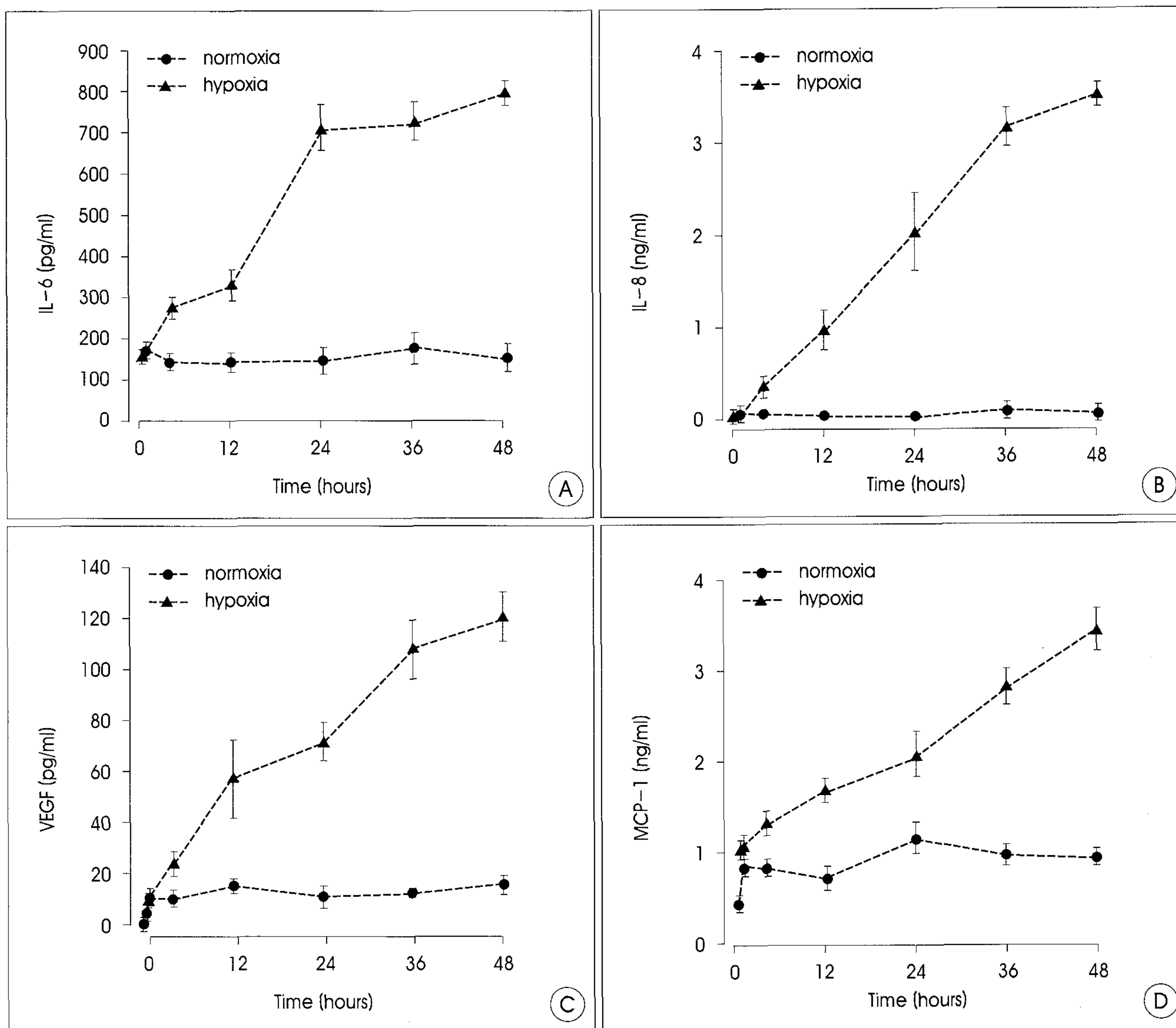

Fig. 3. Cytokine secretion by hypoxic HUVECs. Cells were incubated with 100μM of $CoCl_2$ for 48 h and concentrations of interleukin-6, interleukin-8, monocyte chemoattractant protein-1 and vascular endothelial cell growth factor in supernatants were measured using enzyme-linked imm-unosorbent assay. Levels of cytokines increase depending on the incubation time, and reached to a peak concentration at 48 h.

cells[13]. Among them, we checked two major molecules, vascular endothelial cell growth factor(VEGF) and nitric oxide (NO). HUVECs exposed to hypoxia released considerable amount of vascular endothelial cell growth factor(VEGF) into the medium (Fig. 3D), whereas NO levels were not elevated significantly (not shown).

Effect of hypoxia on endothelial permeability

Vascular or endothelial permeability is regulated by endothelial cell-to-cell junctions, which consist of both tight junctions and adherence junctions. Adherence junction, in particular cadherin is the target of the signaling pathway of agents that increase vascular permeability, and is an important determinant of microvascular integrity[9]. We evaluated the endothelial permeability by protein expression of cadherin-catenin complexes. As shown in (Fig. 4), hypoxic exposure to endothelial cells induced the time-dependent down regulation of cadherin and catenin expression. The amount of pan-cadherin and VE-cadherin protein expressed was transiently increased at 12h, but reduced after prolonged hypoxic condition. The expression of β- and v-catenin proteins also decreased with the increasing time of $CoCl_2$ exposure. These results indicate that hypoxia decreased the protein expression and organization of cadherin-catenin complexes at intercellular junctions of endothelial cells, thereby increasing endothelial permeability.

Effect of the conditioned medium from hypoxic HUVECs on nerve and glial cell damage

To test the possibility that cytokines or other components secreted from hypoxic endothelial cells might have direct cy-

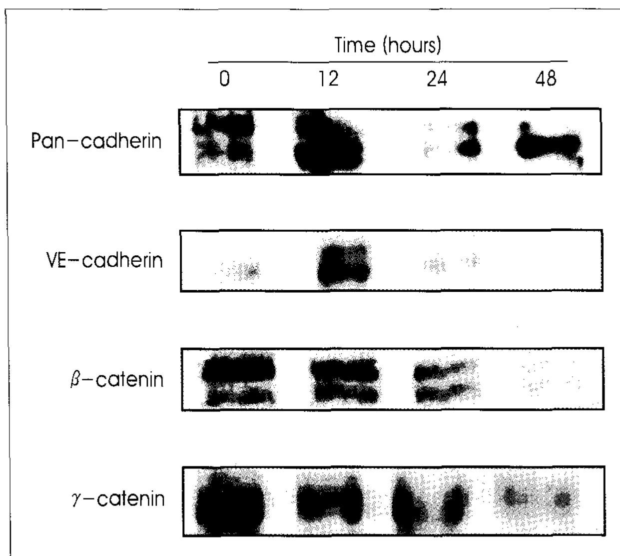

Fig. 4. Hypoxia-induced down-regulation of cadherin-catenin complexes in HUVECs. Cells were incubated with 100μm of $CoCl_2$ for 48 h. The quantitative analysis of cadherin-catenin complex protein was studied using western blot. Hypoxia induced transient increase in pan-cadherin and VE-cadherin protein expression, but prolonged hypoxia decreased the expressions of both cadherins and catenins.

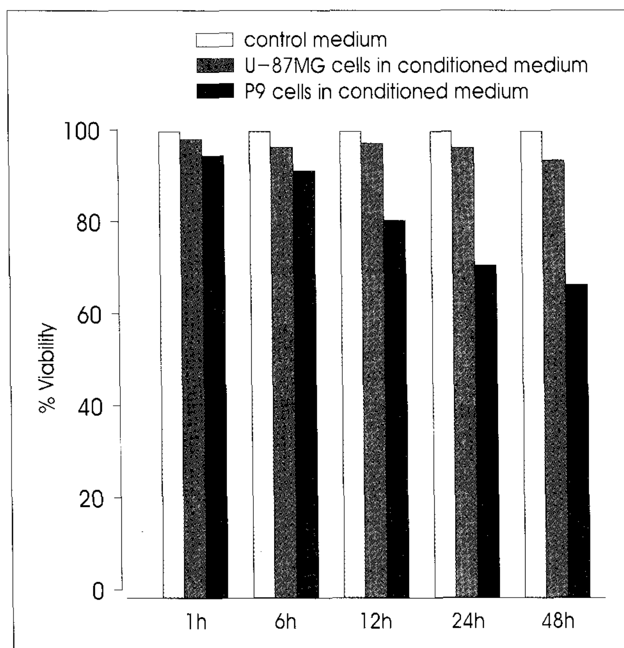

Fig. 5. The cytotoxic effect of conditioned medium from hypoxic HUVECs in U-87MG glioblastoma cells and P9 neuroblastoma cells. HUVECs were incubated with 100μm of $CoCl_2$ for various time and the supernatants were saved as conditioned medium for cytotoxicity study. U-87MG cells and P9 cells were grown for 48 h in poly-N-methyl methacrylimide medium containing 25% of conditioned medium obtained from hypoxic HUVECs. Conditioned medium from hypoxic HUVECs for 48 h had a significant cytotoxic effect on P9 cells to decrease viability to 73%, but not on U-87MG cells.

totoxic effects on nerve and glial cells if delivered into brain interstitial medium across the BBB, we next investigated the cytotoxic effects of the conditioned medium from hypoxic HUVECs. As reported in (Fig. 5), supernatants taken from hypoxic HUVECs at various times had a significant cytotoxic effect on P9 neuroblastoma cells, but not glioblastoma cells. The cytotoxicity of supernatants on neuroblastoma cells was increased as the incubation time of $COCl_2$ treatment in HUVECs was prolonged, which is relatively proportional to the concentration of secreted products from hypoxic HUVECs. Conditioned medium from hypoxic HUVECs for 48h had a significant cytotoxic effect on P9 cells to decrease viability to 73%. In comparison, U-87MG glioblastoma cells were not affected by the conditioned medium even from 48h hypoxic HUVECs. This result suggests that endothelial-derived components in hypoxia directly induce nerve cell damage.

Discussion

Many investigators have studied the role of inflammatory cells on ischemic brain injury because inflammation during reperfusion is believed to be one of the most important mechanisms of neuronal damage in ischemic stroke. The present investigation has focused on the role of endothelial cells on ischemic brain injury, via the direct effect of hypoxia without reperfusion. We demonstrated that hypoxia activates endothelial cells to secrete cytokines and other products such as IL-6, IL-8, MCP-1 and VEGF. It was also shown that the cadherin-catenin complexes at intercellular junctions decreased to increase endothelial permeability, and that the conditioned medium containing all the secretions by hypoxic endothelial cells had a significant cytotoxic effect on nerve cells. These results demonstrate the direct neurotoxic effect of hypoxic endothelial products, which provides the novel mechanism of neuronal injury in hypoxia mediated directly by endothelia cells.

In this experiment, in vitro chemical hypoxia was induced by $CoCl_2$ treatment. It has been suggested that cobaltous ions substitute for ferrous ions in heme, causing a conformational change in heme protein O_2 sensor and reducing heme oxygen binding[14]. $CoCl_2$ is known to activate transcriptions of HIF-1 regulated genes, such as erythropoietin, glycolytic enzymes and VEGF during normoxia[20,34,40], which occurs as the adaptive response to hypoxia. There is a report that $CoCl_2$ mimics physiological hypoxia by augmenting the formation of reactive oxygen species in cells via a nonmitochondrial mechanism[7]. Though the underlying mechanism of O_2 sensing during hypoxia is not understood, mitochondria have been considered as possible O_2 sensors. A change in mitochondrial redox during hypoxia could alter the production of reactive oxygen species, which are known to participate in other transcriptional responses. Therefore, $CoCl_2$ induced hypoxia model represents certain aspects of hypoxia in which reactive oxygen species act as signaling elements.

The effects of hypoxia on cytokine production have been extensively studied in a variety of cell types. It was demonstrated that hypoxia stimulates to release IL-1a, and IL-6 in vascular smooth muscle cells, as well as TNF-a, IL-1, IL-6 and IL-8 in monocytes. In our HUVEC study, prolonged hypoxia up to 48 h resulted in the increased production of IL-6, IL-8 and MCP-1, but not of IL-1α and TNF-α. This finding is consistent with that of Ali MH et al[2], who documented increased secretion of IL-6 and IL-8, but not IL-1a via reactive oxygen species generated in hypoxic HUVECs. There is confliction on whether hypoxic endothelium produce IL-1; some reported that hypoxia induce IL-1 activity in endothelial cells[36], but others observed endothelial IL-1 production after hypoxia followed by reoxygenation[1,8].

VEGF is an angiogenic peptide that is released in response to hypoxia from developing or neoplastic tissue and acts on endothelial cells to promote the sprouting of blood vessels[23]. It is also known as vascular permeability factor(VPF), by virtue of its permeability enhancing effects that enhanced the permeability of normal venules and small veins with potency 50,000 times that of histamine[35]. VEGF gene expression can be induced by hypoxia in a number of cell types, including vascular smooth muscle cells and endothelial cells[22,37]. Hypoxic dependent increase in VEGF expression seems to be controlled at the transcriptional level, probably by HIF-1 binding to cis-acting element[17]. In our HUVECs, peak activation of HIF-1 was at 6h and subsequent secretion of VEGF was observed after 12h of chemical hypoxia (Fig. 2, 3).

NO, a potent vasodilator, is synthesized by endothelial cells form arginine and promotes relaxation of vascular smooth muscle[25]. In hypoxia, release of NO by endothelial cells is inhibited due to a decreased constitutive endothelial NO synthase(eNOS) activity[27]. However, recent data indicate that, unlike eNOS, inducible NO synthase(iNOS) is activated by hypoxia in pulmonary artery endothelial cells[24]. Thus, the overall result of these changes is still controversial. In our system, down-regulated eNOS activity seemed to be dominant over the up-regulation of iNOS, thereby NO release was not significant.

The endothelial cell monolayers form semi-permeable barriers that regulate transit of molecules across the monolayer. This barrier function depends, in part, on the cadherin complexes that form the intercellular junctions, called zonular adherens. The cadherins are transmembrane proteins that homotypically binds to cadherin molecules on neighboring cells through calcium-dependent association. The stability of these cell-cell junctions is modulated by the catenins; β-catenin, γ-catenin and p120-catenin bind directly, but a-catenin indirectly binds to the cadherins[5]. VE-cadherin, an endothelial cell-specific cadherin localizes at the intercellular adherens junctions formed by endothelial cells[16]. Although the extracellular domain of VE-cadherin is necessary for homotypic adhesion and clustering, the intercellular association to catenins and the actin cytoskeleton is required for the stabilization of the complex and a full control of junctional permeability[10]. Adherens junctions and, in particular VE-cadherin, are targets of the signaling pathway of agents that increase vascular permeability such as VEGF, histamine and thrombin[9,39].

The conditioned medium taken form hypoxic HUVECs had a selective cytotoxicity in neuroblastoma cells, but not in glioblastoma cells (Fig. 5). It is uncertain which component in the conditioned medium has cytotoxicity and further investigation is required. Candidate mediators of this cytotoxicity are inflammatory cytokines, such as tumor necrosis factor-a (TNF-a) and IL-1β, which are known as key contributors to neuron dysfunction and death in cerebral ischemia and trauma[11]. But these cytokines are not detected in our hypoxic HUVECs. TNF-α and IL-1β are thought to be secreted in response to hypoxia by other cell types, such as macrophages, lymphocytes and glial cells, and exert tissue injury primarily through microvascular inflammatory reaction following ischemia[18,19]. IL-6 and MCP-1, which are increased in our system, seems not to be candidates, because they are involved mainly in neutrophil- and macrophage- mediated reperfusion injury[31]. In addition, it was reported that brain endothelial cells release IL-6 in response to hypoxia and mediate neurotrophic and neuroprotective functions[30]. IL-8 is a potent neutrophil chemotactic factor, and might contribute ischemic and reperfusion damage via neutrophil migration and infiltration. Interestingly, Terui Y et al[38] documented that activated endothelial cells induce apoptosis in leukemic cells by endothelial IL-8. Applying this finding to our system, released endothelial IL-8 from activated HUVECs might be responsible for the neurotoxicity. Cyclooxygenase-2 (COX-2) reaction products are also believed to be destructive and contribute to cytotoxicity due to reactive oxygen species and toxic prostanoids[11]. Recently VEGF is reported to exert neuroprotective effect in in vitro ischemia[15]. This might explain the lack of higher cytotoxic effect of conditioned mediums after 12h hypoxia, presumably due to increased concentration of VEGF in the medium. Glial cells were resistant to the cytotoxic effect of conditioned medium in this study, which seems to be reasonable considering that brain ischemia and trauma produce a reaction of activation and proliferation of microglia, called gliosis[11].

Conclusion

The present study demonstrates that endothelial cells, upon stimulation of hypoxia, release IL-6, IL-8, MCP-1 and VEGF. Hypoxia induces disorganization and down regulation

of cadherin-catenin complexes, suggestive of increased endothelial permeability. The conditioned medium from hypoxic HUVECs exerts cytotoxic effect selectively on neuroblastoma cells. These data suggest that secreted products from hypoxic endothelial cells, even without the involvement of inflammatory reaction, may play a significant role in breakdown of endothelial barrier and eventual neuronal damage observed in ischemic brain injury. Further study is to be done for identifying specific functions of individual components in conditioned medium, which would suggest the potential role of certain cytokines and other mediators as the novel therapeutic targets for cerebral ischemia.

References

1. Ala Y, Palluy O, Favero J, Bonne C, Modat G, Dornand J : Hypoxia/reoxygenationstimulates endothelial cells to promote interleukin-1 and interleukin-6 production. Effects of free radical scavengers. **Agents Actions 37** : 134-139, 1992
2. Ali MH, Schlidt SA, Chandel NS, Hynes KL, Schumacker PT, Gewertz BL : Endothelial permeability and IL-6 production during hypoxia : role of ROS in signal transduction. **Am J Physiol 277** : L1057-L1065, 1999
3. Ali MH, Schlidt SA, Hynes KL, Marcus BC, Gewertz BL : Prolonged hypoxia alters endothelial barrier function. **Surgery 124** : 491-497, 1998
4. Arnould T, Michiels C, Remacle J : Increased PMN adherence on endothelial cells after hypoxia : involvement of PAF, CD18/CD11b, and ICAM-1. **Am J Physiol 264** : C1102-C1110, 1993
5. Barth AI, Nathke IS, Nelson WJ : Cadherins, catenins and APC protein : Interplay between cytoskeletal complexes and signaling pathways. **Curr Opin Cell Biol 9** : 683-690, 1997
6. BradburyMW : The blood-brain barrier. **Exp Physiol 78** : 453-472, 1993
7. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT : Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. **Proc Natl Acad Sci USA 95** : 11715-11720, 1998
8. Clark ET, Desai TR, Hynes KL, Gewertz BL : Endothelial cell response to hypoxia-reoxygenation is mediated by IL-1. **J Surg Res 58** : 675-681, 1995
9. Corada M, Mariotti M, Thurston G, Smith K, Kunkel R, Brockhaus M, et al : Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. **Proc Natl Acad Sci USA 96** : 9815-9820, 1999
10. Dejana E, Valiron O, Navarro P, Lampugnani MG : Intercellular junctions in the endothelium and the control of vascular permeability. **Ann N Y Acad Sci 811** : 36-43, 1997
11. del Zoppo G, Ginis I, Hallenbeck JM, Iadecola C, Wang X, Feuerstein GZ : Inflammation and stroke : putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. **Brain Pathol 10** : 95-112, 2000
12. Dore-Duffy P, Balabanov R, Beaumont T, Hritz MA, Harik SI, LaManna JC : Endothelial activation following prolonged hypobaric hypoxia. **Microvascular Res 57** : 75-85, 1999
13. Faller DV : Endothelial cell responses to hypoxic stress. **Clin Exp Pharmacol Physiol 26** : 74-84, 1999
14. Goldberg MA, Dunning SP, Bunn HF : Regulation of the erythropoietin gene : evidence that the oxygen sensor is a heme protein. **Science 242** : 1412-1415, 1988
15. Jin KL, Mao XO, Greenberg DA : Vascular endothelial growth factor : direct neuroprotective effect in in vitro ischemia. **Proc Natl Acad Sci USA 97** : 10242-10247, 2000
16. Lampugnani MG, Resnati M, Raiteri M, Pigott R, Pisacane A, Houen G, et al : A novel endothelial-specific membrane protein is a marker of cell-cell contacts. **J Cell Biol 118** : 1511-1522, 1992
17. Levy AP, LevyNS, Wegner S, Goldberg MA : Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. **J Biol Chem 270** : 13333-13340, 1995
18. Liu T, Clark RK, Mcdonnell PC, Young PR, White RF, Barone FC, et al : Tumor necrosis factor αexpression in ischemic neurons. **Stroke 25** : 1481-1488, 1994
19. Liu T, Mcdonnell PC, Young PR, White RF, Siren AL, Barone FC, et al : Interleukin-1β mRNA expression in ischemic rat cortex. **Stroke 24** : 1746-1750, 1993
20. Liu Y, Cox SR, Morita T, Kourembanas S : Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. **Circ Res 77** : 638-643, 1995
21. Michiels C, Arnould T, Remacle J : Endothelial cell responses to hypoxia : initiation of a cascade of cellular interactions. **Biochim Biophys Acta 2** : 1-10, 2000
22. Namiki A, Brogi E, Kearney M, KimEA, Wu T, Couffinhal T, et al : Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. **J Biol Chem 270** : 31189-31195, 1995
23. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z : Vascular endothelial growth factor(VEGF) and its receptors. **FASEB J 13** : 9-22, 1999
24. Palmer LA, Semenza GL, Stoler MH, Johns RA : Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. **Am J Physiol 274** : L212-L219, 1998
25. Palmer RM, Ferrige AG, Moncada S : Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. **Nature 327** : 524-526, 1987
26. Park JH, Okayama N, Gute D, Krsmanovic A, Battarbee H, Alexander JS : Hypoxia/aglycemia increases endothelial permeability : role of second messengers and cytoskeleton. **Am J Physiol 277** : C1066-C1074, 1999
27. Phelan MW, Faller DV : Hypoxia decreases constitutive nitric oxide synthetase transcript and protein in cultured endothelial cells. **J Cell Physiol 167** : 469-476, 1996
28. Plateel M, Dehouck MP, Torpier G, Cecchelli R, Teissier E : Hypoxia increases the permeability of the blood-brain barrier endothelial cell monolayer. **J Neurochem 65** : 2138-2145, 1995
29. Plateel M, Teissier E, Cecchelli R : Hypoxia dramatically increases the nonspecific transport of blood-borne proteins to the brain. **J Neurochem 68** : 874-877, 1997
30. Reyes TM, Fabry Z, Coe CL : Brain endothelial cell production of a neuroprotective cytokine, interleukin-6, in response to noxious stimuli. **Brain Res 851** : 215-220, 1999
31. Sawa Y, Ichikawa H, Kagisaki K, Ohata T, Matsuda H : Interleukin-6 derived from hypoxic myocytes promotes neutrophil-mediated reperfusion injury in myocardium. **J Thorac Cardiovasc Surg 116** : 511-517, 1998
32. Schweitzer CM, van de Loosdrecht AA, Jonkhoff AR, Ossenkoppele GL, Huijgens PC, Drager AM, et al : Spectrophotometric determination of clonogenic capacity of leukemic cells in a semisolid microtiter culture system. **Exp Hematol 21** : 573-578, 1993
33. Semenza GL : HIF-1 : mediator of physiological and pathophysiological responses to hypoxia. **J Appl Physiol 88** : 1474-1480, 2000
34. Semenza GL, Roth PH, Fang HM, Wang GL : Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. **J Biol Chem 269** : 23757-23763, 1994
35. Senger DR, Connolly DT, Van de Water L, Feder J, Dvorak HF : Purification and NH2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability. **Cancer Res 50** : 1774-1778, 1990
36. Shreeniwas R, Koga S, Karakurum M, Pinsky D, Kaiser E, Brett J, et al : Hypoxia-mediated induction of endothelial cell interleukin-1 alpha. An autocrine mechanism promoting expression of leukocyte adhesion molecules on the vessel surface. **J Clin Invest 90** : 2333-2339, 1992
37. Stavri GT, Hong Y, Zachary IC, Breier G, Baskerville PA, Yla-Herttuala S, et al : Hypoxia and platelet-derived growth factor-BB synergistically upregulate the expression of vascular endothelial growth factor in vascular smooth muscle cells. **FEBS Lett 358** : 311-315, 1995
38. Terui Y, Ikeda M, Tomizuka H, Kasahara T, Ohtsuki T, Uwai M, et al : Activated endothelial cells induce apoptosis in leukemic cells by endothelial interleukin-8. **Blood 92** : 2672-2680, 1998
39. Ukropec JA, Hollinger MK, Salva SM, Woolkalis MJ : SHP2 association with VE-cadherin complexes in human endothelial cells is regulated by thrombin. **J Biol Chem 275** : 5983-5986, 2000
40. Wang GL, Semenza GL : Molecular basis of hypoxia-induced erythropoietin expression. **Curr Opin Hematol 3** : 156-162, 1996