Abstract. Cerebral ischemia is caused by reduced blood supply at the microcirculatory level. In the microvessels, the main elements of the reperfusion injury following brain ischemia are the transformation of endothelial cell-surface from anticoagulant to procoagulant property, leukocyte adhesion, sludge or clot formation. There is a paucity of information on how hemostatic factors, cytokines, lipoprotein(a) (Lp(a)) and endothelin-1 (ET-1), being responsible for ischemic/reperfusion injury, interact with human brain microvessel endothelium (HBEC). There are no data furthermore about the expression of complement proteins of HBEC influenced by cytokines or fibrinolytic factors. Previously we established optimal conditions for culturing HBEC. Cell contraction induced by thrombin, plasmin, miniplasmin was recorded. The reassembly of F-actin was observed after thrombin treatment. ICAM-1 upregulation was measured following TNF-α, IL-1-α and thrombin incubation. Plasmin and miniplasmin downregulated the ICAM-1 in our cell culture system. Lp(a) modulated the thromboresistant cell-surface by reduction of t-PA and u-PA, but PAI-1 remained unchanged. Lp(a) modulated the ET-1 production by early increasing and late decreasing, in a bimodal manner. The increased secretion of ET-1 by cytokines (TNF-α, IL-1-α) was reduced in the presence of Lp(a). Gradual increase of complement proteins (factor H, factor B, C4) was induced by cytokines. Plasmin and miniplasmin augmented a rapid increase of C4. Some factors of complex relationship between regulators and modulators of endothelial adhesion molecules have been demonstrated in a human cell culture system prepared from brain microvessel endothelium. A unified concept of sequential events of ischemia/reperfusion in the brain has not yet developed. (Keio J Med 45 (3): 203-206, September 1996)

Key words: human brain endothelium, ICAM-1, Lp(a), ET-1, complement proteins

Introduction

Microvascular endothelium in the brain is a vital interface between the circulating blood and brain tissue. Occupying the important location and having the ability to respond to change in its physical, chemical and humoral environment by the production of an array of biological active substances, the normal endothelium modulates the tone of underlying vascular smooth muscle cells, maintains a nonadhesive luminal surface, mediates hemostasis, cellular proliferation and inflammatory and immune mechanisms in the vascular wall.

In the brain capillary segments, endothelial cells are interconnected by the belt-like continuous tight junctions, and the cells have a low profile vesicular transport activity.1–3 These are the structural basis of the blood-brain barrier (BBB), which allows the stable milieu in the central nervous system. The thromboresistant luminal surface of the endothelial cells is in a close approximation with blood constituents, with circulating plasma factors, and cells, platelets, leukocytes and red blood cells. The abluminal surface of the endothelial cells is intimately opposed by pericytes or astroglia end-feet, in the capillary segments, and by smooth muscle cells in the non-capillary...
vascular bed.

Aggregation, (sludge formation) of blood cells
impending flow in the microcirculation, affected microvascular flow regulation and transformation of the endothelial surface from anticoagulant to procoagulant properties have been demonstrated in the ischemic brain.\(^4\) The endothelium-platelet-leukocyte system reacts in a complex manner.\(^7\) Leukocyte depletion reduced the reperfusion injury following temporal ischemic event in the brain.\(^5\) Endothelium-derived vasodilators (NO, EDHF, PG-I\(_2\), PG-E\(_2\)), and vasoconstrictors (free radicals, TX-A\(_2\), leukotriens C\(_4\)/D\(_4\), ET-1, AT II), procoagulants (TF, PDGF, vWF, IL-1-\(\alpha\)) and anticoagulants (thrombomodulin, protein S, plasminogen activator, heparan sulfate), finally adhesion modulation molecules (IL-1-\(\beta\), vWF, PAF, TGF-\(\beta\), ICAM-1, ELAM-1, VCAM-1, GMP-140, PECAM-1) are in a delicate balance. Interrelation among these factors and their relation to others like the complement proteins are likely to occur under these conditions e.g. histamine, thrombin, oxygen-derived free radicals. IL-1, TNF-\(\alpha\), and activated complement factors induce, in a distinct time course, the (transient) expression of leukocyte adhesion molecules P-selectin, E-selectin, ICAM-1, VCAM-1 on the endothelium. Only VCAM-1 is specific to monocytes, all the others mediate binding and subsequent extravasation of both monocytes and granulocytes. The response to the relevant inflammatory mediators, except extracellularly produced free radicals, is coupled via specific receptors on the surface of the endothelium to specific signal transduction pathways.

Protein Kinase C induced phosphorylation of transcription factors is often shown to be involved except P-selectin.

Methods of isolating microvessels, isolating and culturing endothelial cells from the brain tissue of experimental animals offer a new technology to study endothelial cell morphology, and metabolic activity of the endothelium-derived factors following different experimental procedure.\(^4\)

Isolation and culture of human brain microvessel endothelial cells (HBEC) makes possible even more relevant observations to the clinical conditions, than cells harvested from animal brain.\(^15\)

In this paper the interactions of HBEC and hemostatic factors, cytokines and Lp(a) will be discussed. We demonstrate cytokines (IL-1, TNF-\(\alpha\)) induced ET-1 release modulated by Lp(a) added to the cell culture medium in a pathological concentration. ICAM-1 production as well as the release of different complement proteins (factor H, factor B, C1 inhibitor, C4) induced by cytokines (IL-1, TNF-\(\alpha\)) or influenced by serine proteases (plasmin, miniplasmin) are demonstrated in this paper.

### Materials and Methods

**Isolation and culture of human brain microvessel endothelium**

The preparation of microvascular endothelial cells from human brain and the cell-culture procedure were published in details previously.\(^14,15\) Our method is a modification of the procedure used for isolation and culture of bovine brain microvessel endothelial cells.\(^16\) The brain tissue samples were obtained from subjects autopsied because of sudden death due to cardiac arrest and transferred to the laboratory within 4 hours post-mortem. The homogenate prepared from the cortex was centrifugated and myelin debris were removed. The microvessel segments containing pellet was digested with collagenase. The pellet was suspended in dextrane solution, capillaries and individual cells were separated afterwards with percoll gradient centrifugation. The endothelial cells and capillaries were collected and suspended in tissue culture medium of 20% FBS, DMEM/Ham's F12 completed with endothelial mitogen and antibiotics. After 48 hrs the tissue culture medium was removed and changed regularly (e.g. 2–3 days).

The cells were characterised by visualisation of von Willebrand factor (vWF) and by uptake of Dil-Ac-LDL. The primary culture contained about 80–95% endothelial cells. The contaminating cells are mostly pericytes. The culture was enriched in endothelial cells by mechanical cloning. The cells cultured on glass coverslips for morphological observations and on 24 and 96 well microplates for ELISAs.

**The cells were treated with cytokines, hemostatic factors and Lp(a)**

Cytokines (TNF-\(\alpha\) 2.7–270 U/ml, IL-1-\(\alpha\) 2–50 U/ml) were added in serum free medium and cells were incubated in different time intervals (7–31 hrs).\(^17\)

Hemostatic factors (human thrombin 10–130 nmol/l, plasmin 20 nmol/l and miniplasmin 20 nmol/l) were added in serum free medium and the cells were incubated for 30 minutes. Afterwards these factors were removed and...
cells were further incubated in medium containing BSA for 18–20 hrs.

Lp(a) in a dose of 360 µg/ml or 450 µg/ml was added to the culture and the cells were incubated for 3–72 hrs.18–20

Detection of ICAM-1, ET-1, fibrinolysis components and complement proteins

ICAM-1 immunoperoxidase staining of endothelial cells with Avidin-Biotin method.20 The cells on glass coverslips were fixed in ice cold ethanol/acetone. After the blocking of non-specific binding sites the cells were incubated first with monoclonal anti-human ICAM-1 then with biotinylated second antibody (sheep) at room temperature. Before Avidin-peroxidase binding the activity of endogen peroxidases was blocked. The ICAM-1 expression was visualized by DAB reaction.

In situ cellular ELISA for quantification of endothelial cell-surface bound ICAM-1. The cells on 96 well microplates were fixed in paraformaldehyde at 4°C. The cells were permeabilised and non-specific binding sites were blocked. The cells were incubated first with monoclonal anti-human ICAM-1 (mouse) then with peroxidase labelled second antibody (rabbit) at room temperature. Endogen peroxidases were blocked and the colour was developed by TMB. The reaction was stopped and the optical densities detected at 450 nm (reference 620 nm).17

PAI-1, t-PA, u-PA, were analysed in the supernatant of our cell culture system using ELISAs (Chromogenix t-PA, PAI-1 and Technoclone u-PA).18

Endothelin-1 (ET-1) was measured from the tissue culture medium by ELISA (R&D Systems).18 Complement proteins (C1 inhibitor, factor H, factor B and C4) were analysed using ELISA. The ELISA plates were coated with antibodies against complement proteins (anti-human polyclonal antibodies from goat, Incstar Comp.). After blocking of non-specific binding sites the supernatant of cell culture was added. Following the binding of peroxidase labelled second antibodies (anti-human polyclonal antibodies from sheep, The Binding Site Ltd.) the enzyme reaction was developed by TMB and optical densities at 450 nm were detected.21

Results and Discussion

Human brain microvessel endothelial cells isolated from cortical tissue were seeded on tissue culture plates or glass coverslips and grown in the tissue culture conditions. The cells became confluent, or quasi confluent, mostly after mechanical cloning. However, closely packed continuous monolayer, characterized with a high electric resistance, has not been produced in solo culture of HBEC.

The expression of ICAM-1 was induced by TNF-α (135 U/ml) or by IL-1α (25 U/ml). With immunostaining of Avidin-Biotin method, ICAM-1 expression augmented by cytokines was visualized in our human cell culture. Upregulation of ICAM-1 by TNF-α or IL-1-α was quantitatively measured by in situ cellular ELISA. There were no significant differences in ICAM-1 expression induced by different concentration of cytokines (TNF-α 2.7–270 U/ml, IL-1-α 2–50 U/ml). We detected the increase of ICAM-1 expression during the period of 31 hrs tested. (Fig 1)

Thrombin (10 and 130 nmol/l for 30 min) increased the ICAM-1 expression in a moderate dose dependent fashion. On the other hand plasmin and miniplasmin markedly reduced the ICAM-1 expression. (Fig 2)

Adhesion molecule upregulation on the luminal surface of the microvessel endothelium initiates the sequential events in the brain microcirculation. Not only the inflammatory diseases,19–21 but the ischemic insult,7,8,22 or other insult, like shear stress23 are characterized by adhesion of leukocytes and platelets on the endothelial cell surface. These phenomena are mediated by several types of adhesion molecules, including leukocyte integrins.
(LFA-1 (CD 11a/CD18), or Mac-1(CD11b/CD18)), and their counter receptors (e.g. ICAM-1, ICAM-2, VCAM-1) on the endothelial surface. Release of cytokines, such as TNF-α, IL-1-α, or IFN-γ and other factors (PAF, LTs, TX, free radicals) at sites of inflammation, immuno-reaction or ischemic insults, causes endothelial cell activation and results in augmented cellular expression of ICAM-1 on the luminal surface, which reach the plateau level after 16–24 hrs of stimulus. In our human cell culture system we detect increased ICAM-1 expression from 7 to 31 hrs following cytokine treatment.

Active proteases (e.g. thrombin, plasmin, miniplasmin) responsible for the delicate balance of thrombosis-fibrinolysis produce significant changes on the endothelial cell morphology.15 Reversible cell contraction has been demonstrated, characterized by increased perimeter and reduced area on the individual endothelial cells in culture. Thrombin receptors of endothelial cell surface were demonstrated, and plasmin, miniplasmin binding sites were recently.24 The number of binding sites of plasmin (8 x 10⁵), and of miniplasmin (7.5 x 10⁶) has been determined by Scatchard analysis. Thrombin induced endothelial cell contraction led the remodelling of cytoskeleton system, visualized by fluorochrome labelled phalloidin (our unpublished observation). It was demonstrated,25 that ICAM-1 activates transducer signals in brain endothelial cells by an actin-binding, cortactin and induces cytoskeleton changes and transendothelia migration of blood-born cells, lymphocytes and leukocytes.

Cytoskeleton structure and function of endothelial cell is a topic of intensive research.26,27 but the exact mechanism of external signaling, the transmembrane signal-transduction and remodelling of the cytoskeleton is far to be fully understood. Thrombin binding to its receptors could signal the phospholipase-C, which through the inositol triphosphate pathway activates F actin or increases actomyosin and results in cell movement. It could be assumed, that thrombin binds to the endothelial cell surface and upregulates ICAM-1, or thrombin acts via upregulating TNF-α and IL-1-α, as it was demonstrated in HUVEC model.28 More controversial findings are, that plasmin, miniplasmin downregulate ICAM-1, however, the initiate similar cell contraction as it was demonstrated with thrombin. It has been observed, that dense peripheral bands of F-actin fibers were decreased and central bundles increased when continuous culture of HUVEC was exposed to thrombin. Other serine proteases (plasmin and trypsin) caused rounding up the cells, but they did not decrease the peripheral bundles of F-actin, although gaps were formed between the cells.29,30 The interrelations between the hemostatic system and the adhesion molecules are still controversial.

Lipoprotein(a) modulates t-PA, u-PA and PAI-1 release into the culture medium. Endothelial cells are active in the maintenance of the coagulation-fibrinolysis balance in the vascular bed. They participate in the lysis of the local fibrin clot. The cells modulate the fibrinolysis by expression of t-PA and PAI-1, furthermore the expression of u-PA. Apolipoprotein(a), the kringle-containing moiety of Lp(a) has a close structural similarity to plasminogen.31 Interaction with local and systemic fibrinolysis may explain how Lp(a) acts as an independent risk factor in ischemic stroke.32–34 We demonstrated how Lp(a) modulates the production of t-PA, u-PA and PAI-1 in our HBEC system. The cells synthesized t-PA during a 72 hrs period in a slowly increasing manner, while the u-PA and PAI-1 expression could be detected from the beginning of the observation period. Lp(a) in a concentration of 450 μg/ml, equivalent to the human pathological serum level, were added to the cell culture for 72 hrs reduced the production of t-PA and u-PA, respectively, while the PAI-1 concentration remained unchanged compared to the control (Fig 3).

Lp(a) competes with plasminogen for binding to endothelial cells.33 This interaction decreases the thrombo-resistant character of the luminal surface by decreasing the t-PA and u-PA expression.

Our HBEC system confirms, that Lp(a) modulates the antithrombotic activity of the brain microvessels increasing the risk of ischemic events in the brain.34

ET-1 is a potent vasoconstrictor peptide. ET-1 of different tissue origin may exert pleiotropic effects on cardiovascular, neuroendocrine, renal, gastrointestinal and pulmonary function. ET-1 produced by endothelial cells may act in paracrine (from endothelial cells to smooth muscle cells) or autocrine (from endothelial cell to itself) way. ET-1 binds to specific receptors in the cerebral vessels, elicits contraction and relaxation, controls the BBB, influences cell proliferation and tissue inflammation.35 In our HBEC system ET-1 level increased gradually with time. The secreted immunoreactivity was characterized by HPLC and proved to be intact ET-1. Lp(a), the highly atherogenic lipoprotein modifies the secretion of ET-1 by HBEC in culture in a bimodal manner. After 24 hrs, the increased ET-1 concentration decreased gradually till 48 hrs compared to control. TNF-α and IL-1-α also enhanced the ET-1 secretion in a dose dependent fashion. Similarly, the cytokines induced ET-1 production was inhibited by Lp(a) (Figs 4, 5).

The significance of ET-1 in the brain pathology has been recently reviewed.35 In the cases of ischemic stroke, SAH, head injury, temporal lobectomy or migraine, increased ET-1 level has been documented. ET-1 secretion modified by Lp(a) and by cytokines is a novel mechanism by means of which ET-1 interrelates to cytokine-adhesin system and to lipid metabolism.

Complement protein (factor H, factor B and C4) synthesis and secretion could be detected in our HBEC system during 72 hr-observation period (Fig 6). Gradual increase was detected except in the case of Cl inhibitor.
TNF-α and IL-1-α induced a significant increase of complement protein production compared to the control. Serum proteases, plasmin and miniplasmin increased rapidly the C4 level in the first period of the incubation time (Fig 7).

Complement is an important effector arm of the human immune response. The importance of characterizing the expression and regulation of complement in the CNS is highlighted by growing evidence that complement plays a significant role in the pathogenesis of a variety of neurological diseases. In vitro studies have demonstrated that astrocytes are capable of expressing or producing majority of the components of the complement system. Complement expression is related to the acute phase response. The class III MHC gene product C4 ex-
pression was increased, in a dose-dependent manner by IFN-γ, but it was unaffected by IL-1-β, IL-6 or TNF-α in human fibroblast cell culture. 37 Upregulation of E-selectin, VCAM-1 and ICAM-1 was documented in patients with SLE. Abnormal expression of these adhesion molecules is most marked in active phase of the diseases characterized by significant elevation of complement C3a. 38

The main components of the possible effective stroke therapy (thrombolysis, neuroprotection and prevention of reperfusion injury) have to be conceptionalised on the basis of the understanding on the interaction of endothelium related cytokines, adhesin molecules, hemostatic factors, lipid metabolites and complement proteins. The cell culture of human brain endothelium appears to be a relevant model-system for these interaction.

References

29. Wong MK, Gotlieb AI: Endothelial monolayer integrity. Per-


Discussion (Zólítan Nagy)

Lindsberg: 1) It was recently shown that anoxia stimulates ICAM-1 expression in cultured human endothelial cells isolated and cultured from the brain. Did you have a chance to test whether anoxia upregulates ICAM-1 in your system? 2) You showed data on the expression of complement proteins in EC’s. Is there any evidence of activation also of the terminal complement pathway (TCC, C5b-C9) in these cells? Perhaps you need the presence of serum as the source of complement proteins to show this?

Nagy: 1) We did not use a hypoxia stimulus for ICAM-1 expression in our tissue culture system. Hess’s group (Stroke 1994, 25: 463–467) has demonstrated that hypoxia (24 h) induced ICAM-1 upregulation in the course of re-oxygenation (4-12-24 h) in human brain microvessel endothelial cells. A peak of ICAM-1 mRNA has been detected after 4 h of re-oxygenation. Using a HUVEC model, hypoxia induced an increase of neutrophil adhesion to the endothelial cell surface. PAF, P-selectin and ELAM-1 upregulation have been demonstrated in the recently published literature. 2) In our culture system, the tissue culture medium is 0.1% BSA in DMEM. There are no data on whether human brain endothelial cells do actually express complement proteins in serum free medium. On the other hand, it has been demonstrated that, in serum free medium, HUVEC can synthesize complement proteins (C3, C5, C6, C8 and C9) (Johnson and Hetland, Scand J Immunol 1991, 33: 667–671). There is a lack of evidence of activation of the terminal complement pathway (TCC) in our system. It has been demonstrated that terminal complement proteins, C5b-9, release basic fibroblast growth factor and platelet-derived growth factor from HUVEC and bovine aortic endothelial cells. These mitogens stimulate DNA synthesis in smooth muscle cells (Benzaquen LR et al, J Exp Med 1994, 179: 985–992). To study the effect on the barrier properties of complement proteins in the brain endothelial systems seems to provide a new perspective.

Raivich: Is the effect of thrombin on endothelial cell shrinkage mediated by adenylate cyclase? Did you look at the effects of cAMP degradation inhibitors (caffeine, theophylline) on thrombin-induced shrinkage?

Nagy: Thrombin-induced endothelial cell contraction is a well-known phenomenon. In brain endothelial models, this phenomenon could reflect on the mechanism of junctional opening of endothelial cells. The cell contraction resulted in opened paracellular avenues. Inhibition of myosin light-chain kinase activity could be a possible mechanism. In our system, we did not use cAMP degradation inhibitors to protect the cells from thrombin-induced shrinkage.

Tanahashi: You showed that thrombin caused shrinkage of human endothelial cells. Is that phenomenon dose-dependent?

Nagy: The thrombin-induced endothelial cell contraction is dose-dependent, as we demonstrated in a previous paper (50 nm/1, 0.44 μm/l, 5.5 μm/l) (Nagy et al, Stroke 1995, 26: 265–270).