Damage and Activation of Endothelial Cells during *in Vitro* Hypoxia

O. A. Antonova, S. A. Loktionova, N. V. Golubeva, Yu. A. Romanov, and A. V. Mazurov

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 144, No. 10, pp. 384-386, October, 2007
Original article submitted January 26, 2007

We studied the effect of hypoxia on activation and stimulation of apoptosis in cultured endothelial cells. The effect of hypoxia was compared to that of apoptosis-inducing agents (tumor necrosis factor and bacterial lipopolysaccharide). Incubation of endothelial cells for 24 h under hypoxic conditions (2% O_2, 5% CO_2, and 93% N_2) increased secretion of von Willebrand factor, but had no effect on the expression of cell adhesion molecule ICAM-1. Tumor necrosis factor and lipopolysaccharide did not stimulate secretion of von Willebrand factor, but significantly increased the expression of ICAM-1. These data attest to significant differences in the mechanisms of endothelium activation under hypoxic conditions and during treatment with tumor necrosis factor or lipopolysaccharide. Hypoxia stimulated apoptosis in endothelial cells, which was seen from the increase in the number of annexin V-binding cells and activation of caspase-3. Similar changes were revealed in the presence of tumor necrosis factor and lipopolysaccharide. Hence, damage to endothelial cells caused by hypoxia and these compounds is mediated by similar mechanisms.

Key Words: *endothelium; hypoxia; apoptosis; cell adhesion molecules; von Willebrand factor*

Tissue hypoxia due to thrombotic occlusion of blood vessels in infarction, stroke, and other disorders is followed by significant changes in the state of cells, including endothelial cells (EC) of the vascular wall. The exposure of EC to hypoxia can lead to not only activation of these cells [5,7], but also their damage and apoptosis [4,6]. Secretion from intracellular granules and variations in protein expression on the cell surface are the major signs of endothelium activation, the process leading to changes in thrombotic and inflammatory properties of the vascular wall. D. J. Pinsky *et al*. [7] showed that incubation of EC under hypoxic conditions increases secretion of von Willebrand factor (platelet adhesion protein) from granules, *i.e.* stimulates one of the key reactions in endothelium activation. Increased expression of cell adhesion molecules and receptors involved in the interaction of EC with leukocytes also serves as the major sign of activation of these cells. However, C. F. Maurus *et al*. [6] failed to reveal changes in the amount of these proteins on the surface of EC during hypoxia. It should be emphasized that these reactions were not studied simultaneously during hypoxia. Moreover, much attention was paid to the reactions characteriziny endothelial activation, while markers of endothelial damage and apoptosis were poorly studied [6,7]. C. N. Lee *et al*. [4] reported that hypoxia stimulates apoptosis in EC, which is mediated by the general mechanism and results in damage to mitochondria and DNA. The signs of EC activation were not evaluated in this study. Hence, the re-

Institute of Experimental Cardiology, Russian Cardiology Research and Production Complex, Russian Ministry of Health, Moscow. ***Address for correspondence:*** calab@cardio.ru. S. A. Loktionova

lationship between EC activation and apoptosis during hypoxia remains unknown. It is unclear whether these processes can be activated simultaneously.

Here we studied the effect of hypoxia on activation and stimulation of apoptosis in EC. The effect of hypoxia was compared with that of apoptosis-inducing agents, tumor necrosis factor (TNF) and bacterial lipopolysaccharide (LPS).

MATERIALS AND METHODS

The culture of EC was obtained from human umbilical vein as described elsewhere [2]. Experiments were carried out on confluent culture of passages 1-3. The cells were cultured in DMEM supplemented with 10% fetal calf serum, 200 μg/ml endothelial growth factor, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5 U/ml heparin. The medium, serum, and additives were from Gibco. Culturing under hypoxic conditions was performed in a Hereaus incubator with simultaneous delivery of CO_2 and N_2. O_2 content in the gas mixture was monitored by an oxygen sensor. Incubation under hypoxic conditions was performed for 24 h using gas mixture containing 2% O_2, 5% CO_2, and 93% N_2. Under conditions of normoxia (21% O_2, 5% CO_2, and 74% N_2), the cells were incubated in the absence or presence of TNF (100 U/ml, Sigma) and LPS (100 μg/ml, Sigma).

After the end of incubation, the medium was sampled for von Willebrand factor assay by enzyme immunoassay [1].

Expression of ICAM-1 and binding of annexin V and propidium iodide (PI) to EC were studied by the method of flow cytofluorometry. The study was performed on a FACS Calibur cytofluorometer (Becton Dickinson). The suspension of EC was prepared with trypsin/EDTA (0.02 and 0.05%, respectively) and placed on ice to study ICAM-1 expression. The cells were prefixed with 0.02% solution of paraformaldehyde for 1-2 min, pelleted, resuspended in a solution containing 10 μg/ml biotinylated monoclonal 10F3B2 antibodies against ICAM-1 [8], and incubated for 30 min. These cells were washed and incubated with phycoerythrin-labeled streptavidin (PE, Caltag) for 30 min. The dilution was selected according to manufacturer's recommendations. Fluorescence of cells and ICAM-1 expression were evaluated from the medians of cell distribution histograms. Fluorescence of cells incubated in the absence of TNF and LPS under normoxic conditions was taken as 100%.

Binding of FITC-labeled annexin V and PI to EC was studied using Annexin V-FITC commercial kit (Immunotech Inc.). The suspension of EC was prepared with trypsin/EDTA (0.02 and 0.05%, respectively), placed on ice, and stained with annexin V-FITC and PI according to the manufacturer's recommendations. The level of fluorescence due to binding of annexin V-FITC and PI to cells was measured by means of flow cytofluorometry. The results were expressed as the percent of stained cells (relative to total cell number).

The suspension of EC was prepared using trypsin/EDTA (0.02 and 0.05%, respectively) and placed on ice to estimate caspase-3 activity. Each study involved standard number of cells ($0.5\text{-}1\times10^6$ in various experiments). The cells were treated with lysing buffer. Caspase-3 activity was measured using Caspase-3 Colorimetric Assay kit (R&D Systems). DEVD-pNA served as the substrate. Caspase-3 activity in cells incubated in the absence of TNF and LPS under normoxic conditions was taken as a unit of enzyme activity.

The results were analyzed by Student's *t* test.

RESULTS

Hypoxia significantly stimulated secretion of von Willebrand factor (Table 1). The concentration of von Willebrand factor in the medium after cell incubation under hypoxic conditions was 4-fold higher than after normoxic incubation. However, TNF and LPS had little effect on this parameter (Table 1). Expression of cell adhesion molecule ICAM-1 on the cell surface remained unchanged during hypoxia, but significantly increased under the influence of TNF and LPS (more than by 14 and 5 times, respectively; Table 1).

For evaluation of the level of stimulation of EC apoptosis, activity of caspase-3, key proteolytic enzyme activated during apoptosis [3], and the number of cells stained with annexin V were determined. Annexin V binds to negatively charged phospholipids. The increase in the amount of these

TABLE 1. Activation of EC during Exposure to Hypoxia, TNF, and LPS: Expression of ICAM-1 and Secretion of von Willebrand Factor (% of Normoxia Level, $M\pm m$, n=7-11)

Conditions	ICAM-1 expression	Secretion of von Willebrand factor
Normoxia	100	100
Hypoxia	102±6	436±43***
TNF	1441±362***	174±44^{++}
LPS	505±156^{+}	180±31^{++}

Note. Here and in Table 2: *p<0.05, **p<0.01, and ***p<0.001 compared to normoxia; $^{+}p\leq0.05$ and $^{++}p\leq0.01$ compared to hypoxia.

TABLE 2. Stimulation of EC Apoptosis during Exposure to Hypoxia, TNF, and LPS: Binding of Annexin V and Activation of Caspase-3 ($M \pm m$, n=6-8)

Conditions	Annexin V^+ cells, % of total cell number	Caspase-3 activity, % of normoxia level
Normoxia	1.5±1.5	100
Hypoxia	11.7±3.9**	316±81
TNF	4.6±0.98+*	233±52*
LPS	7.3±0.8***	217±39*

compounds on the outer cell surface is a early manifestation of apoptosis [9]. Caspase-3 activity increased by more than 3 times after incubation of cells under conditions of hypoxia (compared to culturing of control cells under normoxic conditions, Table 2). The increase in caspase-3 activity in the presence of TNF and LPS was less pronounced compared to that observed during hypoxia (by 2-2.5 times). The number of cells stained with annexin V—FITC significantly increased during hypoxia and under the influence of TNF and LPS. The percent of annexin V-positive (annexin V^+) cells after incubation under hypoxic conditions was slightly higher compared to that observed after addition of TNF and LPS (12, 5, and 7%, respectively). This percent is consistent with the results of studying apoptosis by the measurement of caspase-3 activity. Enzyme activity was also highest under hypoxic conditions. The ratio of cells in which nucleic acids were stained with PI (marker of membrane integrity and cell necrosis) corresponded to 70-85% of annexin V^+ cells under various conditions of incubation. The presence of these cells was probably associated with transition from apoptosis to necrosis during culturing. Moreover, cell and preparation of samples could also be accompanied by damage to these cells.

The exposure of EC to hypoxia was followed by activation of these cells and stimulation of apoptosis. Activation of EC under hypoxic conditions differed from that induced by TNF and LPS. Hypoxia was followed by an increase in secretion of von Willebrand factor, but had no effect on the concentration of cell adhesion molecule ICAM-1 on the cell surface. Activation induced by TNF and LPS increased expression of ICAM-1, but did not stimulate secretion of von Willebrand factor.

This work was supported by the Russian Foundation for Basic Research (grant No. 06-04-48312a).

We are grateful to M. M. Pekalo for antibodies.

REFERENCES

1. E. V. Yanushevskaya, E. A. Barkevich, S. G. Khaspekova, *et al*., *Gematol. Transfuziol*., **50**, No. 2, 20-25 (2005).
2. A. S. Antonov, M. A. Nikolaeva, T. S. Klueva, *et al*., *Atherosclerosis*, **59**, No. 1, 1-19 (1986).
3. G. M. Cohen, *Biochem. J*., **326**, No. 1, 1-16 (1997).
4. C. N. Lee, W. F. Cheng, M. C. Chang, *et al*., *Apoptosis*, **10**, 887-894 (2005).
5. C. Michiels, T. Arnould, and Y. Remacle, *Biochem. Biophys. Acta*, **1497**, No. 1, 1-10 (2000).
6. C. F. Maurus, D. Schmidt, M. K. Schneider, *et al*., *Eur. J. Cardiothorac. Surg*., **23**, No. 6, 976-983 (2003).
7. D. J. Pinsky, Y. Naka, H. Liao, *et al*., *J. Clin. Invest*., **97**, No. 2, 493-500 (1996).
8. O. Yu. Printseva, M. M. Peclo, and A. M. Grown, *Am. J. Pathol*., **140**, No. 4, 889-896 (1992).
9. I. Vermes, C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger, *J. Immunol. Methods*, **184**, No. 1, 39-51 (1995).