Proliferative Activity of Retinal Vascular Cells in Newborn Rat at Different Oxygenation Modes

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We evaluated the relationship between the percentage of oxygen in inhaled air and alternation of this parameter and proliferative activity of cells in retinal vessels of normal newborn rats. The relationships between these parameters and the mean diameter of retinal vessels were evaluated. The study was carried out on total retinal preparations and tangential sections of the retina by the immunoperoxidase and immunofluorescent methods. Hypoxia and hyperoxia significantly suppressed proliferative activity, while alternation of hyperoxia and normoxia significantly increased both proliferative activity of vascular cells and the mean diameter of retinal vessels.

Key Words: *proliferation; hyperoxia; hypoxia; retina*

Increased incidence of severe retinal diseases associated with pathological angiogenesis in preterm babies necessitated investigation of the main processes underlying the development of retinal vessel.

The formation of vessels in fetal retina and neovascularization in retinal diseases are associated with the release of vasoproliferative factor in response to hypoxia [1]. On the other hand, hyperoxygenation is one of the main risk factors for neonatal retinopathy [2,3,5]. These mutually excluding facts are explained using the notion of "relative hypoxia", because pathological new vessels in newborn retina appear after the end of oxygen therapy [3,4].

We studied factors essential for proliferative activity of retinal vessels of newborn rats under different oxygenation conditions.

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MATERIALS AND METHODS

Newborn Wistar rats (aged no more than 12 h) were placed into a pressure chamber for 9 days. The animals were divided into 4 groups depending on the level and protocol of oxygenation. Group 1 animals were exposed to atmosphere with 11% oxygen (hypoxia) for 9 days; group 2 animals were exposed to hyperoxia (50% oxygen); group 3 animals were exposed to atmosphere with oxygen levels alternating every 24 h (from 50 to 11%); and group 4 pups were exposed to 100% oxygen for 5 days, after which they were transferred into common room air. Control group consisted of animals of the same age kept under conditions of normoxia.

Proliferative activity of retinal vascular cells was studied by the immunofluorescent and immunoperoxidase methods.

For immunofluorescent studies total preparations of the retina were fixed by transcardial perfusion of 4% paraformaldehyde solution, washed, and treated with antibodies to Von Willebrand factor (vWF; Chemicon), Ki-67, and PCNA (Novo Castra). The preparations were then treated with second anti-mouse antibodies conjugated with Cy-2

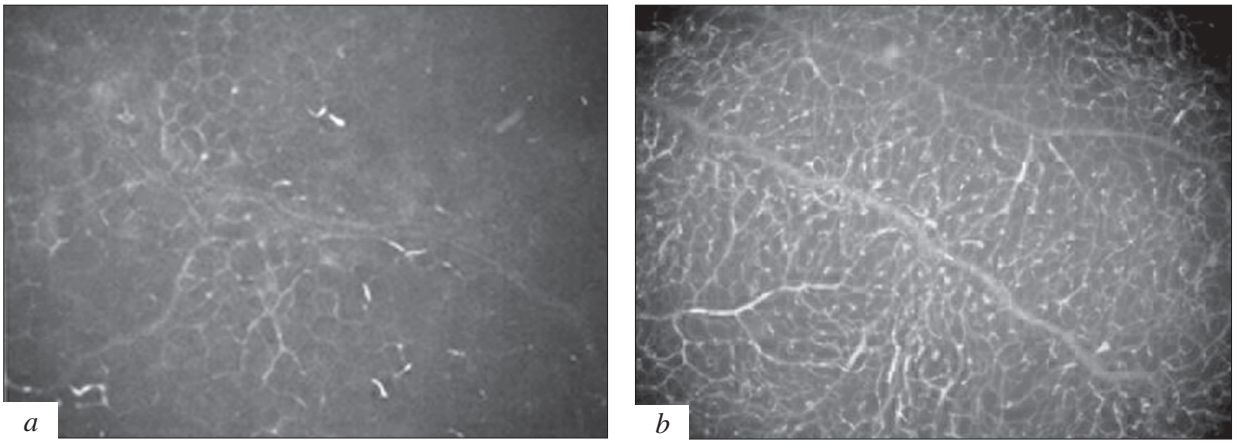

Fig. 1. Fluorescent microscopy of total preparations of the retina with antibodies to Ki-67 and PCNA (×80). *a*) hyperoxia (group 2); *b*) hyperoxia alternating with normoxia (group 4).

fluorescent dye and anti-rabbit antibodies conjugated with Texas Red fluorescent dye (Jackson). Proliferative activity of vascular cells was evaluated by measuring the mean fluorescence intensity of retinal vascular cells using morphodensitometry software (Lucia Morpho, Nikon).

Immunoperoxidase staining was carried out on tangential serial paraffin sections with first antibodies to Ki-76 and PCNA, which were visualized by the EnVision system (anti-mouse and anti-rabbit, DAKO) by the standard protocol. The sections were post-stained with Mayer's hematoxylin (2-5 min). Proliferative activity was analyzed by the semiquantitative method. Independent operators evaluated the counts of Ki-67- and PCNA-immunopositive cells in the vascular wall in 4 representative retinal sections using a 5-point system.

In order to measure the diameter of retinal vessels, the fragments of the retina fixed in 2% glutaraldehyde and routinely processed were embedded in Epon-Araldite resin. The minimum diameter of the vessels was evaluated on stained semithin sections using morphometry software (Lucia Morpho, Nikon).

RESULTS

Analysis of tangential sections showed that proliferative activity of retinal vascular cells in animals exposed to hypoxia and hyperoxia was significantly below the normal. On the other hand, proliferative activity was at least 2-fold higher in animals exposed to hyperoxia or hypoxia alternating with normoxia. Hence, proliferative activity of vascular cells was minimum in group 2 and maximum in group 4. In group 1 these parameters were significantly below the control and much lower than in two other groups (Table 1).

Analysis of total preparations of the retina by fluorescent microscopy showed similar results (Fig. 1). The fluorescence intensity was 39 arb. units (pixels) in control group, 37 arb. units in group 1, 29 in group 2, 44 in group 3, and 47 arb. units in group 4. Hence, we observed a certain reduction of proliferative activity of retinal vascular endothelial cells in group 1 compared to the control and a pronounced decrease in this parameter in group 2 in comparison with the control and group 1. Proliferative activity increased in group 3 compared to

TABLE 1. Numbers of Labeled Cell Nuclei in Rat Retinal Vessels (Score; *M*±*m*)

Group	Sections				Summary
	1	2	3	4	
Control (*n*=8)	1.30±0.33	0.8±0.3	1.40±0.24	1.6±0.4	5.20±1.06
1 (*n*=14)	0.70±0.15	1.00±0.14	0.70±0.21	0.75±0.16	3.50±0.37
2 (*n*=14)	0.12±0.12	0	0	0	0.12±0.12
3 (*n*=8)	2.0±0.4	2.25±0.25	2.00±0.40	1.75±0.25	8±1
4 (*n*=10)	2.30±0.21	2.10±0.16	2.50±0.22	2.50±0.22	10.00±0.42

Note. Number of animals is shown in parentheses.

the control and groups 1 and 2, and in group 4 in comparison with all other groups.

The mean diameter of retinal vessels in control animals was 84.0±1.4 μ, in group 1 91.0±1.6 μ, in group 2 77.0±1.1 μ, in group 3 94.0±0.9 μ, and in group 4 98.0±1.6 μ. The maximum diameters were observed in groups exposed to alternating oxygenation, particularly after sharp discontinuation of high (100%) oxygen concentrations. This can indicate significant vasodilatation resulting in more intensive perfusion of the retina and stimulation of proliferative activity of its vessels in animals experiencing "rigid" discontinuation of high oxygen exposure.

The study showed that hypoxic exposure did not stimulate the retinal angiogenesis in newborn animals. The data suggest that reactive hyperemia resulting from sharp discontinuation of exposure to high oxygen concentrations in inhaled gas mixture is an important factor increasing proliferative activity of retinal vessels, including that in neonatal retinopathy.

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