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Inhibition of Vascular Endothelial Growth Factor-Induced Retinal Neovascularization by Retinoic Acid in Experimental Retinopathy of Prematurity

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Summary

Vascular endothelial growth factor (VEGF) has an important role in the pathogenesis of retinopathy of prematurity (ROP) and inhibition of VEGF expression in the neovascular phase might prevent destructive neovascularization in ROP. It is suggested that retinoids exert a highly potent antiangiogenic activity by inhibiting VEGF expression. The aim of this study was to demonstrate the preventive effect of retinoic acid (RA) on the VEGF-induced retinal neovascularization in a rat model of ROP. Wistar albino rats were placed into incubators at birth and exposed to an atmosphere alternating between 50 % and 10 % O_2 every 24 hours. After 14 days, the animals were removed to room air and received either an intraperitoneal injection of RA (5 mg/kg/day) (n=9) or saline (n=4) daily for six days, and sacrificed at 21 days. Other rats (n=4) were raised in room air and served as age-matched controls. The globe of each eye was cut through the cornea and embedded in paraffin. Serial sections were stained with hematoxylin-eosin for quantification of neovascular nuclei. The avidin-biotin peroxidase method was performed for evaluation of VEGF expression. The average number of neovascular nuclei was significantly lower in the control group compared to that in the ROP groups. In addition, it significantly decreased in the RA-treated ROP group compared to that of the saline-administrated ROP group. VEGF immunostaining was overall negative in room air-exposed rats. The VEGF immunostaining score significantly decreased in the RA-treated ROP group compared to that in the saline-administered ROP group. RA treatment might be beneficial in preventing neovascularization resulting from oxygen-induced retinopathy by downregulation of VEGF expression.

Key words

Vascular endothelial growth factor • Retinoic acid • Retinopathy of prematurity

Introduction

Retinopathy of prematurity (ROP) is a common retinal neovascular disorder and major cause of blindness, despite current treatment of late-stage ROP (The Cryotherapy for Retinopathy of Prematurity Cooperative

Group 2002). Because the visual disorders after treatment are often poor, preventive therapy for ROP is still lacking (The Laser ROP Study Group 1994).

Although ROP is a multifactorial disease, the altered regulation of vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF-1) have recently been implicated in the pathogenesis of ROP (Pierce *et al*. 1996, Robbins *et al*. 1998). The vascular endothelial growth factor (VEGF) is a hypoxia-inducible cytokine and a vascular endothelial cell mitogen (Plate *et al*. 1992, Shweiki *et al*. 1992, Kim *et al*. 1993). If VEGF is suppressed, normal vessel growth is inhibited, but if in excess, retinal neovascularization is precipitated. This indicates that VEGF is a critical factor in retinal neovascularization (Smith *et al*. 1994, Pierce *et al*. 1995, Aiello *et al*. 1995, Robinson *et al*. 1996).

Inhibition of VEGF at the neovascular phase might prevent destructive neovascularization (Adamis *et al*. 1996). However, the choice of any intervention for the inhibition of VEGF should be taken into account very carefully, because VEGF also promotes normal physiological development of blood vessels in many tissues. In addition, this intervention can be applied to all preterm infants when potential side effects are almost minimal. Vitamin A has been used in this population prophylactically for chronic lung disease with the large doses and no reported significant adverse effect exists (Tyson *et al*. 1999). It is suggested that vitamin A-retinoids and their active metabolite, retinoic acid (RA) have highly potent antiangiogenic activity by inhibiting VEGF expression (Oikawa *et al*. 1989, Majewski *et al*. 1995, Pal *et al*. 2000) However, the significance of RA administration has not been investigated to our knowledge in an experimental ROP model. The aim of this study was to demonstrate the preventive effect of retinoic acid (RA) on the VEGF-induced retinal neovascularization in a rat model of ROP.

Methods

Animal model and treatment

The present study was performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by Animal Care and Use Committee of Dokuz Eylul University, School of Medicine. Wistar albino rats with dated pregnancies were maintained at the same center and housed in individual cages with free access to water and laboratory chow.

The rat pups (n=13) were delivered spontaneously and within 4 h after birth were placed in a Plexiglas chambers with the mother. The environment within the incubator was adjusted to 50 % oxygen in which oxygen concentration was monitored twice daily, humidity was maintained at more than 80 % and CO_2 was removed by soda lime absorption. After 24 h, the oxygen concentration was rapidly reduced to 10 % every 24 h for 14 days. The animals were then removed from the incubator to room air (21 % oxygen). The first of total 6 doses of intraperitoneal injection was started on day of removal from the incubator and continued through postnatal day 20, the time of peak retinal neovascularization in this model (Holmes and Duffner 1996, Shafiee *et al*. 2000) Pups in Group 1 (n=9) received intraperitoneally all trans-retinoic acid (RA) in diluents (500 µg/kg) (Sigma, St. Louis, USA) whereas pups in Group 2 (n=4) received only saline in same volume. Additional pups (n=4) served as controls and kept in room air (Group 3). Nursing mothers were rotated between litters in room air and hyperoxia/hypoxia every 24 h. Room air controls were raised in the same room as the animals exposed to hyperoxia/hypoxia and maintained under normal vivarium conditions. On postnatal day 21, animals from each group were sacrified by intraperitoneal injection of pentobarbital sodium (200 mg/kg/day).

Quantification of neovascular proliferative retinopathy

The eyes were enucleated and fixed in 10 % buffered formalin overnight. The globe of each eye was cut through the cornea and parallel to the optic nerve and embedded in paraffin. Serial sections (6 µm) were cut sagitally and stained with hematoxylin-eosin. In the central half of the globe, between two and four sections (6 µm) on each side of the optic nerve (30 to 90 µm apart) were counted for neovascularization. This sampling method yielded between four and eight sections per eye that were within 10 % of the mean retinal length. Nuclei of the new vessels could be distinguished from other structures in the retina and counted in cross-sections with light microscopy (magnification x400). Vascular cell nuclei, identified under light microscopy with hematoxylin-eosin staining, were considered to be associated with new vessels, if they were found on the vitreal side of the internal limiting membrane (Smith *et al*. 1994)

Immunohistochemistry of VEGF antibody

The sections were mounted on poly-L-lysin-

coated slides. The avidin-biotin peroxidase method was performed using the primary monoclonal antibody against VEGF (1:50 dilution, Neomarkers, Fremont, USA). Briefly, the sections were deparaffinized and endogenous peroxidase activity was blocked using a 0.3 % solution of hydrogen peroxidase in PBS at room temperature for 10 min. After microwave treatment, primary antibody was applied for 30 min at room temperature and washed in PBS. Linking antibody and streptavidin-peroxidase complex (Neomarkers, Fremont, USA) were added consecutively for 10 min at room temperature and washed in PBS. The peroxidase activity was visualized with diaminobenzidine (Sigma, St. Louis, USA) applied for 5 min. Appropriate positive and negative controls were also labeled with the primary antibody. The most representative area of the section fulfilling the criteria of neovascularization was selected and marked for analyses. For immunoscoring of VEGF antibody, the degree of positive staining was evaluated by semi-quantitative scoring on a scale of 1 to 4 for intensity (I) and for distribution (D). Tissues with I x D less than or equal to 4 were considered weakly positive, and those with I x D greater than 4 were considered as strongly positive (Ozer *et al*. 2000).

Statistics and analysis of inter-observer variation

The data were statistically analyzed with Mann Whitney-U and χ^2 tests (SPSS 10.0, Chicago, USA). The probability level $P<0.05$ was chosen to represent statistical significance. Fisher's exact test was used to calculate P values, as the cell frequencies were too small for the standard χ^2 test to be accurate. To assess inter-observer variation, the histological counting and immunoscoring were performed under code by two pathologists (E.O. and B.L.) who had no prior knowledge of the results of measurement performed by other pathologist. The results of two assessments were compared and there was no significant difference by paired t-test. In addition, to test the significance of the most representative area, the results in randomly selected five animals were statistically compared and there was also no significant difference.

Results

The results of quantification of neovascular nuclei are demonstrated in Table 1. The average number of nuclei extending into the internal limiting membrane per 6 μm retinal cross section in the control group, the saline-administrated ROP group, and the RA-treated ROP group were 0.7 ± 0.9, 72.0 ± 10.4, and 30.8 ± 6.5, respectively (Fig. 1. A-C). Statistical analysis revealed that the neovascular nuclei in the RA-treated group were significantly more frequent than those in the control group and significantly less frequent than those in the saline-administered group (Table 1)

The VEGF immunostaining was generally negative in the control group, whereas all animals in the saline-administered group showed strong VEGF immunostaining (Table 2, Fig. 2 A,B). Of nine animals in the retinoic acid-treated group, VEGF immunostaining scores were negative in three (33.3 %) animals, weak in five (55.5 %) and strong in one (11.2 %) (Table 2, Fig. 2C). VEGF immunostaining was significantly decreased in this group compared to that in the saline-administered group (p=0.02).

Table 1. Quantification of neovascular proliferative retinopathy (average number of neovascular nuclei)

Group	
Room air-exposed control group	0.70 ± 0.90
Saline-administered ROP group	72.0 ± 10.4*
Retinoic acid-treated ROP group	30.8 ± 6.5*#

Data are mean ± SD. Significant differences ($p<0.005$): * vs. room air-exposed controls, # vs. saline-administered ROP group

Table 2. Quantification of VEGF immunostaining scores

Group	VEGF Immunostaining Scores		
	None	Weak	Strong
Room air-exposed control group	4	0	0
Saline-administered ROP group	0	0	4
Retinoic acid-treated ROP group	3	5	1

Saline-administered ROP group differed significantly ($p<0.02$) from both other groups.

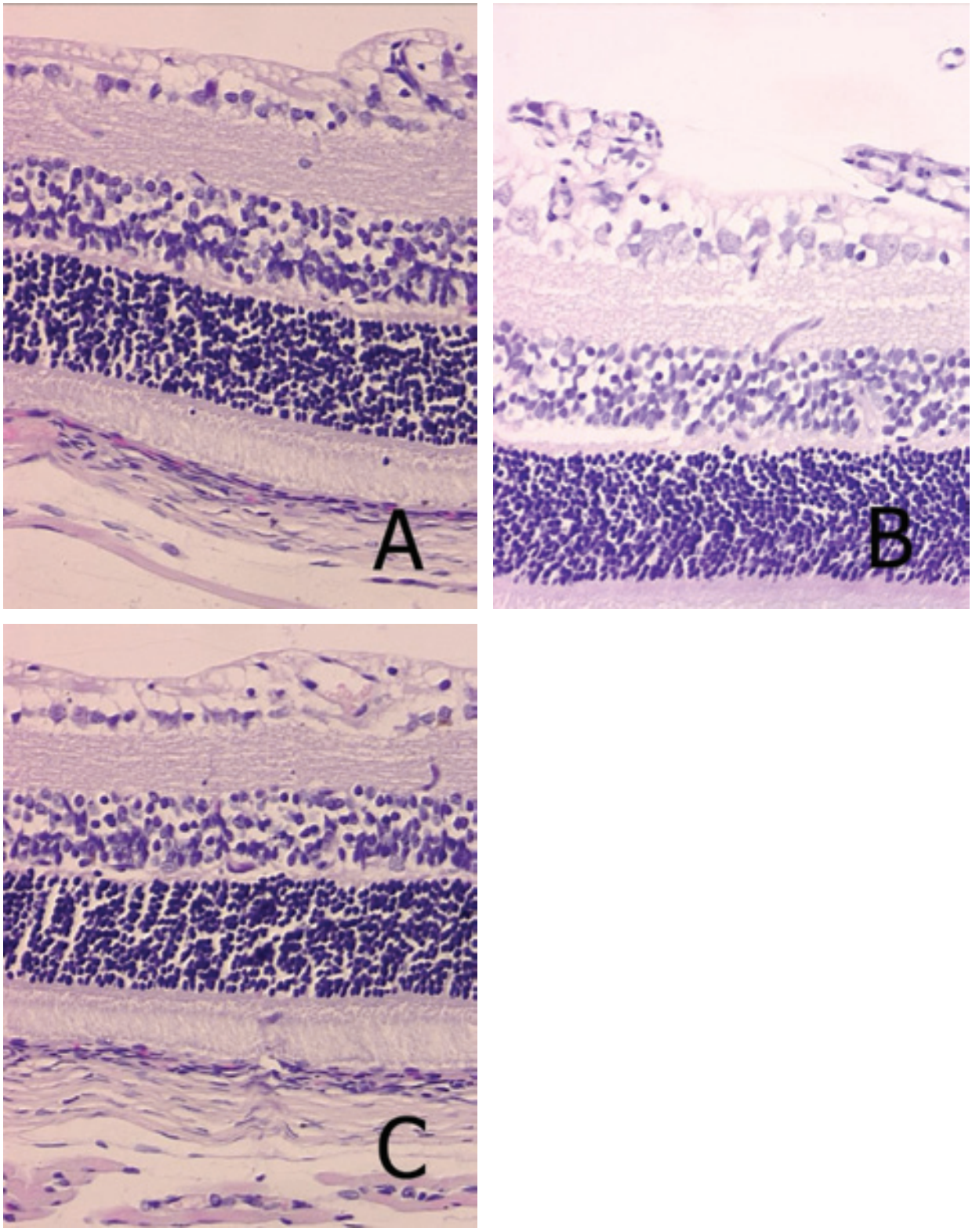

Fig. 1. The number of nuclei extending into the internal limiting membrane in the RA-treated ROP group (A) is less than that in the saline-administered ROP group (B), but more than that in the control group (C). Hematoxylin-eosin staining, 200x (original magnification).

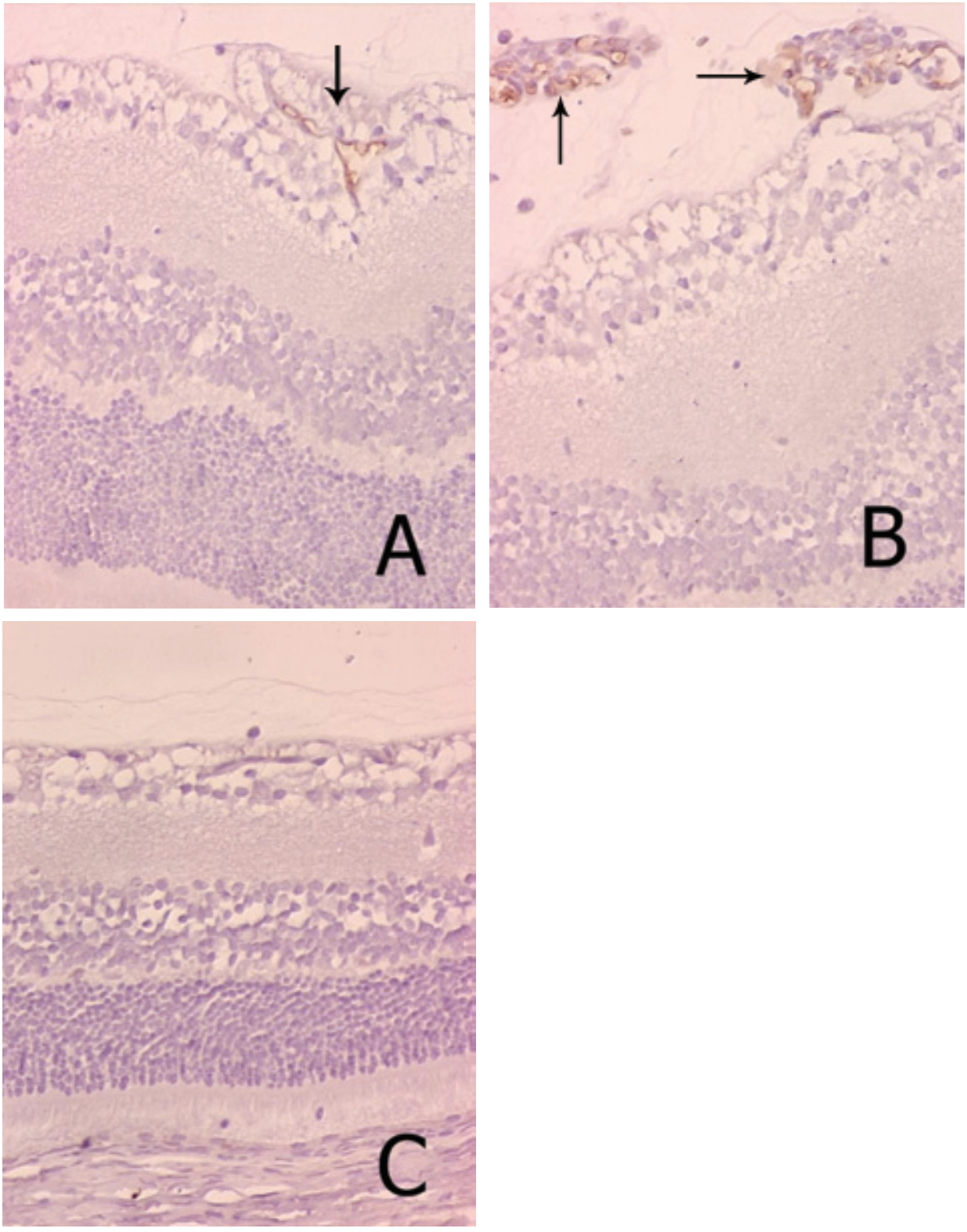

Fig. 2. VEGF immunostaining was significantly decreased in the RA-treated ROP group (A) compared to that in the saline-administered group (B). Note the negative immunostaining in the control group (C). Immunoperoxidase staining, 200x (original magnification).

Discussion

Many factors may play a role in the pathogenesis of ROP. Infants born prematurely have incompletely vascularized retinas, with a peripheral avascular zone. In these infants, administration of supplemental oxygen may lead to sustained hyperoxia, with associated vaso-obliteration and cessation of vessel growth. VEGF expression may also be downregulated. Thus, in the first stage of ROP, normal vessel growth is suppressed due to immaturity and hyperoxia. As the premature infant matures, the developing but non-vascularized immature retina becomes hypoxic and may overproduce VEGF pathologically. VEGF increases in the retina and the vitreous body. Therefore, high levels of VEGF stimulate neovascularization of the retina in the second phase of ROP. If elevated levels of VEGF persist, further neovascularization and fibrosis leading to retinal detachment may occur, however, if VEGF levels decrease, then ROP may regress (Pierce *et al*. 1996, Smith *et al*. 2003). Repeated cycles of hyperoxia and hypoxia also favor the progression of ROP (Saito *et al*. 1993, Penn *et al*. 1995a,b).

The present experimental model affords several important advantages (Penn *et al*. 1994, 1995a,b). Exposure to variable hyperoxia in an attemp to resemble more closely the arterial oxygen of preterm infants, has been shown to be a much more effective stimulus of proliferative retinopathy in the newborn rat than exposure to constant hyperoxia (Phelps 1990, Penn *et al*. 1995a,b) Raising rats in an environment cycling between 50 and 10 % oxygen results in a much higher incidence and severity of retinopathy than raising them in an 80/40 % cycle. This provides evidence that episodic hypoxia may play critical role in the pathogenesis (Penn *et al*. 1994).

This protocol produces retinal neovasculari-zation in 100 % of the eyes removed at 20 day after the six additional days in room air and in none for the first 14 days. (Zhang *et al*. 2003). Once the animals are returned to room air, the retina becomes hypoxic because of its attenuated vasculature, systemic oxygen levels return to normal, and neurogenesis progresses at some level putting demands on the ambient retinal oxygen available. At this point, the vasoproliferative stage of the disease occurs (Chan-Ling *et al*. 1995, Lutty and McLeod 2003). For this reason, in the present study, RA treatment was started on day 14 before neovascularization and continued six days in room air after variable oxygen exposure during the appearance of neovascularization at the second phase of ROP.

Although care must be taken not to overestimate the significant histological outcome of RA treatment in this study due to inclusion of relatively small number of animals, RA treatment resulted in a significant decrease of retinal neovascularization and VEGF immunostaining in the experimental model of ROP. Anti-VEGF strategies have been used successfully to inhibit neovascularization in oxygen-induced retinopathy. However, none of these strategies completely inhibits angiogenesis and has aroused significant concerns about promoting normal physiological development of both blood vessels and other tissue in preterm infants. Additionally, most of these agents were applied with intravitreal injections, which are not available in clinical use (Aiello *et al*. 1995, Robinson *et al*. 1996, Sone *et al*. 1999, McLeod *et al*. 2002, Penn and Rajaratnam *et al*. 2003.)

The retinoids are a family of natural and synthetic derivatives of vitamin A (retinol) that potently modulate cell growth and differentiation (Jones *et al*. 1997). Retinoids have been found to encourage terminal cell differentiation, to prevent cell transformation in tissue culture, and to delay the onset of cancer in experimental animals exposed to chemical carcinogens (Wan *et al*. 1999). In recent years, evidence has accumulated to show that retinoids have an additional important antitumor activity, because they inhibite tumor-induced angiogenesis (Majewski *et al*. 1995, Lingen *et al*. 1998, Ribatti *et al*. 2001, Kini *et al*. 2001, Akiyama *et al*. 2002). RA is their active metabolite that has antiangiogenic effect by inhibiting angiogenic factors, such as fibroblast growth factor-1 and 2, platelet-derived growth factor, transforming growth factor β-1, interleukin-8, and VEGF (Lingen *et al*. 1996). In a recent study, it has been shown that RA selectively blocks VPF/VEGF-induced microvascular permeability and angiogenesis and also identifies VPF/VEGF as a major target of RA action (Pal *et al*. 2000). These data suggest that RA and certain of its isomers may have a therapeutic use in inhibiting angiogenesis, particularly that induced by VPF/VEGF. To the best of our knowledge, our study is the first investigating the effect of RA treatment on retinal neovascularization and VEGF expression in an experimental model of ROP.

Several evidences also suggest that free radicals play a role in the pathogenesis of ROP (Tin *et al*. 2001, Smith 2002). Premature human infants probably have an increased susceptibility to free radical-mediated retinal injury, as all major antioxidant systems are deficient in

the immature retina (Oliver and Newsome 1992, Chen *et al*. 1999). Exposure of cells to high concentrations of oxygen leads to increased production of free radicals, which can react with cellular components causing cell and tissue injury (Wispe *et al*. 1985). Oxygen exposure also reduces retinal antioxidant levels, creating an additional burden for retinal tissues responding to oxidative stress (Penn *et al*. 1992). In animal models, products of lipid oxidation have been identified, strongly supporting a link between oxygen exposure, ROP, and free radical-mediated cellular injury (Cunningham *et al*. 2000). Although retinoids are well known as an antioxidant and the investigation of their antioxidant effect in the ROP model remains beyond the scope of our study, we think that further studies will address whether RA treatment might protect the retina from damage by oxygen free radicals generated through direct oxygen exposure.

The choice of any intervention for the prevention or treatment of ROP should be considered very carefully to promote normal physiological development of both blood vessels and other tissues in the fragile preterm baby. Several studies have been undertaken to assess whether vitamin A supplementation beyond that routinely given in multivitamin preparations can reduce the incidence of chronic lung disease. The meta-analysis suggests that supplementation with vitamin A results in benefit in terms of reducing death or oxygen consumption at one month of age and oxygen requirement at 36 weeks post-menstrual age. The meta-analysis suggests a trend towards reduced ROP incidence in vitamin A-supplemented infants, but clinical or biochemical evidence of toxicity of vitamin A-supplementation has also been reported (Darlow *et al*. 2002).

It is important to note that timing is critical to any intervention in clinical setting, since the two phases of ROP require very different approaches. Although ROP is a multifactorial disease, inhibition of VEGF early after birth can detrimentally alter normal blood vessel growth and precipitate the disease, whereas inhibition at the second neovascular phase might prevent destructive neovascularization. We conclude that RA treatment may yield benefits in ROP and may be a safety choice for potential use in the clinical practice. However, further investigations are warranted on the optimal dose and timing of RA to achieve full efficiency and sufficient safety.

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