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Assessment of serum levels of angiogenic factors in dizygotic twin infants with and without retinopathy of prematurity

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ABSTRACT

The aim of the study was to evaluate the plasma concentrations of vascular endothelial growth factor (VEGF-A), its soluble receptors VEGFR-1 and VEGFR-2, insulin like growth factor (IGF-1) and soluble Tie-2 in twin infants with and without retinopathy of prematurity (ROP). Eleven pairs of twin infants (total 22 infants) were included in the study: There were 11 infants with advanced stage ROP in the study group and 11 infants with no ROP in the control group. Before time-point measurements, the peripheral venous blood samples were first centrifuged, and the plasma was then stored at -70°C. The plasma concentrations of the angiogenic factors were measured by using high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits. The average gestational age of the infants at the time of birth and the time at which the serum samples were collected were 30.5±2.3 and 37.5±2.5 weeks, respectively. The average serum levels of all the angiogenic factors were higher in infants with ROP, but the differences were not statistically significant ($p>0.05$). In this study, serum samples of twins with and without ROP were evaluated simultaneously. The time of serum sampling in the study group corresponded to the period of phase 2 ROP. Therefore, there appeared to be no difference between the ROP group and the control group.

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1. Introduction

Retinopathy of prematurity (ROP), defined by abnormal vascular development of the vascular peripheral retina, is the most important cause of preventable blindness in premature infants. The abnormal vascularity can cause retinal-vitreal hemorrhage, retinal traction, optical disc traction, and macular or retinal detachment. Early and timely treatment is of benefit in most cases (Salvin et al., 2010). ROP is commonly treated with argon laser photocoagulation, but in some cases, this treatment may not yield any benefit. In these cases, anti-vascular endothelial growth factor (anti-VEGF) therapy may be beneficial (Salvin et al., 2010; Mintz-Hittner and Best, 2009).

In ROP, the development of pathological revascularization in the periphery of the retina is thought to occured principally due to angiogenic factors (Romagnoli, 2009). The same factors playing important roles in the stages of normal development of the retinal vasculature are likely involved with the pathological neovascularization (Biglan et al., 1986; Salvin et al., 2010; Hard and Hellstrom, 2011). Several angiogenic inhibitors have been identified which effectively inhibit pathological neovascularization, but today the effects of such antiangiogenic factors on normal and pathologic retinal vascular development are not clear.

The pathophysiology of ROP consists of two sequential phase; a hypoxic phase followed by a neovascular response. In phase 1, exposure of premature infants to hyperoxia causes vaso-obliteration and cessation of normal retinal blood vessel development. This vaso-obliteration can lead to a reduction of oxygen, especially in the peripheral retina. And, this situation bring about the production of molecules that cause the abnormal blood vessels developments. Phase 2 begins with the appearance of pathological neovascularization depending on these mediators that release from hypoxic retina. Significant changes in serum levels of vasoactive mediators may observed during the formation of ROP. Although there is observed changes in serum levels of angiogenic factors, retina and vitreous levels of these factors are changed significantly compared their serum levels. (Modanlou et al., 2006). In ROP, serum levels of VEGF and insulin like growth factor (IGF-1) are reduced in phase 1 and increased in phase 2 (Leske et al., 2006; Sylvester, 2008). Although VEGF secreted in the ischemic retina is known to play an important role in the formation of ROP, the roles of vascular endothelial growth factor receptors 2 (VEGFR-2) and vascular endothelial growth factor receptors 1 (VEGFR-1) are not clear (Budd et al., 2010). Many studies have demonstrated that VEGF regulation of IGF-1 also plays a role in the formation of ROP, but the effect of IGF-1 on ROP not clear as VEGF (Leske et al., 2006; Modanlou et al., 2006). Studies have revealed a role for IGF-1 in abnormal angiogenesis, basal membranes, and extracellular matrix degradation and migration caused by proteolysis (Grant et al., 1993). The angiogenic factor angiopoietin-2 promotes VEGF induced neovascularization (Asahara et al., 1998). Angiopoietin-2 acts by binding to the endothelium-specific receptor tyrosine kinase 2 (Tie-2), which is a soluble form of the Tie-2 receptor present in human biological fluids (Chung et al., 1993). Angiopoietin integrates with Tie-2 in the body to promote autophosphorylation of the Tie-2 receptor, vascular remodeling and maturation, maintenance of the integrity of the blood vessels, and regulation of their functions (Asahara et al., 1998; Fiedler et al., 2003). Therefore, Tie-2 may play a role in pathological neovascularization via its effect on normal vascular growth and regulation.

We investigated the potential role of serum levels of angiogenic factors (VEGF, VEGFR-1, VEGFR-2, IGF-1 and Tie-2) in the development of ROP in dizygotic twins with severe ROP requiring treatment; we used the non-ROP spouses of these dizygotic twins as a control group.

2. Material and methods

Study characteristics

This was a prospective study conducted in Ondokuz Mayis University, Ophthalmology Department that was performed in accordance with the tenets of the Helsinki Declaration. Before starting the study, approval was received from the local ethics committee. Four hundred-fifty infants who underwent routine ROP screening protocol in our clinic between May 2008 and January 2010 were included in the assessment. All babies born at or before the 34th gestational week or with a birth weight (BW) of less than 1,500 g were screened.

Twenty-five pairs of dizygotic twins were included in the sample, and 12 pairs fulfilled the criteria for inclusion in the study. One of the subjects in the non-ROP control group developed aggressive ROP (stage 3 ROP in zone 1 with plus disease) one week later; he and his twin with ROP were excluded from the study. Their results were also evaluated.

Procedure for ROP screening

Informed consent was obtained from the parents before the ophthalmological examination for ROP. The mother was instructed not to feed the baby for at least 60 minutes before pupillary dilation. Pupillary dilatation was achieved by cyclopentolate 0.5% and phenylephrine 1%. Following pupillary dilatation, 0.5% of topical proparacain hydrochloride was applied to induce topical anesthesia, and the patients were examined using a pediatric eyelid speculum. Anterior segment and pupillary examinations were performed with a pen light, and the retinal examination was performed with an indirect ophthalmoscope with a +20 D fundus lens. Scleral depression was performed as necessary to observe the peripheral retina. Topical antibiotic drops were applied to both eyes following the examination for infection prophylaxis.

The ROP examination included an assessment of pupillary dilatation, vitreous clarity, presence of plus disease, ROP stage, ROP location, and the extent of the ROP disease. ROP was classified according to the International Classification of Retinopathy (The Committee for the Classification of Retinopathy of Prematurity, 1984). The ophthalmological examination recorded the location of abnormal vasculature (zone-1, 2, or 3), the extent of the disease (by clock hours), the severity (stage 1, 2, 3, 4, or 5), and the presence of plus disease. Treatment criteria were set according to revised indications for the treatment of ROP derived from the results of the Early Treatment for Retinopathy of Prematurity Randomized Trial (Early Treatment For Retinopathy Of Prematurity Cooperative Group, 2003).

Laboratory procedure

Approximately 1 cc of peripheral blood was collected simultaneously from the twins in each group. Before the time-point measurements, the blood samples were first centrifuged, and the plasma was stored at -70°C until the assay was performed. Plasma concentrations of the angiogenic factors were measured using high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits. A human specific double-antibody ELISA (Bender MedSystems, Vienna, Austria) was used to determine the concentration of VEGF, VEGFR-1, VEGFR-2 and Tie-2. The plasma level of IGF-1 was measured using the Equine IGF-1 immunoassay (DRG International, New Jersey, USA).

Statistical analysis

After coding of the data obtained by the investigation, they were transferred to a computer and analyzed by the SPSS 15.0 packet program. Normality tests were applied for all measurement variables in the statistical analysis. Among the measurement variables, for those with a normal distribution, the t-test was used to compare the groups. For the variables that did not show a normal distribution, the Mann-Whitney U test was used. The statistical significance level was set as $p<0.05$ for all the tests.

3. Results

Both eyes of 24 infants (12 pairs of twins) were screened during the study period. The mean age at birth in both study

Table 1. General demographic characteristics

	Without ROP	With ROP	p
Number of infants	11	11	
Gender (F/M)	6/5	9/2	0.17
Birth date (week)	30.5±2.3	30.5±2.3	
Time at which the serum samples were collected (week)*	37.5±2.5	37.5±2.5	
Birth weight (g)	1339±328	1286±280	0.34

* Time at which the serum samples were collected, defined according to the gestational week of birth

groups and in the control group was 30.5±2.3 (26-31) weeks. The mean birth weight of the infants in the study group and in the control group was 1.286±280 (900-1.730 g) and 1.339±328 (1.040-1.790 g), respectively (p>0.05). When the gestational age (GA) was 37.5±2.5 weeks, approximately 7 weeks after birth, serum samples were taken in both groups.

The study group, 7 infants had threshold ROP, 4 infants had stage 2 ROP in zone 2 without plus, and 1 infant had type 1 pre-threshold ROP (stage 3 ROP in zone 1 with plus). In the control group, 9 infants had no ROP, and 3 infants had stage 1 ROP in zone 3. The retinal examination findings in the control group did not progress in the follow-up time, except one. Aggressive ROP (stage 3 ROP in zone 1 with plus disease) developed one week later in the infant; he and his twin (have threshold ROP) were excluded from the study. After one infant was removed for each group, statistical analysis was carried out with total of 22 infants. In the study group, 7 of 11 infants had a plus. There was no infant in the control group with plus disease. The demographic characteristics of the infants in the study are presented in Table 1.

Table 2. Assessment of the average serum levels of angiogenic factors in the twin infants with and without ROP

	Without ROP	With ROP	p
VEGF (pg/ml)	121.8±96.2	115.9±73.8	0.256
VEGFR-1 (ng/ml)	0.82±0.33	0.79±0.25	0.834
VEGFR-2 (ng/ml)	4.96±4.84	4.19±3.32	0.325
IGF-1 (ng/ml)	123.9±35.3	74.9±30.0	0.514
Tie-2 (ng/ml)	36.0±6.0	33.8±8.8	0.718

VEGF; vascular endothelial growth factor, VEGFR; vascular endothelial growth factor receptor, IGF; insulin like growth factor, Tie; tyrosine kinase receptor.

The serum levels of the angiogenic factors in the study group and in the control group are presented in Table 2. The mean serum levels of VEGF, VEGFR-1, VEGFR-2, IGF-1, and Tie-2 were higher in the study group than in the control group, but no parameter was found to be statistically significant (p>0.05). The results of the twin infant to exclude our study were analyzed separately and presented in Table 3. The first serum sample was taken when the gestational age 34 weeks in both infants. Serum samples were collected again at follow-up a week later. Although there was no significant change in the serum levels of the other angiogenic factors, VEGF and VEGFR-2 levels increased approximately 3-fold.

4. Discussion

Retinopathy of prematurity is an abnormal vascular proliferative disorder of the retina that occur only premature infants. Studies to elucidate the etiology of this disease have implicated several angiogenic factors (Romagnoli, 2009). The majority of the information on ROP has been obtained from experimental animal models (EAMs). However, the disease in humans differs in a number of ways. First, to generate ROP in EAMs are needed higher oxygen concentrations than human infants. Second, most EAMs use continuous oxygen, but human infants' oxygen saturation exhibits fluctuations. Such fluctuations may play a role in the development of ROP in humans (Asahara et al., 1998). Third, the retinal vasculature does not extend to the ora serrata in premature human infants, but the inner retinal vasculature is completely vascularized to the ora serrata at birth in term infants. Fourth, in EAM of ROP, to time to the formation of ROP is shorter than that seen in humans. Therefore, data obtained from the EAMs may not be applicable to humans. Conducting studies on human newborn infants is difficult in terms of ethics. Thus, our study is important because it provides information on human infants.

Angiogenesis is a complex process mediated by several factors. It is known that VEGF is the dominant proangiogenic factor, which can increase vascular permeability and promote abnormal neovascularization (E, 2012). In phase 1 ROP (hyperoxic phase), VEGF and IGF-1 levels are decreased in the serum. In phase 2 ROP (hypoxic phase), the VEGF serum level is increased. To better understand the impact of this factor on ROP, serum samples must be taken concurrently. In our study, all the factors, such as VEGF in serum samples, were obtained simultaneously.

Table 3. Serum levels of one twin infant whose results excluded from our study because of development of aggressive ROP. Change in the serum levels of the angiogenic factors one week later

	VEGF (pg/ml)	VEGFR-1 (ng/ml)	VEGFR-2 (ng/ml)	IGF-1 (ng/ml)	Tie 2 (ng/ml)
Week 34	129.39	0.764	1.338	35.33	25.6
Week 35	467.98	0.888	3.388	56.95	28.4

VEGF; vascular endothelial growth factor, VEGFR; vascular endothelial growth factor receptor, IGF; insulin like growth factor, Tie; tyrosine kinase receptor

Although the physiologic elevation of VEGF in the neonatal period decreases rapidly if oxygen saturation is normal, VEGF elevations persist if systemic hypoxia is present (Himeno, 2003). VEGF is also important for normal vascular development via VEGFR-1 induced vascular stability. Endothelial proliferation and migration of VEGFR-2 is known to cause neovascularization (Smith, 2003). Sennlaub and Chemtob (2004) asserted that VEGFR-1 activation by VEGF can prevent the formation of ROP. Kim and colleagues (2009) stated that it is effective in the inhibition of VEGFR-2. However, both studies were performed on mice. Many studies have shown that VEGFR-2 activation causes pathological neovascularization such as coroidal neovascular membrane (Gu et al., 2010), tumoral formations (Hamerlik et al., 2012), and ROP (Pieh et al., 2008). Pathological neovascularization of ROP disease is caused by VEGF via VEGFR-2 in particular. Studies on EAMs show that the serum level of VEGF and VEGFR-2 was significantly higher with retinopathy of prematurity. However, in that studies found no difference at VEGFR-1 serum levels (Sarlos et al., 2003; Stone et al., 1996). Studies performed on human premature infants with ROP have also reported an increase in the plasma level of VEGFR-2 (Pieh et al., 2008). Pieh et al., (2008) examined the

relationship between the serum levels of VEGF-A, VEGFR-1, Tie-2, and VEGFR-2 and ROP. They reported that the levels of VEGFR-2 and Tie-2 were increased, but that those of VEGF-A and VEGFR-1 were not increased significantly. In our study, compared with the control group, VEGF, VEGFR-1, and VEGFR- 2 serum levels were higher in infants with ROP. However, the difference was not statistically significant.

Another factor that is known to have an effect on vascular development of ROP is IGF- 1, but the effect on ROP is not as obvious as that of VEGF. The IGF-1 level has been reported to increase with gestational age and to play a substantial role in fetal growth and development (Machalinska et al., 2009). IGF-1 deficiency, even in the presence of normal levels of VEGF, has been shown to prevent the formation of normal blood vessels in mice (Hellstrom et al., 2001). One study has shown that the deficiency in IGF-1 levels appears to block the interaction of VEGF-activated protein kinase B, thereby affecting the continuity of the endothelial cells (Hellstrom et al., 2001). Low levels of IGF-1 prevent the development of blood vessels. As the retina continues to mature, the combination of an avascular retina and increased metabolic activity leads to hypoxia (Smith, 2003). The levels of VEGF increase with resultant retinal neovascularization. When IGF-1 levels reach a critical level, retinal neovascularization begins. In the study made by Brock et al. (2011) on the prevention of oxygen-induced retinopathy in a rat model with an IGF-I analog (JB1), they showed that early short-term exposure to systemic JB1 treatment normalizes retinal abnormalities. In addition, subjects completed retinal vascular maturation with IGF-1 having no effect on retinal neovascularization. Another study reported no significant effect of IGF-1 on diabetic proliferative retinopathy (Lee et al., 1994). From these results, the prevention of ROP depends on continued normal vascular development and sufficient levels of IGF-1 levels after birth. In our study, although the ROP group had slightly higher average serum values of IGF-1, the levels were not statistically significant between the groups with and without ROP. This is consistent with the literature data, which suggest that IGF-1 deficiency occurs in the hypoxic phase of ROP.

The VEGF and angiopoietin families of ligands are important regulators of blood vessel formation (Cai et al., 2008; Singh et al., 2009). VEGF binds to the receptor tyrosine kinases of the VEGF-receptor family to activate signaling pathways leading to endothelial migration, proliferation, and survival, whereas the angiopoietins interact with the Tie receptor tyrosine kinases to control vessel stability, survival, and maturation (Singh et al., 2009). Activation of human endothelial cells with VEGF has been reported to result in a 4-fold stimulation of tyrosine phosphorylation of Tie-2 (Singh et al., 2009). Immunoprecipitation analysis on receptors demonstrated no physical interaction between VEGF receptors and Tie-2 (Singh et al., 2009). However, Tie-2 interacts with the related receptor tyrosine kinase Tie-1, and this receptor was found to be essential for VEGF activation of Tie-2 (Singh et al., 2009). Tie-2 is a receptor tyrosine kinase (RTK) essential for aspects of both normal and pathological angiogenesis (Sturk et al., 2010). According to one study, the highest VEGF and (Angiopoietin) Ang-2 levels were seen among patients with preproliferative and proliferative retinopathy, but there was no relation between Tie-2 and the severity of retinopathy (Lip et al., 2004). Umeda et al. (2003) reported that after vitrectomy in patients with stage 5 ROP, the levels of VEGF, Ang-2, and Tie-2 were significantly higher in patients with ROP; they found no significant change in the level of Ang 1. Pieh et al. (2008) reached similar conclusions in their study. Their study included infants with and without ROP; they reported that the serum levels of Tie-2 were higher in the group with ROP. However, in our study, the serum levels of Tie-2 were not significantly different between the groups with and without ROP.

There are many limitations in our study. First, our study was performed with a small sample size. Second, in our study were taken a serum sample from the babies. In the ROP group, if the serum samples were taken before and after beginning of ROP disease, the data would be more valuable. Therefore, we might see a change before and after the disease in infants with ROP. Unfortunately, we were not performing these for ethical reasons.

5. Conclusion

In this study, we evaluated the twin premature infants with and without ROP. The levels of all angiogenic factors (VEGF, VEGFR-1, VEGFR-2, Tie-2 and IGF-1) were not statistically significant between the two groups. In the case of aggressive ROP with plus disease compared to all other angiogenic factors the level of VEGF and VEGFR-2 increase at 3-fold, so compared the other evaluated angiogenic factors these two factors are the foreground in the aggressive ROP disease.

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