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Original Articles

The Relationship Between Cytokines Serum Levels at Postnatal 4-6 Weeks and Retinopathy of Prematurity

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Abstract ***Objective:*** To investigate the relationship between cytokines serum levels at postnatal 4-6 weeks and retinopathy of prematurity (ROP). ***Method:*** Six premature infants having stage 2 or more ROP were enrolled as ROP group and four premature infants without ROP as the control group. The factors including gestational age, birth weight and gender were not significantly different in the two groups. Human antibody arrays were used, including 507 cytokines, to identify which cytokine serum levels were different in ROP group and control group at 4-6 weeks postnatal age. SPSS 16.0 was carried out to analyse the data. ***Result:*** The serum levels of 26 cytokines in ROP group are significantly different from those in the control group at 4-6 weeks postnatal age by AAH-BLG-1, including chemotacic factors, inflammatory factors etc. Eighteen cytokines serum levels were higher and 8 were lower in ROP group. ***Conclusion:*** The serum levels of cytokines at 4-6 weeks postnatal age are related to ROP, including chemotacic factors, inflammatory factors etc.

Key words Cytokines; Human antibody arrays; Retinopathy of prematurity

Introduction

Retinopathy of prematurity (ROP) that occurs in the incompletely vascularised retina of premature infants and low birth weight infants is a leading cause of childhood blindness around the world. With the rapid development of perinatal medicine in recent 20 years, the number of newborns with ROP has risen because of the increasing survival rate of low birth weight and premature infants.

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ROP is caused by many factors, first reported by Terry in 1942.[1] Many researches about the risk of ROP have shown that the gestational age and birth weight are associated with ROP, and the use of oxygen after birth is also a very important cause of ROP.[2-5]

The pathological process of ROP consists of two phases: Phase 1: hyperoxia-vasocessiondelayed normal retinal vascularisation-the formation of normal retinal vasculature ceases, even with loss of the developed vessels. Phase 2: hypoxia-vasoproliferation-new vessels pathologically grow, leading to retinal detachment and blindness. Many cytokines such as angiogenic and antiangiogenesis factors take part in both phases,[6,7] including vascular endothelial growth factor (VEGF), insulin-like growth factor1 (IGF-1), erythropoietin (EPO), pigment epithelium derived factor (PEDF), inflammatory factors, etc. In the first phase high oxygen pressure restrains angiogenic factors expression and elevates antiangiogenesis factors expression in retina, inhibiting the formation of normal retinal vessels. In the second phase the overproduction of angiogenic factors results in an uncontrolled neovascularisation, leading to retinal detachment.

At present ROP is mainly diagnosed by the routine ophthalmic examination, but routine ocular examinations cannot be carried out in primary hospitals because of some limitation posed by its characteristics. The examination is invasive and should be performed sterilely by specialised ophthalmologists. As ROP is vasoproliferative, a complex network of cytokines, extracellular matrix components, and growth factors are involved in the process of it. It is a hot issue whether serum levels of the cytokines are related to ROP. It's verified that VEGF,[8,9] IGF-1,[8,10-12] and some inflammatory factors[13-15] levels in serum after birth are associated with ROP. However, the result varies in these studies. So our study is to explore serum levels of cytokines at 4-6 postnatal weeks and their relationship with the development of ROP.

Material and Methods

Study Design and Population

Approved by Children's Hospital of Fudan University ethics committee, this case-control study was conducted at this hospital in China from March 2014 to September 2014, with written informed parental consent obtained in all cases. The inclusion criteria for .case and control subjects were infants with gestational age <32 weeks and birth weight <2000 g admitted to Children's Hospital of Fudan University. The exclusion criteria were as follows: (1) infants died before 38 weeks postmenstrual age, (2) infants without complete ROP screening examinations, (3) infants with chromosome abnormality and major congenital anomaly. The patient group consisted of 6 premature infants, and the control group consisted of 4 premature infants. Because the purpose of this study was to select the cytokines, the infants with mild ROP (including stage 1 and 2 ROP without plus) were excluded. It's ensured that there was no significant difference in gestational age, birth weight and the proportion of male infants between two groups. Data on delivery modes, and some neonatal parameters were also collected from both groups. Sepsis indicated both blood culture positive and signs of serious infection with some abnomal laboratory tests (C-reactive protein, WBC count, PLT count, procalcitonin, etc).

The Classification of ROP

The screening examination for ROP followed the guidelines proposed by the Ministry of Health in China, and the first examination was performed 4 weeks after birth or 32 weeks of corrected gestational age. Retinopathy of prematurity was classified according to the International Classification of Retinopathy of Prematurity.[16] The stage of ROP was defined as the highest stage during the ocular examinations.

Laboratory and Procedures

Blood samples were drawn at 4-6 weeks after birth. In every case, 0.5 mL of peripheral blood was extracted in a tube without additional agent. Samples were then centrifuged at 3000 revolutions per minute for 10 minutes, and the prepared serum was stored at -80°C until assay. Soluble proteins in the sera of the premature infants were measured by Biotin Label-based Human Antibody Array 1 (AAH-BLG-1, Raybiotech, United States) in accordance with the recommended protocols. This semi-quantitative multiplex array consists of 507 human proteins, including inflammatory factors, chemokines, growth and differentiation factors, angiogenic factors, adipokines, adhesion molecules and matrix metalloproteases, as well as binding proteins, inhibitors and soluble receptors to these proteins.[17] In this array, these human antibodies were immobilised in specific spot locations on glass slides. All samples were biotinylated, and then incubated in Antibody Array. Data were normalised to the positive controls in the individual slide. Raw data were collected by fluorescence detection (GenePix 4000 b, Axon Instruments, United States), and the GenePix Pro 6.0 software (Axon Instruments, United States).

Statistical Analyses

Statistical analysis were performed using SPSS statistical software, version 16.0 (SPSS, Inc., Chicago, IL, USA) for Windows. Data was presented as mean ± SD. Student's *t*-test was used to compare serum levels of cytokines in two groups when the distribution of serum levels of cytokines in two groups was normal, and Mann-Whitney U test was used when the serum levels of cytokines within two groups failed to show a normal distribution. Fisher Exact test were used for qualitative variables. A two tailed *P* value of less than 0.05 was considered statistically significant.

Results

Demographic Characteristics

This study involved 6 premature infants with ROP (ROP group) and 4 without ROP (control group). Among the 6 infants in the ROP group, One had stage 2 in zone II with plus±, one had stage 2 in zone II with plus+, one had stage

3 in zone II without plus, one had stage 3 in zone II with plus±, two had stage 3 in zone II with plus±. Only one patient (patient Number 6) in this section of our study was given laser therapy, other nine people did not require treatment and there was no difference between the two groups (P=1.0). The demographic data of ROP and control group are summarised in Table 1. There were no significant differences in gender, birth weight, gestational age, and other parameters between two groups (Table 2).

Different Biotin Signal Values of Cytokines in Two Groups

As shown in Table 3, 26 cytokines were found differently expressed in the serum between two groups at 4-6 weeks after birth (P<0.05). Eighteen cytokines levels (GM-CSF, MMP-2, AR, Endostatin, Ubiquitin, Chem R23, GDF11, TLR4, IFN-gamma, IL-17, NRG1 Isoform GGF2, BMP-6, SMDF/NRG1 Isoform, Dkk-4, IL-8, IP-10, HVEM/TNFRSF14, TGF-beta2) in serum were higher in ROP group than those in control group, and 8 cytokines levels (TECK/CCL25, VEGF-C, Lck, PF4/CXCL4, NAP-2, IL-1 R9, Tarc, PECAM-1/CD31) were lower in ROP group.

Discussion

Pathogenic pathway of ROP is characterised as abnormal retinal vascular development, and can be separated into two phases. Many vascular-related cytokines are involved in these two phases. Recently cytokines involved in ROP has become hot topics of studies on ROP, including the aspects on genetic variation of these cytokines, intraocular and systemic levels of mRNA and protein of these cytokines. However, it is only proteins that execute biological function

Table 1 General demographic data

Number	Gender	Gestational age (weeks)	Birth weight (grams)	Disease status
1	Male	29.857	1400	3 stage in zone II with plus±
2	Male	31.714	1625	3 stage in zone II with plus±
3	Female	29.143	1150	3 stage in zone II without plus
4	Female	27.429	810	2 stage in zone II with plus±
5	Male	27.286	1030	2 stage in zone II with plus±
6	Male	30.429	1350	3 stage in zone II with plus±
7	Male	29.857	1510	No ROP
8	Male	28.000	1400	No ROP
9	Female	28.857	1180	No ROP
10	Female	29.857	970	No ROP

Table 2 Demographic characteristics of the infants in this study

	ROP group, n=6	Control group, n=4	*P* value
Gestationalage, week	29.3±1.73	29.1±0.90	0.865
Birth weight, grams	1227.5±290.6	1265.0±239.8	0.837
Sex, male	4 (66.7%)	2 (50.0%)	1.000
Delivery mode, cesarean	3 (50.0%)	2 (50.0%)	1.000
Antenatal steroid	4 (66.7%)	2 (50.0%)	1.000
Surfactant at any time	5 (83.3%)	1 (25.0%)	0.190
Mechanical ventilation	3 (50.0%)	0 (0.0%)	0.200
BPD	2 (33.3%)	0 (0.0%)	0.467
Sepsis	4 (66.7%)	0 (0.0%)	0.076

BPD: bronchopulmonary dysplasia; ROP: retinopathy of prematurity

in human body. It has been demonstrated that transcription of cytokines may alter under different physiological and pathologic conditions so as to produce different types and levels of cytokine proteins. So the levels of proteins can show the condition of human body.

As is known to all, this is the first study to directly show association of so many cytokines serum levels and ROP. Cytokines are important mediators involved in the complex pathogenesis of ROP, and the abnormal expression of cytokines in serum and ocular tissue is an important risk factor of ROP. It has been confirmed that abnormal serum levels of some cytokines at postnatal 4-6 weeks predict the development of ROP in many studies. In our study we used human antibody array to discuss whether vascular genesis-related cytokines levels in peripheral circulation are different in ROP group and non-ROP group at 4-6 postnatal weeks.

It was reflected in our study that 26 cytokines levels in serum at 4-6 postnatal weeks were significantly associated with ROP. Eighteen cytokines serum levels were higher in preterm infants with ROP in comparison to those without ROP which were GM-CSF (granulocyte macrophage colony-stimulating factor), MMP-2 (matrix metallopreteinase 2), AR (amphiregulin), endostatin, ubiquitin, Chem 23, GDF11

Table 3 Different biotin signal values of cytokines in ROP group and control group

	ROP group (n=6)	Control group (n=4)	*P* value
TECK/CCL25	9.7±3.4	19.5±4.8	0.009
GM-CSF	56.5±12.6	35.4±10.2	0.010
VEGF-C	15.6±4.6	25.4±5.1	0.011
MMP-2	19.4±5.9	8.5±5.6	0.012
AR	7626±1971	4984±1095	0.013
Lck	74.3±23.1	112.6±21.2	0.015
Endostatin	43.0±23.8	14.8±10.6	0.019
Ubiquitin	37.5±15.2	16.8±11.6	0.022
Chem R23	12.6±8.6	2.7±4.2	0.022
PF4/CXCL4	341±135	528±111	0.024
GDF11	78.1±23.9	52. 5±8.1	0.024
TLR4	52.1±14.7	36.1±6.7	0.025
NAP-2	43.8±14.0	63.1±12.0	0.026
IFN-gamma	40.4±7.3	24.2±11.2	0.027
IL-17	17.3±6.6	8.6±5.5	0.030
NRG1 Isoform GGF2	33.9±8.1	19.7±10.3	0.032
BMP-6	12.0±6.1	4.6±4.8	0.034
SMDF/NRG1Isoform	37.2±9.6	17.8±14.4	0.034
IL-1 R9	19.2±11.1	36.6±13.0	0.036
Dkk-4	93.2±51.1	41.9±26.6	0.037
Tarc	11.8±4.7	21.2±7.2	0.037
IL-8	76.9±12.8	55.9±16.0	0.037
IP-10	52.2±14.6	34.6±12.1	0.038
HVEM/TNFRSF14	27.3±11.0	15.0±8.6	0.043
PECAM-1/CD31	11.8±3.8	16.6±3.6	0.044
TGF-beta 2	61.8±38.4	25.3±14.8	0.047

Data are given as mean ± SD. Serum levels (biotin signal values) of MMP-2 and TGF-beta 2 in two groups failed to show a normal distribution, so Mann-Whitney U test was used to compare serum levels of MMP-2 and TGF-beta 2 in two groups. Student's *t*-test was used to compare serum levels of other cytokines in two groups.

(growth differentiation factor 11), TLR4 (toll like receptor 4), IFN-γ (interferon-gamma), IL-17 (interleukin 17), NRG1 Isoform GGF2 (neuregulin 1 glial growth factor 2), BMP-6 (bone morphogentic protein 6), SMDF/NRG1 Isoform (neuregulin 1 sensory and motor neuron-Derived factor), Dkk-4, TGF-β2 (transforming growth factor β2), IL-8 (interleukin 8), IP-10 (interferon inducible protin-10), HVEM/TNFRSF14 (tumour necrosis factor receptor super family 14). Eight cytokines serum levels were lower in ROP group which were TECK/CCL25, PF4/CXCL4, VEGF-C (vascular endothelial growth factor C), Lck (lymphocyte protein tyrosine kinase), NAP-2 (neutrophil alkaline phosphatase 2), IL-1 R9 (interleukin 1 receptor 9), Tarc, PECAM-1/CD31 (platelet endothelial cell adhesion molecule 1).

Chemokines are a panel of proteins with low molecular weight (8-10 kDa), composed of 70-90 amino acids. Chemokines and their receptors have multiple actions. When pathogens and agents invade human organism, chemokines induce the migration of various leucocytes (including macrophages and monocytes, etc.) in combination with their receptors, regarded as mediators of inflammatory responses. Meanwhile they also play a vital role in developmental and immunologic process of human.[18] Of these 26 cytokines, serum concentration of chemokines including TECK/CCL25, PF4/CXCL4, Tarc and IP-10 was significantly different in two groups, showing serum levels of chemokines were related to ROP. This may be due to the association with inflammation and ROP. In these 4 chemokines, IP-10 can have anti-angiogenic action, inhibiting angiogenesis and vascular remodeling by decreasing VEGF and bFGF expression.[19] In clinical research, it has been found IP-10 vitreous level is significantly higher in both vascularly active and inactive ROP eyes.[20]

Inflammatory factors play important roles in physiological and pathological process of ROP. As we know, some inflammatory factors including TGF-beta, IL-4 are involved in intraocular inflammation. Some inflammatory factors including IL-1, IL-18, TNF-α have been reflected that their anti-inflammatory action is associated with pro-angiogenic role. Inhibition of TNF-alpha significantly reduces neovascularisation in a murine model of oxygen-induced retinopathy.[21] Sato et al found some inflammatory factors including IL-6, IL-7, IL-15 expressed differently in ROP and non-ROP eyes.[20] In previous study, Sood et al[13] found several cytokines remained significantly different in ROP and control group. Among these cytokines, in early time periods after birth (D0-3) IL-6 and IL-17 levels were different in patient group and control group; at D7-21 TGF-beta expressed differently among groups; IL-18, neurotrophin-4, C-reactive protein expressed differently among groups in both early and later time periods. Other studies also indicated that the levels of inflammatory factors in peripheral circulation were related to ROP.[22,23] Our study showed a coordinated pattern of 8 higher serum cytokines levels and 2 lower serum cytokine levels at 4-6 weeks postnatal age in ROP group compared with non-ROP group; GM-CSF, IL-17, IL-8, TGF-beta 2, TNFRSF14, IFN-gamma, TLR-4, and Chem R23 serum levels in ROP group were higher, IL-1 R9 and NAP-2 serum levels in ROP group were lower. This suggested that inflammatory factors may be involved in the pathogenesis of ROP.

Matrix metalloproteinase 2, a leading member of MMPs family, is the most widely distributed. MMP-2 plays important roles in inflammation processes and in the regulation of endothelial cell migration and extracellular matrix remodeling during neovascularisation. However, in the process of angiogenesis, the formation of new blood vessels begins with the disruption of the endothelial cell basement membrane and extracellular matrix, and then continues with endothelial cell migration and extracellular matrix remodeling. So MMP-2 is involved in angiogenesis and has an important function in this process. Hoffmann et al showed VEGF could stimulate MMP-2 secretion.[24] In mice of oxygen-induced retinopathy without MMP-2 gene, retinal neovascularisation was significantly reduced.[25] Then Barnett et al[26] found that when intravitreal injection of MMP-2 inhibitors after variable-oxygen exposure, retinal neovascularisation in rat would be inhibited to some degree, and suggested that MMP-2 had a vital role in retinal neovascularisation and the suppression of MMP-2 expression could offer an innovative strategy for ROP. In this report, it was demonstrated that serum levels of MMP-2 were higher in ROP patients than in non-ROP patients, reflecting serum levels of MMP-2 is likely associated with ROP.

Belonging to the family of epidermal growth factors, AR can activate intracellular signal way through interactions with its receptor which has pleiotropic roles such as the regulation of cell proliferation in the mature of mammary gland and the bone. Its elevated expression is associated with inflammation and neoplasia. It has been found that AR can regulate the expression of VEGF in angiogenesis, and AR-siRNA can down-regulate VEGF protein expression.[27] In our research, it is demonstrated that serum levels of AR were higher in ROP patients than in non-ROP patients, indicating serum levels of AR at postnatal 4-6 weeks are possibly associated with the development of ROP.

Lck is a transmembrane protein and a member of Scr protein kinase family, produced by T cell and natural killer cell. It has been shown the disruption of the Lck gene produces serious consequences on the retina in mice and the retinal pathology is similar to ROP.[28] In our study the serum concentration of Lck was lower in patients with ROP compared to patients without ROP, and this result accorded with that in animal study.

As an endogenous anti-angiogenic factor, endostatin can significantly inhibit the proliferation and migration of endothelial cell. It has been confirmed as an effective inhibitor of angiogenesis in tumour in animal and clinical study.[29] The serum level of endostatin is related to the prognosis of some tumours.[30] It acts through inhibiting the tyrosine phosphorylation of VEGF receptor. In ocular diseases, intravitreal injection of endostatin results in decreased expression of VEGF, reflecting endostatin may be a novel therapeutic drug for diseases characterised by retinal neovascularisation.[31] In our study the serum levels of endostatin were higher in ROP group than non-ROP group. This result may be due to body protection by itself.

Other cytokines which are NRG1 Isoform GGF2, SMDF, PECAM-1, VEGF-C, BMP-6, GDF11, DDK and Ubiquitin are not discussed here, as no significant relevance are found to the formation of new vessels and ROP in previous studies.

The serum levels of some cytokines such as VEGF, IGF-1 and other inflammatory factors that have been reported to have strong association with ROP in literature were not demonstrated to be related to ROP. This may be due to a small sample size in our study, so future research needs to be based on a larger sample size.

The sample size is very small, and it is a limitation for our study to draw a definitive conclusion. Future research including a larger number of cases with severe ROP, mild ROP and cases without ROP is required.

In summary, the research has shown a coordinated pattern of higher and lower serum levels of 26 cytokines at postnatal 4-6 weeks in premature infants with ROP compared with premature infants without ROP. The demonstration of the significant association between ROP and these cytokines serum levels at postnatal 4-6 weeks not only offers a window of opportunity to explore the aetiology of ROP, but also finds a way for studies on the diagnosis and treatment of ROP.

Competing Interests

The authors declare no conflict of interest.

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