·Basic Research·

Expressions of survivin and vascular endothelial growth factor in a Murine model of proliferative retinopathy

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Abstract

• AIM: To examine the expression of survivin and vascular endothelial growth factor (VEGF) during the development of retinal neovascularization (NV) in a mouse model.

• METHODS: A well-characterized murine model of retinal NV was used to study the expression of survivin and VEGF. NV of the retina was induced in mice by exposure to 75% O_2 from postnatal day P7 to P12, followed by return to room air from P12 to P17. Expression of survivin and VEGF protein was analyzed by Immunohistochemistry. In addition, mouse model of proliferative retinopathy was analyzed by retinal fluorescein angiography and quantification analysis.

• RESULTS: The normal mice had both superfiekal and deep vascular layers that extended from the optic nerve to the periphery. In intraocular pressure (IOP) mice were characterized by represent a typical pattern of pathological retinal NV. There are less or little nuclei of new vessels vascular endothelial cell breaking through the inner retinal than in retinopathy of prematurity (ROP) mice, large clusters of blood vessels were adherent to the internal limiting membrane(ILM) (0.27± 0.20 *vs*23.38± 1.027, t=9.454, P< 0.001). During the angiogenic period from P13 to P17, survivin and VEGF protein expression increased in experimental retinas compared with control samples (2.56± 0.46 *vs* 3.34 ± 0.40, t =17.43, P <0.01: 2.18 ± 0.75 *vs* 4.34± 0.25, t=19.61, P<0.01). Protein levels of VEGF and survivn has significantly positive correlation(P<0.05, r=0.411).

• CONCLUSION: Correlation was made at the protein levels of survivin expression compared with that of VEGF in a murine model of retinal NV, which suggests a temporal role for survivin and VEGF in new vessel formation in response to hypoxic stimulation.

• KEYWORDS: retinal neovascularization; survivin; vascular endothelial growth factor
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INTRODUCTION

Retinopathy of prematurity (ROP) in laboratory animals is a widely used method to study diabetic retinal microvascular complications, because as proliferative diabetic retinopathy, it is characterized by hypoxia-induced retinal angiogenesis[1-4].

Several pathogenetic factors are implicated in the development of both proliferative diabetic retinopathy and ROP and include vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), connective tissue growth factor, and angiotensin II [5-7]. However, despite blockade of these cytokine pathways in ROP, complete prevention of retinal angiogenesis does not always occur [4, 6]. This has led to a search for other factors that may participate in formation of new blood vessels in diabetic retinopathy and other angiogenesis associated retinal diseases.

Inhibition of apoptosis may be involved in the pathogenesis of cancer by prolonging cell life and facilitating retention of deleterious mutations. Several inhibitors oapoptosis related to the baculovirus inhibitors of apoptosis (IAP) gene have been identified [7]. Survivin is unique for its expression in fetal tissue and in a variety of human cancers [8,9]. Survivin is involved in the regulation ocellular proliferation and angiogenesis in cancer [10,11]. Remarkably, increased survivin expression has been observed in the most common human neoplasm, including oesophageal cancer [13], ovarian carcinoma [14]. Most of these studies have demonstrated a positive correlation between survivin expression and poor prognosis of the disease. In this study, we assessed the expression of survivin and VEGF in NV and their correlations.

MATERIALS AND METHODS

Materials

Mouse model of proliferative retinopathy Litters of C57Bl/6J mice were placed with their nursing mothers in an incubator maintained at 75%±2% oxygen from postnatal day (P)7 to P12, as described previously [15]. Oxygen levels were continuously monitored using a portable oxygen analyzer. At P12, mice were removed from the incubator to room air (n= 14). Control litters were maintained in room air only (n= 14). Mice were killed at P17(n=10 per group),and both eyes were immediately enucleated and either fixed for histology.

Methods

Retinal fluorescein angiography and visualization of retinal vascularization Animals were anesthetized and perfused with pluorescein via intraventrice injection of 50g/L of 2×10^6 molecular weight fluorescein isothiocyanate-dextran (Sigma,.St. Louis, MO) (n=4 per group). The animals were immediately killed. The eyes were enucleated and fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 minutes. The retinas were then isolated from the eyecup and fixed with 4% paraformaldehyde for 3 hours. The retinas were flat-mounted on a gelatin-coated slide. The vasculature was then examined under a fluorescein microscopy.

Quantification of neovascular proliferative retinopathy Mice were sacrificed and the eyes were enucleated, immersed in 4% paraformaldehyde in PBS for at least 24 hours, and embedded in paraffin. Serial sections (6m) of whole eyes were cut sagittally through the cornea and parallel to the optic nerve and stained with hematoxylin. Approximately 20 serial sections were cut down from each eye. Between two and four sections on each side of the optic nerve, 30m to 90m apart, were counted for neovascularization, cross-sections that included the optic nerve were excluded. Vascular cell nuclei were considered to be associated with new vessels, they were found on the vitreal side of the internal limiting membrane.

Immunohistochemistry for survivin and VEGF Ten sections of four groups were randomly chosen from each mouse (n = 10 mice per group). According to the SABC, the protein expression of VEGF and Survivin were method by the immunohistochemisty. The sections incubated with PBS instead of the primary antiserum were used as the negative control. The positive cells of VEGFand Survivin were light yellow or dark brown in the cytoplasm. The integrated A of VEGF and Survivin were analyzed on computer (The antibodies should be provided by BOSTER).

Statistical Analysis All values were expressed as mean ± SEM. All analyses were performed with appropriate software (SPSS). Comparisons between groups were performed by use of an unpaired Student' s t test. At the meantime,correlation analysis between VEGF and survivin was performed by use of an liner correlation. P<0.05 was considered statistically significant.

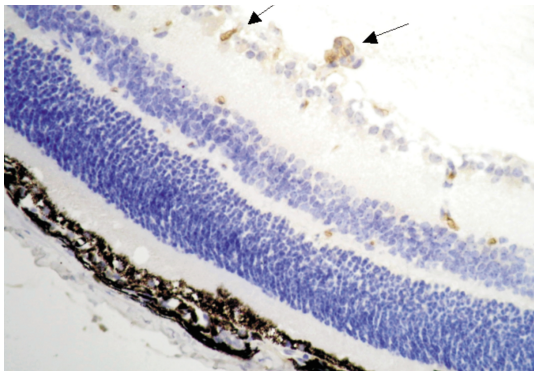

Figure 1 Immunohistochemistry staining of survivin of oxygen–treated group There are protein expression of survivin in the ganglion cell layer and the neovascularization breaking through the inner retinal (arrows noted DAB×400).

RESULTS

Hyperoxia –Induced Proliferative Retinopathy The pattern of vascular development and neovascularization were seen readily in retinal flat-mounts after fluorescein-dextran perfusion.The normal untreated mice had both superfiekal and deep vascular layers that extended from the optic nerve to the periphery. The retinal vascular patterns in the mice exposed to hyperoxia was characterized by the neovascular tutes, non-perfusion regions, microaneurism and hemorrhage that represent a typical pattern of pathological retinal neovascullariza-tion.

Quantification of Proliferative Retinopathy The degree of hyperoxia-induced neovascularization was quantified in serial paraffin cross-sections by counting the number of vascular cell nuclei on the vitreal side of the internal limiting membrane. In normal untreated mice, there are less or little nuclei of new vessels vascular endothelial cell breaking through the inner retinal than in ROP mice,large clusters of blood vessels were adherent to the internal limiting membrane (ILM) (0.27±0.20 vs 23.38±1.027, t=9.454, P<0.001).

Survivin and VEGF Immunohistochemistry In normal mice, there is no protein expression of survivin in each layer tissue of retinal . In ROP mice, there are protein expression of survivin in the ganglion cell layer and the neovascularization breaking through the inner retinal (2.56± 0.46 vs 3.34±0.40, t=17.43, P<0.01) (Figure 1). The protein of the VEGF is expressed in the outer nucler layer, less in ganglion cell layer and some cells of inner nuclear lay er in normal mice. In ROP mice, VEGF is expressed in the inner nuclear layer, the ganglion cell layer and the neovascularization breaking through the inner retinal (2.18± 0.75 vs4.34±0.25, t=19.61, P<0.01) (Figure 2) .

Correlaion Analysis There is significantly positive correlation between expression levels of VEGF and survivn protein (P<0.05, r=0.411).

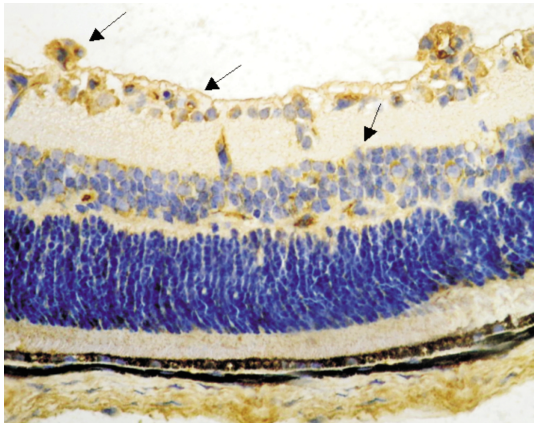

Figure 2 Immunohistochemistry staining of VEGF of oxygen-treated group VEGF is expressed in the inner nuclear layer, the ganglion cell layer and the neovascularization breaking through the inner retinal (arrows noted DAB×400).

DISCUSSION

This study demonstrates that expression of survivin is consistent with expression of VEGF in NV.Survivin is the smallest member of the Inhibitor of Apoptosis (IAP) gene family [16]. Originally described as cell survival factors that target caspases, we now know that IAPs have a much broader portfolio of functions, encompassing signaling pathways, cell division, metabolism and adaptation to unfavorable environments [16]. These 'survivin networks' are dramatically exploited in cancer, and survivin is unanimously viewed as one of the most prominent cancer genes [17]. Overexpressed in virtually every human tumor, survivin expression has been consistently associated with disease progression, metastatic dissemination,

Survivin, VEGF, Bcl-XL and Hsp27 have been shown to be elevated following the hypoxic episode and all these proteins have been reported to help protect cells by inhibiting the processes leading to apoptotic death.

Survivin is a member of the inhibitor of apoptosis protein family (IAP family) and is reported to diminish apoptosis by interfering with the activity of caspase-3, caspase-7, and caspase-9 [18-21]. Additionally, Survivin can increase cell survival through its effects on mitosis and cell cycle progression[22-26] .

In this study, we found that the protein expression of survivin in retinal tissues was positively correlated with formation of the NA. Over-expression of survivin may play some roles in NA pathogenesis. As mentioned above, survivin expression was previously reported to significantly correlate with poor prognosis in a range of malignant tumors, such as colorectal cancer [27], bladder cancers [28], lymphoma[29], soft-tissue sarcomas[30]. However, so far, there were only few reports about the correlation of survivin expression in NA. Our results showed that in normal mice, there is no expression of survivin in each layer tissue of retinal. It is consistent with overexpressed in virtually every human tumor, but undetectable or present at very low levels in most normal adult tissues [31]. Accordingly, survivin promoter activity is basically silent in normal cells, but strongly expressed in tumor cells[32].

In ROP mice, there are expression of survivin in the ganglion cell layer and the neovascularization breaking through the inner retinal. The protin expression of VEGF are loated in the inner nuclear layer,the ganglion cell layer and the neovascularization.The space of the experssion is more extensive than the expression of survivin.It is indicated that there are not only autocrine but also paracrine of the VEGF in the formation of the NV. It is conformity with mentioned earlier that VEGF has been reported to have anti-apoptotic properties by up-regulation of Survivin, Akt and Bcl-2, key anti-apoptotic proteins[33-35].

VEGF is considered to be the most cardinal vascular growth factor prompting retinal angiogenesis. We demonstrate further that both VEGF addition and survivin overexpression can promote the formation of NV by preserving the microtubule network. As such, survivin induction by VEGF may ensure the integrity of microtubule dynamics.These results strengthen the rationale for targeting EC survival pathways to enhance the pathologic NV.

Furthermore, we found that over-expression of survivin was significantly positively correlated with over expression of VEGF in retinal NV. These results indicated that co-analysis of VEGF and survivin protein expression in retinal NVtissues was more valuable for prognosis evaluation of NV disease. To our knowledge, the present data firstly provide a compelling case confirming a correlation among the survivin expression, VEGF expression and the emerge of NV.

Previous studies also reported that survivin as one of the target genes induced by VEGF in endothelium, which was associated with prominent up-regulation of survivin in newly formed blood vessels during angiogen-esis *in vivo*[36]. Taking together, we assume that in NV, over-expression of suvivin and VEGF, enhance retinal angiogenesis, EC cell infiltration and invasion, and inhibit apoptosis of EC cells, result in poor prognosis. However, further investigation about this is needed.

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