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Effects of endostatin in proliferative diabetic retinopathy

Hiroshi OZAKI[1], Yoshiya KATSURA[1], Masayuki NORITAKE[1], Kazuhisa MATSUNO[2], Tadashi OKANO[2], Hiroshi KOSANO[3], Hideo NISHIGORI[3], Takeshi MATSUOKA[1]

[1]Fifth Department of Internal Medicine, Tokyo Medical University
[2]Department of Ophtalmology at Kasumigaura Hospital, Tokyo Medical University
[3]Faculty of Pharmaceutical Sciences, Teikyo University

Abstract

Aims : To determine whether endostatin is elevated in the vitreous fluid of patients with proliferative diabetic retinopathy (PDR).

Methods : Vitreous fluid samples were obtained at the time of vitreoretinal surgery from 27 eyes of PDR patients and from 16 eyes of non-diabetic patients (control subjects). All control subjects had been diagnosed with macular holes (MH) but without any associated vascular proliferation. Endostatin concentration in the vitreous fluids was determined by a competitive enzyme immunoassay.

Results : Intravitreous concentrations of endostatin (median [range]) were significantly higher ($p<0.0001$) in diabetic patients with PDR (26.47 [2.31-52.06] ng/ml) than in control patients (9.36 [1.95-17.75] ng/ml). The ratio of endostatin concentration (expressed in ng/ml) to vitreous protein concentration (expressed in mg/ml) was also significantly elevated ($p<0.0001$) in the PDR patients (4.57 [0.82-9.70] ng/mg protein) when compared with the control group (1.64 [0.72-3.60] ng/mg protein).

Conclusions : We found that levels of endostatin in vitreous fluid of PDR patients are significantly higher than those in non-diabetic patients. This evidence suggests that endostatin, one of the angiogenic inhibitors, may play a role in the neovascularization of PDR.

Introduction

Diabetic retinopathy is a major cause of blindness when it progresses to the stage of proliferative diabetic retinopathy (PDR) characterized by intraocular angiogenic disease. There is considerable evidence that various growth factors are involved in initiating and perpetuating the neovascularization process of PDR. PDR has been shown to be associated with increased levels of various growth factors within the vitreous, including basic fibroblast growth factor (bFGF)[1], insulin-like growth factor type I (IGF-I)[2,3], hepatocyte growth factor (HGF)[4] and vascular endothelial growth factor (VEGF)[5,6].

In 1996, Hanahan et al proposed that the angiogenesis could be regulated both by inducers and inhibitors of endothelial cell proliferation and migration. Moreover, the balance of the angiogenic inhibitors and inducers appear to govern the angiogenic switch[7]. They suggested that a net balance of inhibitor over inducer maintains the switch in an off position and a shift to an excess of activating stimuli turns on angiogenesis[3].

Endostatin, originally isolated from the medium of hemangioendothelioma cells, is generated from collagen

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Reprint requests to : Hiroshi Ozaki, Fifth Department of Internal Medicine, Tokyo Medical University, 3-20-1 Chuo, Ami-machi, Inasiki-gun, Ibaraki, Japan 300-0395. Tel 0298-87-1161 ext 1128. Fax : 0298-88-3463

XVIII through cleavage by proteases, and potently inhibits angiogenesis and tumor growth *in vivo*[8]. From the evidence that collagen XVIII is localized mainly in a perivascular position around blood vessels[8], it has been speculated that endostatin acts as a powerful endothelial inhibitor within protein localized to blood vessels and provides a regulatory mechanism for vessel growth.

In the past, PDR has been studied exclusively from the angiogenic point of view. However, we believe that it is also important to investigate the inhibitory factor(s) for the angiogenic process. Since collagen XVIII is abundant in basal laminae of the retina[9], there is a possibility that endostatin plays a specific role in the pathophysiology of PDR in relation to the balance of angiogenic factors and inhibitors.

In the present study, we attempted to measure the concentration of endostatin in vitreous fluid from PDR patients and discuss the relationship between endostatin and other angiogenic factors such as HGF and VEGF.

Patients and Methods

All patients gave prior informed consent. Twenty-seven vitreous fluid samples from PDR patients and 16 vitreous fluid samples from non-diabetic ocular disease patients (MH) were obtained at random. The mean age of the diabetic patients (21 men and 6 women) was 53.6±10.3 years (mean±SD) and the group had a mean glycosylated hemoglobin (HbA1c) of 7.11±2.0 %. The mean age of the 16 patients with MH (7 men and 9 women) was 64.7±7.0 years. Undiluted vitreous fluid samples (50-100 μl) were collected at the start of vitreoretinal surgery and frozen at −80°C until required for laboratory analysis. Before the vitreoretinal surgery, photocoagulation was performed on all the PDR patients except one. The stage of PDR was classified as active (19 eyes) if there were perfused preretinal new capillaries or quiescent (8 eyes) if the vasoproliferation consisted of only large vessels within the membranes at surgery. Before analysis, the vitreous samples were thawed and divided into aliquots containing appropriate volumes for subsequent assays and then immediately refrozen at −80°C. Aliquots for individual assays were thawed and used in the assay.

Concentrations of endostatin in vitreous samples were measured using a competitive enzyme immunoassay for the detection of total human endostatin (Cytimmune, College Park, MD, USA). The assay was carried out according to the manufacture's instruction with some modifications. Briefly, 40 μl of undiluted vitreous fluid was diluted with 60 μl of assay diluent and then analyzed. Precoated goat anti-rabbit endostatin polyclonal antibody was used to capture a specific endostatin complex in each well that consisted of endostatin antibody, biotinylated endostatin and the samples. The detection limit of this assay was 1.95 ng/ml. For data processing, we allocated the minimum value detected by enzyme-linked immunosorbent assay (ELISA) to all samples with concentrations below the detection threshold.

Concentrations of HGF and VEGF in vitreous fluid were measured by ELISA for HGF (Otsuka, Tokushima, Japan) and VEGF (Amersham, Buckinghamshire, UK) as previously described[4]. Protein in vitreous fluid was determined using a Pierce protein assay kit (Pierce, Rockford, IL, USA)

Statistical analysis

The Mann-Whitney U test was used to compare intravitreous concentrations of protein, endostatin, VEGF and HGF. Correlations between intravitreous concentrations of endostatin, HGF and VEGF were examined by Spearman's rank correlation test. A p value of less than 0.05 was considered to indicate a statistically significant difference.

Results

Intravitreous concentrations of endostatin (median [range]) were significantly elevated ($p<0.0001$) in samples from PDR patients as compared to samples from MH patients (26.47 [2.31-52.06] versus 9.36 [1.95-17.75] ng/ml) (Fig. 1A). The ratio of endostatin concentration (expressed in ng/ml) to protein concentration (expressed in mg/ml) was significantly elevated ($p<0.0001$) in samples from PDR patients as compared to samples from MH patients (4.57 [0.82-9.69] versus 1.64 [0.72-3.60] ng endostatin/mg protein) (Fig. 1B). However, there was no significant difference between vitreous concentrations of endostatin in active PDR patients and quiescent ones (Fig. 2A, B).

Intravitreous concentrations of VEGF and HGF were significantly elevated in samples from PDR patients as compared to samples from MH patients. Intravitreous protein concentration (median [range]) showed no significant difference between diabetic patients with PDR (5.28 [0-14.41 mg/ml]) and the control group (4.76 [0.79-7.32 mg/ml]). Also in the samples from PDR patients, there was no significant relationship between intravitreous concentrations of endostatin and VEGF ($p=0.092$) (Fig. 3A) or HGF ($p=0.78$) (Fig. 3B).

There was only one case without photocoagulation before surgery. The intravitreous concentration of endostatin was 16.67 ng/ml and we did not consider it an exception.

Discussion

In this study, we report for the first time that the level

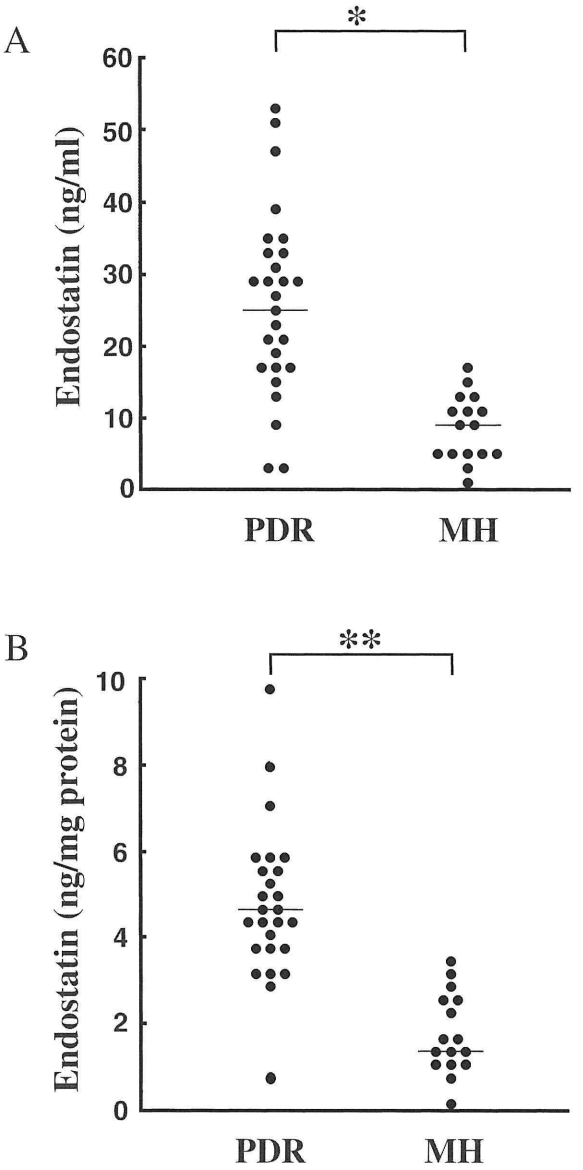

Fig. 1 A : Endostatin concentrations in the vitreous body of patients with PDR and of those with PDR and of those with MH. *P<0.0001. B : Endostatin concentrations per milligram of protein in the vitreous body of patients with PDR and of those with MH. **P< 0.0001. The horizontal lines refer to median values.

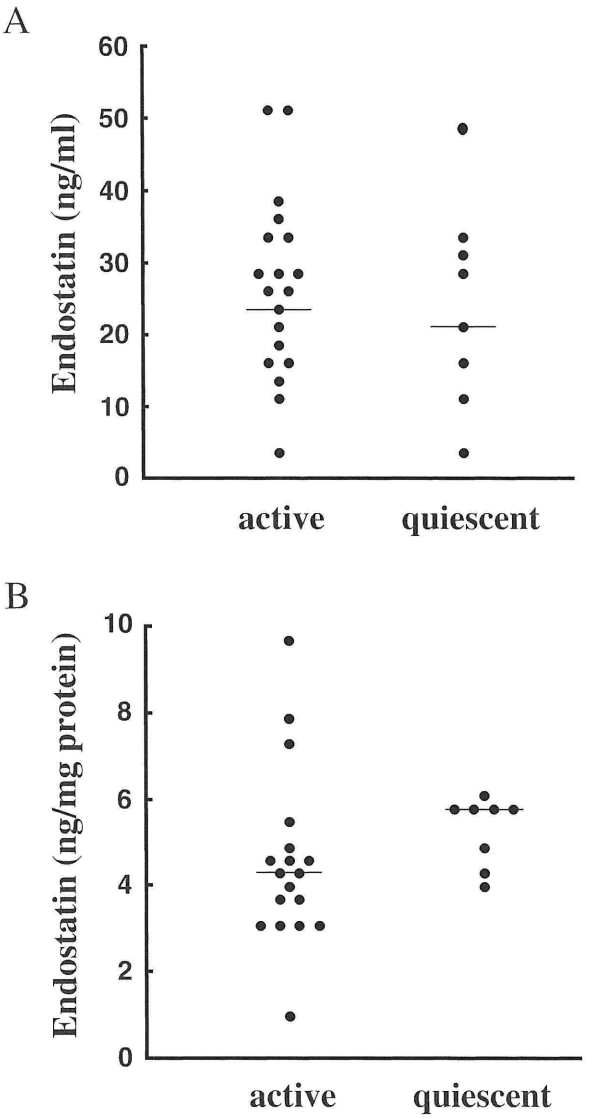

Fig. 2 A : Endostatin concentrations in the vitreous body of patients with active or quiescent PDR. B : Endostatin concentrations per milligram of protein in the vitreous body of patients with active or quiescent PDR. The horizontal lines refer to median values.

of endostatin is significantly higher in vitreous fluid of patients with PDR versus those with MH. There are many studies that have reported on the documented roles angiogenic factors play in the PDR process[1–6]. However, angiogenesis can be regulated not only by inducers but also by inhibitors of endothelial cell proliferation[7]. Studies of tumor growth show that various factors play a role in initiating and promoting angiogenesis. For example, these processes are controlled by a variety of endogenous inhibitors, such as angiostatin[10], thrombospondin[11], platelet factor 4[12] and endostatin. Based on our results, we propose that there is a new aspect of neovascularization that needs to be considered in PDR.

The main question that needs to be answered is why endostatin, an angiogenesis inhibitor, is elevated in vitreous fluid with PDR, an intraocular angiogenic disease. One possibility is that the inhibitor is produced as a reaction product against the angiogenic stimulation. The angiogenic inhibitor may be produced in the response to excessive angiogenesis but the activity of endostatin is not sufficient to restrict the angiogenesis in PDR. A second possibility is that the proteolytic activity that accompanies neovascularization in PDR may also mobilize angiogenesis inhibitors from precursor proteins that are not inhibitory themselves. However, the produced endostatin may have some effect on angiogenesis. Angiogenesis in PDR is not induced

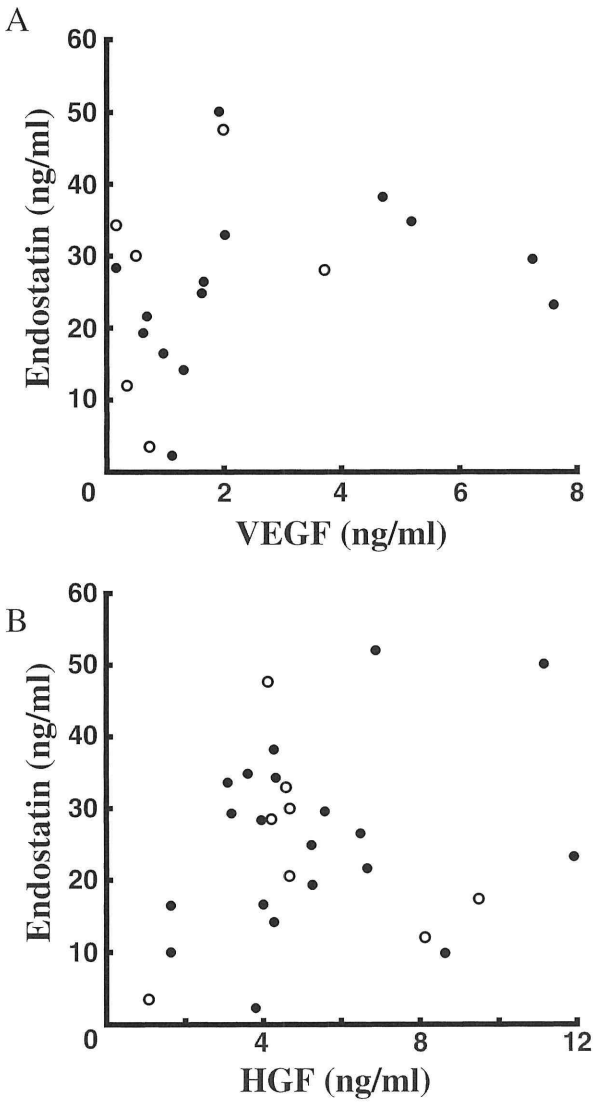

Fig. 3 A : The relationship between the vitreous concentration of endostatin and VEGF in patients with active PDR (black circles) and quiescent PDR (white circles). B : The relationship between the vitreous concentration of endostatin and HGF in patients with active PDR (black circles) and quiescent PDR (white circles).

by the depletion of endostatin, an angiogenic inhibitor. It is likely that it is induced by the dominance of an angiogenic stimulator, which is in greater concentrations than the increased levels of angiogenic inhibitor that occurs.

Endostatin is a C-terminal fragment of collagen XVIII derived through cleavage of the Ala-His linkage. Wen et al. reported that the generation of endostatin from collagen XVIII has at least two steps, a metal dependent early step and an elastase activity-dependent final step[13]. Matrix metalloproteinases (MMPs) are considered to be able to process collagen XVIII into an endostatin precursor (e.g. NC1). Elastase activity is then involved in the cleavage of NC1 to form endostatin. Felbor et al. proposed that cathepsin L generated endostatin directly from collagen XVIII[14].

Collagen XVIII is the core protein of a heparan sulfate proteoglycan in vascular and epithelial basement membranes. Collagen XVIII has been reported to be abundant in the basal laminae of epidermis, pia, cardiac and striated muscle, kidney, lung, blood vessels and retina[9]. It has also been found that there is a 300 kDa collagen XVIII in the chick vitreous body and that collagen XVIII is present in the inner limiting membrane of the retina, the pigment epithelium and the lens capsule of the chick[9]. Therefore it is possible that the neovascularization in PDR occurs in a milieu that is abundant in areas that contain collagen XVIII. Angiogenesis is an invasive process that requires controlled release and activity of extracellular proteases. We have previously reported that the expression of MMP-9 activity in the vitreous fluid of PDR patients was significantly higher than that for non-diabetic patients[15]. Hence there is a possibility that the elevated MMP-9 in vitreous fluid with PDR affects collagen XVIII degradation and produces endostatin to prevent neovascularization. In this study, we measured the MMP-9 activities of vitreous fluid with PDR or MH. However, we could not find any significant relationship between the levels of endostatin in vitreous fluids in PDR cases where MMP-9 was positive and where MMP-9 was negative (data not shown).

Moreover, we also found no relationship between vitreous concentrations of endostatin and VEGF or HGF in PDR patients. This finding may suggest that the neovascularization caused by VEGF or HGF does not induce endostatin production directly.

The mechanism of endostatin action is still unknown. A possible mechanism is competition for the heparin/heparan sulfate binding sites, which act as co-receptors for several cytokines, such as FGF-2 and VEGF[16,17]. PDR has been shown to be associated with increased intravitreous levels of FGF[1] and VEGF[5,6]. On the other hand, it has also been reported that endostatin causes apoptosis of endothelial cells *in vitro*[18]. Based on our results, endostatin in vitreous fluid with PDR could have a role in the neovascularization process.

It has been reported that some angiogenic inhibitor have some roles in PDR. The concentrations of pigment epithelium derived factor in patients with PDR were lower than in control[19]. On the other hand, angiostatin was more frequent in the vitreous of PDR patients than in those of control[20]. Recently, Funatsu et al. reported that mean vitreous level of endostatin in diabetic patients was not higher than their mean level in nondiabetic patients and that the vitreous level of VEGF was significantly correlated with the vitreous level of endostatin[21]. These results are different from ours. However, the mean concentrations of vitreous Endos-

tatin and VEGF in our study were 25.9 ng/ml and 2444 pg/ml, and those in the study by Funatsu were 7.24 ng/ml and 812 pg/ml. They also stated that vitreous endostatin levels significantly correlated with the clinical severity of diabetic retinopathy. The reason of these differences may be attributable to the PDR stages of study patients. Further investigations for collagen XVIII and protease are required to elucidate the products of endostatin and the roles of angiogenic inhibitors in PDR.

Conclusion

In the present study, we found that the concentration of endostatin in the vitreous fluid was significantly higher in PDR patients than in control patients. Angiogenesis is thought to be regulated by both inducers and inhibitors of endothelial cell proliferation and migration. Further research into the mechanism of endostatin production and its role in vitreous fluid of PDR patients is needed to clarify the mechanisms.

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Endostatinの増殖糖尿病網膜症における役割

尾崎浩史[1)] 桂善也[1)] 則武昌之[1)]
松野員寿[2)] 岡野正[2)] 小佐野博史[3)]
西郡秀夫[3)] 松岡健[1)]

[1)]東京医科大学内科学第五講座
[2)]東京医科大学霞ヶ浦病院眼科
[3)]帝京大学薬学部薬物治療学教室

【要旨】 Endostatinはコラーゲン XVIIIのC末端フラグメントであり、腫瘍性の血管新生を特異的に抑制する作用をもつと言われる。腫瘍性の血管新生はVEGFなどの増殖促進因子とEndostatinなどの抑制因子とのバランスによって制御されているとの説が有力である。他方、血管新生が病態に深く関与するとされる増殖糖尿病網膜症 (以下PDR) の発症進展にはVEGFなどの増殖因子・サイトカインの関与が報告されており、Endostatinの血管新生抑制作用がPDRの病態にも関与することが考えられる。このためPDRにおける硝子体液中でのEndostatin増加の有無を検討した。方法はPDR群27例、黄斑円孔 (以下MH) 群16例より眼科手術中に硝子体液を採取し、Endostatin濃度、VEGF濃度、HGF濃度を測定した。硝子体液Endostatin濃度はPDR群26.47 [2.31-52.06] ng/ml、MH群9.36 [1.95-17.75] ng/mlとPDR群で有意に高値を示した ($p<0.0001$)。さらに硝子体液蛋白濃度で補正したが同様にPDR群で有意に高値を示した (PDR群4.57 [0.82-9.70] ng/mg protein、MH群1.64 [0.72-3.60] ng/mg protein) ($p<0.0001$)。今回、硝子体液Endostatin濃度がPDRで高値を示すことが明らかとなったことから、EndostatinがPDRの病態に関与する可能性が示唆された。PDRにおける血管新生も腫瘍性血管新生と同様に、促進と抑制のバランスで制御されていることが考えられる。

〈**Key words**〉 Endostatin、血管内皮増殖因子、血管新生、糖尿病網膜症