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Expression and Possible Roles of Activin A in Proliferative Vitreoretinal Diseases

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Purpose: To examine the expression of activin A in eyes and to determine the possible functions of activin A in proliferative vitreoretinal diseases.

Methods: The activin A concentration in vitreous specimens obtained from eyes with or without retinal ischemia was measured by a bioassay using erythroid differentiation factor effects of activin A. The expression of activin A and activin receptors in the preretinal membranes was observed by immunohistochemical analysis.

Results: The mean concentration of activin A in the eyes with proliferative diabetic retinopathy was 1.50 ± 1.27 ng/mL (mean ± SD; n = 10), and that in the nondiabetic eyes without retinal ischemia (macular hole and epiretinal membrane) was 0.90 ± 0.55 ng/mL (n = 5). Neither difference was significant. Activin A and its receptors were detected in the vascular endothelial cells, fibroblast-like cells and round-shaped macrophage-like cells in preretinal proliferative membranes by immunohistochemical analysis.

Conclusions: Activin A is involved in the proliferative membrane formation in both ischemic and nonischemic vitreoretinal proliferative diseases. Activin A, a member of TGF-β superfamily, regulates angiogenesis and tissue fibrosis in the wound healing process. **Jpn J Ophthalmol 2000;44:221–226** © 2000 Japanese Ophthalmological Society

Key Words: Activin A, activin receptors, diabetic retinopathy, proliferative membranes, vitreoretinal diseases.

Introduction

Vitreoretinal proliferative membranes cause vitreoretinal proliferative diseases, such as proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR). Proliferative membranes are composed mainly of connective tissue and new vessels.[1,2] The connective tissue components are formed by the functions of various growth factors and cytokines, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2 or basic FGF), transforming growth factor-β (TGF-β) and others.[2–6] These growth factors also cause intraocular angiogenesis, which occurs in various ischemic retinal diseases, including diabetic retinopathy and retinopathy of prematurity. Michaelson proposed in 1948 that some chemical angiogenic factor, denoted as "factor X," is released from the ischemic retina and causes retinal new vessel formation.[1] At least 15 angiogenic growth factors and cytokines have been reported.[3] Among them, vascular endothelial growth factor (VEGF) is known to play an important role in angiogenesis in diabetic retinopathy.[4–6]

TGF-β stimulates connective tissue formation and regulates vascular endothelial proliferation.[7–10] These observations show that TGF-β plays important roles in the pathogenesis of proliferative membranes. Activin A, a member of the TGF-β superfamily, has also been reported to inhibit vascular endothelial cell proliferation[11–14] and to be involved in tissue fibrosis.[15–17] Activin A is produced by cultured human retinal pigment epithelium,[18] and ocular cells express activin receptors.[19] These findings suggest that activin A may be involved in the proliferative mem-

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brane formation in vitreoretinal diseases. In this study, we examined the expression of activin A in eyes with vitreoretinal diseases, and discussed the possible functions of activin A in the pathogenesis of vitreoretinal diseases.

Materials and Methods

Subjects and Specimens

We obtained 28 vitreous specimens and preretinal proliferative membranes from 25 patients during vitreous surgery. We explained the purpose of this study and obtained informed consent from all patients before surgery. This study was performed in accordance with the principles of the Declaration of Helsinki of the World Medical Association, and was approved by the Ethics Committee at the Toho University Sakura Hospital.

Patients with retinal ischemia and ocular new vessels included 15 patients (17 eyes) with PDR. The nondiabetic patients without new ocular vessels included 5 patients (6 eyes) with PVR, 3 patients (3 eyes) with epiretinal membranes, and 2 patients (2 eyes) with macular hole. The clinical backgrounds are presented in Tables 1 and 2.

Activin A concentration was measured in the vitreous samples obtained from 15 patients (10 cases with PDR, 3 cases with epiretinal membrane, and 2 cases with macular hole). The average age of the PDR patients was 46.0 ± 10.4 years (mean ± SD; n = 10), and that of the nondiabetic patients was 60.6 ± 9.7 years (n = 5). The average age of the PDR patients was significantly lower than that of nondiabetic patients (Student's *t*-test, $P = .049$). The expression of activin A and activin receptors was observed immunohistochemically in the preretinal membranes obtained from 12 cases (7 cases of PDR, 5 cases of PVR). The average ages of PDR and PVR groups were 47.0 ± 15.5 years (n = 7) and 40.8 ± 7.3 years (n = 5), respectively. This difference was not significant.

Bioassay of Activin A Concentration

Activin A concentration was measured by Dr. Yuzuru Eto of the Central Research Laboratories, Ajinomoto Company, Inc., Japan, using the erythroid differentiation factor assays.[20] Briefly, serially diluted samples were added to Friend cells at a cell density of 1×10^3 cells per well of 96-well plates in 100 mL of RPMI 1640 medium. After incubation for 5 days at 37°C, the cells were stained with o-dianisi-

Table 1. Patient Data: Quantification of Activin A in Vitreous Samples from Diabetic and Nondiabetic Patients

Patient No.	Retinal Diseases*	Age (years)	Sex†	Vitreous Bleeding‡	RD§	Retinal Ischemia	Rubeosis Iridis	Activin A Concentration (ng/mL)‖
1	PDR	56	F	0	0	+	−	2.5
2	PDR	47	F	1	2	+	+	1.5
3	PDR	57	F	1	1	+	+	4.0
4	PDR	35	M	1	1	+	−	0.5>
5	PDR	36	M	0	2	+	−	0.5>
6	PDR	26	F	0	2	+	+	0.5>
7	PDR	50	F	0	1	+	+	0.5>
8	PDR	48	M	1	1	+	−	0.5>
9	PDR	56	F	2	0	+	+	1.5
10	PDR	49	F	1	1	+	−	3.0
11	PVR	44	M	0	2	−	−	1.0
12	MH	58	F	0	0	−	−	0.5>
13	MH	60	F	0	0	−	−	0.5>
14	ERM	46	M	0	0	−	−	0.5>
15	ERM	68	F	0	0	−	−	1.5
16	ERM	71	M	0	1	−	−	1.5

*PDR: proliferative diabetic retinopathy, PVR: proliferative vitreoretinopathy, MH: macular hole, ERM: epiretinal membrane.

†F: female, M: male.

‡Severity of vitreous bleeding—0: no bleeding, 1: fundus was visible, 2: fundus was invisible due to bleeding.

§Extent of retinal detachment (RD)—0: no retinal detachment, 1: partial retinal detachment, 2: total retinal detachment.

‖0.5 > = under detection level (0.5 ng/mL>).

Table 2. Expression of Activin A and Activin Receptors in Proliferative Membranes, Determined Immunohistochemically

Patient No.	Retinal Pathology*	Age (years)	Sex†	Vitreous Bleeding‡	Retinal Ischemia	RD§	Immunohistochemical Expression				
							Activin A	Activin Receptors			
								R-I	R-IB	R-II	R-IIB
1	PDR	26	F	0	+	2	+	+	+	+	+
2	PDR	48	M	1	+	1	+	ND‖	+	+	+
3	PDR	56	F	2	+	0	−	+	+	+	−
4	PDR	51	F	2	+	0	+	+	−	+	−
5	PDR	57	M	1	+	0	+	+	+	+	+
6	PDR	66	M	0	+	1	+	+	+	+	ND
7	PDR	26	M	1	+	1	+	+	+	+	+
8	PVR	30	M	1	−	2	+	+	+	+	+
9	PVR	42	M	1	−	2	+	+	+	−	+
10	PVR	38	M	0	−	2	+	+	+	+	+
11	PVR	45	M	0	−	2	+	+	+	+	+
12	PVR	49	M	0	−	2	+	+	+	+	+

*PDR: proliferative diabetic retinopathy, PVR: proliferative vitreoretinopathy.
†F: female, M: male.
‡Severity of vitreous bleeding—0: no bleeding, 1: fundus was visible, 2: fundus was invisible due to bleeding.
§Extent of retinal detachment (RD)—0: no retinal detachment, 1: partial retinal detachment, 2: total retinal detachment.
‖Not determined.

dine,[20] and the percentage of differentiated cells was determined. Activin A induced the differentiation of Friend cells at a low concentration range in a dose-dependent manner. In the range of 1–10 ng/mL, the proportion of the differentiated cells increased in a monotonic fashion, and the minimum concentration at which Friend cells differentiated was 0.5 ng/mL.[20]

Immunohistochemical Analysis

The immunohistochemical observation of the expression of activin A and activin receptors was performed by the following procedures. The membranes were frozen immediately after the operation in Tissue-Tek OCT compound® (Miles Laboratories, Naperville, IL, USA), and 6-μm thick sections were mounted on 3-aminopropyl triethoxysilane-coated glass slides. The sections were fixed in ice-cold acetone for 10 minutes and then rinsed in phosphate-buffered saline (PBS; 0.1 M, pH 7.4). The sections were treated with 3% H_2O_2 in PBS for 15 minutes to block the endogenous peroxidase activity, rinsed in PBS, and then followed by incubation in normal goat serum for 20 minutes at room temperature (RT) to avoid nonspecific binding of the antibodies. Sections were then incubated with the primary antibody overnight at 4°C in a moist chamber. The binding sites of the primary antibody were detected by the streptoavidin-biotin-peroxidase method using the Histofine SAB-PO kit® (Nichirei, Tokyo) according to the manufacturer's protocol. The streptoavidin-biotin-peroxidase complexes were visualized with 3,3′-diaminobenzidine tetrahydrochloride. As the negative control, the samples were treated with nonimmunized IgG in place of the primary antibodies.

The antibody to activin A was a generous gift from Dr. Yuzuru Eto of the Central Research Laboratories of Ajinomoto Company, Inc. (Kawasaki, Kanagawa).[21] The antibodies to activin type I receptor, type IB receptor, type II receptor, and type IIB receptor were generous gifts from Dr. Kohei Miyazono and Dr. Mitsuyasu Kato of the Department of Biochemistry, the Cancer Institute (Tokyo).[22,23]

Statistics

Differences in the mean of activin A concentration in the vitreous samples were analyzed statistically using Wilcoxon's rank sum test. To investigate the clinical factors relevant to activin A concentration, multivariate regression analysis was performed using a logistic model. The factors that were significantly relevant were selected by stepwise method.

Results

Activin A Concentration in Vitreous Samples

The concentration of activin A in 10 vitreous samples from 10 eyes with new vessel disease (PDR) ranged from <0.5 ng/mL (undetectable) to 4 ng/mL

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with a mean of 1.50 ng/mL (± 1.27, SD). In this analysis, 0.5 ng/mL was used for the calculations when the level was undetectable. The vitreous from the 5 eyes with diseases other than new vessel disease (macular hole and epiretinal membrane) contained activin A ranging from the undetectable level to 1.5 ng/mL with a mean of 0.90 ± 0.55 ng/mL. There was no statistically significant difference between the activin A concentrations in the eyes with and without new vessels or retinal ischemia (Wilcoxon's rank sum test, $P = .173$). The average of activin A concentration of women patients was 1.6 ± 1.2 ng/mL (n = 10), and that of men patients was 0.67 ± 0.26 ng/mL (n = 6). The mean concentration of activin A in the women tended to be higher than in male patients (Wilcoxon's rank sum test, $P = .053$).

Multivariate regression analysis was performed to investigate the factors relevant to the concentration of activin A. In this analysis, the dependent variable was the concentration of activin A, and the independent variables were age, sex (female = 1, male = 0), retinal detachment and vitreous hemorrhage (grades are listed in Table 1), retinal ischemia (+ = 1, − = 0), rubeosis iridis (+ = 1, − = 0). The concentration of activin A did not correlate with any of the examined clinical factors.

Expression of Activin A and Activin Receptors in Proliferative Preretinal Membranes

Activin A and activin receptors (activin type I, IB, II, IIB receptors) were observed immunohistochemically to be expressed in the cells of the preretinal proliferative membranes (Figures 1 and 2). The endothelial cells of the new vessels expressed activin A and activin receptors (Figures 1 and 2). In addition, the fibroblast-like cells and the round-shaped cells (macrophage-like cells) in the extracellular matrix expressed activin A and activin receptors (Figures 1 and 2).

Activin A was detected in all but one proliferative neovascular membrane obtained from eyes with PDR, and in all non-neovascular membranes obtained from eyes with PVR (Table 2). Activin type I receptor and type II receptor were detected in all the examined membranes obtained from eyes with PDR (Table 2). Activin type IB receptor was detected in 6 of 7 membranes, and activin type IIB receptor was

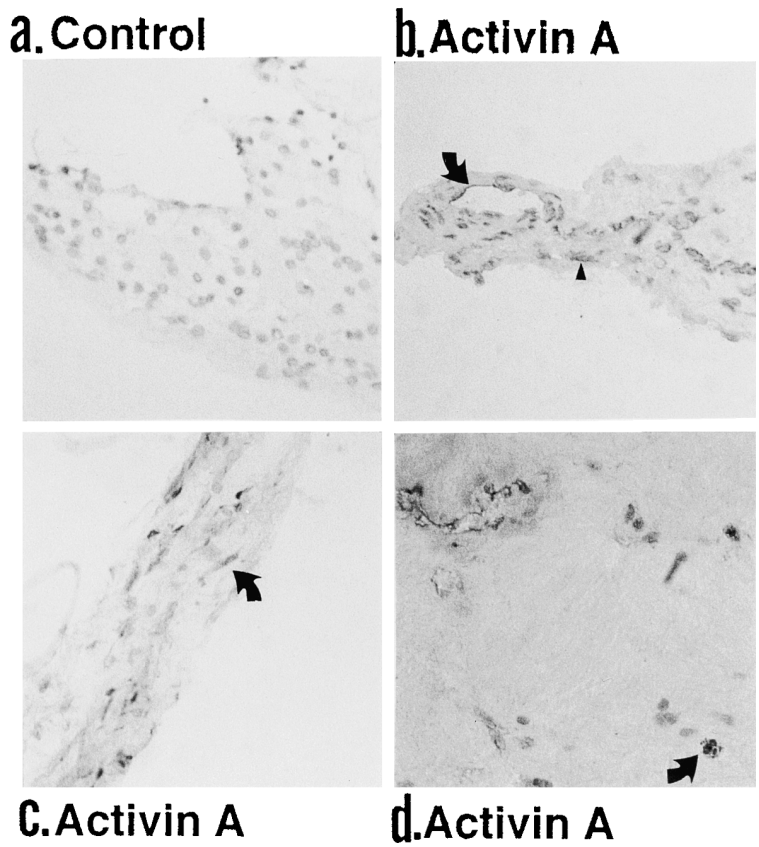

Figure 1. Immunohistochemical observation of activin A expression in preretinal membranes. Original magnification: ×200. (**a**) Negative control using nonimmune IgG instead of anti-activin A. (**b**) Preretinal membrane obtained from proliferative diabetic retinopathy case 4 in Table 2. Vascular endothelial cells (arrow) and fibroblast-like cells in extracellular matrix (arrowhead) are positively stained. (**c**) Preretinal membrane obtained from proliferative diabetic retinopathy case 1 in Table 2. Fibroblast-like cells in extracellular matrix (arrow) are positively stained. (**d**) Preretinal membrane obtained from proliferative diabetic retinopathy case 1 in Table 2. Round-shaped cells in extracellular matrix (arrow) are positively stained.

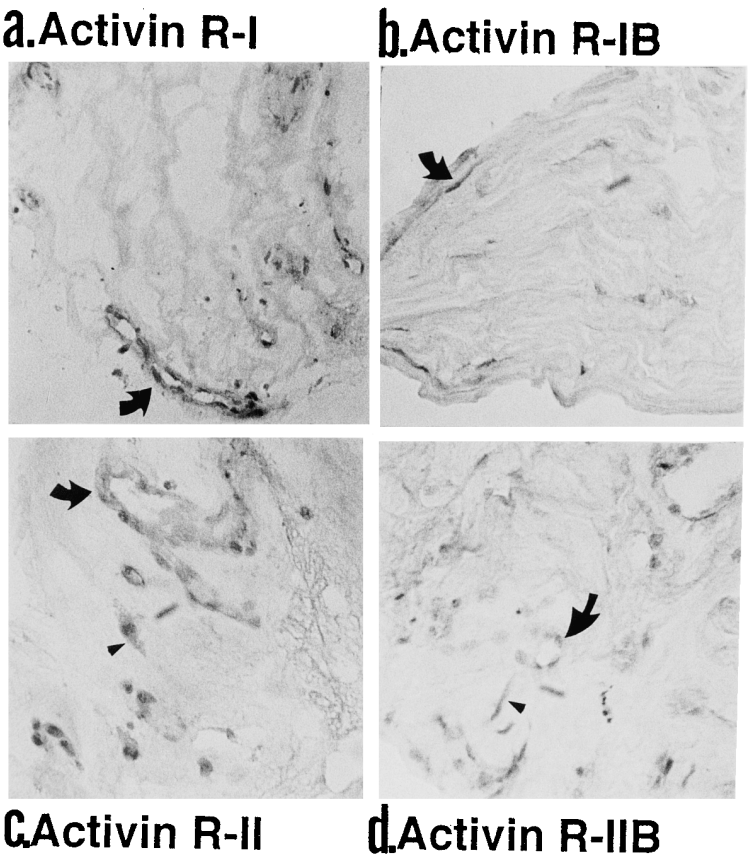

Figure 2. Immunohistochemical observation of activin receptors expression in preretinal membranes. Original magnification: ×200. (**a**) Expression of activin type I receptor in preretinal membrane obtained from proliferative diabetic retinopathy case 4 in Table 2. Vascular endothelial cells (arrow) are positively stained. (**b**) Expression of activin type IB receptor in preretinal membrane obtained from proliferative diabetic retinopathy case 3 in Table 2. Fibroblast-like cells in extracellular matrix (arrow) are positively stained. (**c**) Expression of activin type II receptor in preretinal membrane obtained from proliferative diabetic retinopathy case 3 in Table 2. Vascular endothelial cells (arrow) and fibroblast-like cells in extracellular matrix (arrowhead) are positively stained. (**d**) Expression of activin type IIB receptor in preretinal membrane obtained from proliferative diabetic retinopathy case 3 in Table 2. Vascular endothelial cells (arrow) and fibroblast-like cells in extracellular matrix (arrowhead) are positively stained.

detected in 4 of 6 membranes from diabetic eyes (Table 2). Activin type I receptor family (type I and type IB) and type II receptor family (type II and type IIB) were detected in all the examined membranes obtained from the eyes with PVR (Table 2). All the examined preretinal membranes from eyes with or without new vessels or retinal ischemia contained the component cells expressing at least one type I receptor (activin type I or IB receptor) and one type II receptor (activin type II or IIB receptor). These findings suggest that all the membranes contained cells responding to activin A.

Discussion

Activin A is a 28-kD dimeric protein and has been identified as a hormone that is produced by the ovary and stimulates follicular stimulating hormone (FSH). However, activin A has been shown to exert various effects on many types of cells in both men and women. Activin A inhibits cell proliferation, accelerates cell differentiation, including erythroid differentiation, stimulates fibrosis, and regulates development and organogenesis.[11,14–17,20] Activin A exerts these functions by binding to specific receptors. Activin A binds to type II receptor (activin type II receptor or activin type IIB receptor), which recruits type I receptor (activin type I or type IB receptor).[24,25] Type I receptor is phosphorylated at the serine and/or threonine residues by the type II receptor and its kinase activity is activated, allowing type I receptors to transduce signals.[24,25]

The present study revealed for the first time that activin A is detected in proliferative vitreoretinal diseases, including both ischemic retinal disease (PDR) and nondiabetic diseases (PVR and epiretinal membrane). The concentration of activin A ranged from nondetectable to 4 ng/mL. The concentration of activin A was not related to age or sex of the patients, or the presence of retinal ischemia according to a multivariate analysis. The components of preretinal proliferative membranes, endothelial cells of new vessels, fibroblast-like cells, and round-shaped macrophage-like cells in the extracellular matrix expressed activin A at the protein level and also expressed activin receptors (type I, type IB, type II, type IIB) at the protein level. These observations suggest that activin A is produced in eyes with diabetic and nondiabetic proliferative vitreoretinal diseases, and exerts some effects on vascular cells and other types of cells.

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A previous report has confirmed the inhibitory effect of activin A on cultured vascular endothelial cells.[12] However, activin A did not induce angiogenesis in vivo in contrast to TGF-β.[6,7,14] These results suggest that activin A may exert some inhibitory effect on ocular angiogenesis. Various angiogenic growth factors, including FGF-2 (βFGF), PDGF, TGF-β and EGF, have been found to stimulate the production of activin A from cultured fibroblasts,[26] which suggests a negative feedback mechanism in new vessel formation in ocular diseases.

Activin A was also detected in eyes without retinal ischemia or new vessels, including PVR and epiretinal membranes. Activin A was produced by fibroblast-like cells and activin receptors were expressed in these cells. In previous reports, activin A was found to be related to lung fibrosis.[15,16] It is also speculated that activin A is involved in granulation tissue formation in dermal wound healing.[17] Another possibility is that activin A is involved in the production of extracellular matrix in the pathogenesis of preretinal proliferative membrane formation. The results of the present study show that activin A was detected in both ischemic and nonischemic vitreoretinal proliferative diseases, which is consistent with the speculation that activin A is involved in fibrosis.[15–17]

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