Graefe's Arch Clin Exp Ophthalmol (1994)
232:477–481

CLINICAL INVESTIGATION

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The significance of vitronectin in proliferative diabetic retinopathy

Received: 19 March 1993
Revised version received:
31 January 1994
Accepted: 18 March 1994

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Abstract Vitronectin, an integrin-binding a-1-glycoprotein with potent cell-adhesion and proliferation-mediating properties, has been shown to be incorporated in surgically removed membranes from patients with proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR) and macular pucker. Therefore we developed an ELISA technique to quantify levels of vitronectin in human vitreous and plasma samples in order to be able to evaluate the significance of vitronectin in these different vitreoretinal diseases. Our results indicate a significant increase of vitronectin in all proliferative disorders except idiopathic macular pucker. Adjustment of all probes to equal total protein content yielded a significant increase only in PDR (type II diabetes). Plasma samples demonstrated a significant increase of vitronectin in patients with type II diabetes suffering from PDR. Therefore, breakdown of the blood-retina barrier appears to be the most likely explanation for the increased levels of vitronectin in the vitreous. Our results indicate that vitronectin may not only be involved in the regulation of epiretinal membrane formation at significantly higher levels in patients with type II diabetes, but the increase of vitronectin in diabetic plasma may also help to explain the pathological alteration of the coagulation cascade in diabetics.

Introduction

The glycoprotein vitronectin, also described as serum spreading factor, promotes the attachment and proliferation of a variety of cells in vitro. It is a regulator of the complement cascade [26] and the coagulation system [27, 28]. Vitronectin is synthesized by platelets and found in the extracellular matrix of surgically removed membranes from patients with proliferative vitreoretinal disorders [40]. Because of its complex functions we quantified levels of vitronectin in vitreous and plasma samples from patients undergoing vitrectomy for a variety of vitreoretinal pathologies.

Material and methods

Vitreous samples were obtained from patients suffering from traumatic proliferative vitreoretinopathy (PVR; $n=10$), idiopathic PVR (PVR following rhegmatogenous retinal detachment; $n=10$), proliferative diabetic retinopathy (PDR; type I diabetes – $n=5$, type II diabetes – $n=10$) and macular pucker ($n=10$) by aspirating 100 ml of vitreous from the center of the vitreous cavity prior to vitrectomy. Patients with apparent intraocular bleeding at the time of surgery were excluded from the study. Control samples ($n=10$) were taken from keratoplasty donor eyes within 24 h post mortem. Plasma samples were obtained prior to surgery. Immunoreactive vitronectin in vitreous and plasma was measured using a noncompetitive ELISA method originally described by Gomez-Lechon and Castell [12] and especially designed for adhesion-promoting proteins, with the necessary modification [34] for the use of vitreous quantification [38] of vitronectin. In brief, micro-ELISA plates were coated with vitreous (1:50) and plasma (1:500) samples and vitronectin standards (1 mg/ml) in triplets

serially diluted over eight steps. After incubation overnight at +4° C and rinsing with PBS-Tween (0.1%, Serva) the plates were exposed to rabbit-derived anti-human vitronectin (Calbiochem 681110) for 1 h at 37° C. Following the addition of anti-rabbit-IgG-F(ab)^2-alkaline-phosphatase-conjugated (1:1000, Sigma 0407) for 1 h, the plates were covered with 50 ml/well 4-nitrophenyl-phosphate (0.5 mg/ml, Merck 6850). Optical density (OD) readings were obtained after 30 min using an ELISA reader (MR 5000, Dynatech). Vitronectin levels were calculated by plotting the OD curves of samples against standards. Statistical analysis was performed by means of the Mann-Whitney *U*-test. Protein levels were measured using the method described by Lowry et al. [21]. In addition to the ELISA we performed sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE; Multiphor II electrophoresis unit, LKB 2117-001) [12] with subsequent western blotting after protein transfer to nitrocellulose [35]. The same antibodies and detection system were used as described above. Naphthol AS-MX phosphate (0.4 mg/ml in H_2O, Sigma N4875) and Fast Red TR-Salt (6 mg/ml in 0.2 M Tris, pH 8.2) served as substrate for the alkaline phosphatase.

Results

SAS-PAGE and western blotting

Vitreous samples of human postmortem eyes did not contain enough vitronectin to be visualized by the assay. However, all samples from patients with proliferative vitreoretinal disorders (Fig. 1, lanes B–E) displayed the typical double band at 65 and 75 kDa with a varying degree of staining indicative of a different concentration of the glycoprotein in the samples. Similarly, vitronectin could be detected in human plasma from normal patients (lane F) and patients with diabetes (lane G).

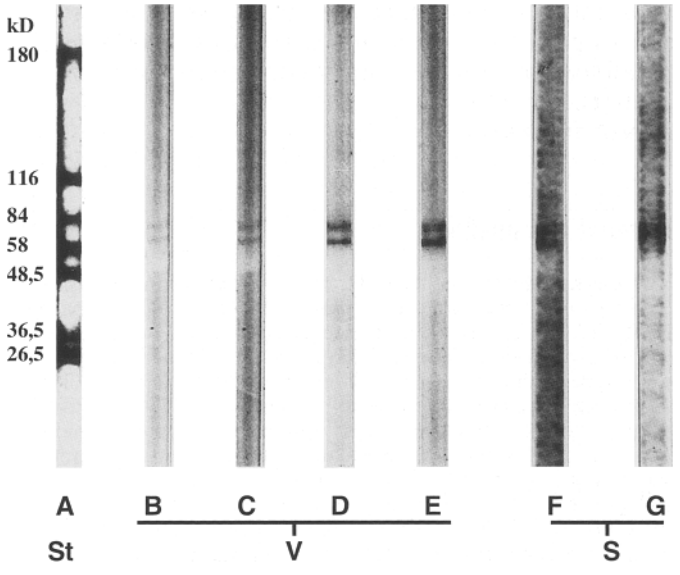

Fig. 1 Western blot: a double band at 65 and 75 kDa indicates vitronectin. *A* Molecular weight standard, *B* macular pucker, *C* idiopathic PVR, *D* traumatic PVR, *E* PDR (type II diabetes), *F* normal plasma, *G* diabetic plasma

Table 1 Vitronectin levels (mean ± SD) in vitreous samples (*PVR* proliferative vitreoretinopathy, *PDR* proliferative diabetic retinopathy)

	n	Concentration (mg/l)	Significance (*P*)
Controls	10	4.22 ± 2.34	
Macular pucker	10	10.77 ± 9.10	NS (0.58)
Idiopathic PVR	10	27.20 ± 17.10	≤0.01
Traumatic PVR	10	21.34 ± 8.60	≤0.01
PDR			
Type I diabetes	5	49.88 ± 5.75	≤0.01
Type II diabetes	10	64.53 ± 26.70	≤0.01

Table 2 Ratio of vitronectin to total vitreal protein

	n	Total protein (mg/l)	Vitronectin/ total protein	Significance (*P*)
Controls	10	1.0	4.21	
Macular pucker	10	2.61	4.12	NS (0.65)
Idiopathic PVR	10	7.18	3.79	NS (0.59)
Traumatic PVR	10	5.08	4.20	NS (0.87)
PDR				
Type I diabetes	5	8.78	5.68	NS (0.73)
Type II diabetes	10	6.53	9.88	≤0.01

Table 3 Vitronectin levels (mean ± SD) in plasma

	n	Concentration (mg/l)	Significance (*P*)
Controls	10	347.3 ± 114.1	
Macular pucker	10	320.2 ± 124.6	NS (0.71)
Idiopathic PVR	5	321.6 ± 83.8	NS (0.73)
Traumatic PVR	10	372.4 ± 72.8	NS (0.91)
PDR			
Type I diabetes	5	422.2 ± 131.1	NS (0.37)
Type II diabetes	10	573.5 ± 154.0	≤0.01

Vitronectin levels in vitreous

There was a significant increase in vitronectin concentration in most of the different proliferative disorders compared with human postmortem eyes; macular pucker was an exception. In control samples only minor amounts of vitronectin could be detected.

Since the concentration of a vitreal protein is influenced not only by local synthesis but also by plasma exudation due to disturbances of the blood-eye barrier, we determined the ratio between total vitreal protein content and vitronectin levels. Protein levels were elevated in all disorders. However, a significant relative

increase of vitronectin levels could be detected only in the vitreous derived from patients with type II diabetes and PDR.

Vitronectin levels in plasma

Compared with healthy control groups and patients with nondiabetic proliferative vitreoretinal disorders, all diabetics displayed an increase of plasma vitronectin levels, but only in patients suffering from PDR with type II diabetes was this difference statistically significant.

Discussion

In 1967, Holmes described an adhesion-promoting protein with a molecular weight of 65–75 kDa in human serum [15]. Because of its high affinity to plastic surfaces and its effects on spreading of various cultured cells (e.g. epithelial cells, fibroblasts), it has been named vitronectin, S-protein or serum spreading factor [1, 2, 13]. Plasma vitronectin is predominantly derived from the liver [30], but platelets [25, 30], megakaryocytes and mesothelial cells also have been shown to synthesize vitronectin [18]. This a-1-glycoprotein belongs to the family of integrin-binding proteins such as fibronectin, fibrinogen, von Willebrand factor and thrombospondin, which feature similar cell-surface receptors [4, 17, 19, 31].

We have recently described the colocalization of vitronectin and fibronectin in the extracellular matrix of membranes surgically removed from patients with different proliferative vitreoretinal disorders [40]. Fibronectin and vitronectin receptors were also equally distributed on cellular surfaces in intravitreal membranes [9, 41]. However, the amount of immunoreactive vitronectin appeared to be higher in patients with PDR than in those with PVR. Since the methods used in this study were qualitative rather than quantitative, our goal was to demonstrate the actual differences by measuring the vitronectin concentration in vitreous. The average level for vitronectin in healthy individuals (347.3 mg/l ± 114.1 mg/ml) correlated well with the results of other authors [30, 34] who reported plasma levels of 250–450 mg/l, indicating the validity of our test.

Vitronectin levels were significantly increased in traumatic and idiopathic PVR but not in eyes with macular pucker. Possible sources of elevated vitronectin levels in the vitreous include local production and breakdown of the blood-retina barrier. Although vitronectin has predominantly been associated with platelets, some authors describe the possibility of vitronectin synthesis by monocytes and macrophages [13]. These cells are thought to be involved in cellular spreading and proliferation as a characteristic feature of the eye's reaction towards vitreoretinal trauma [22, 42] and have been immunohistochemically demonstrated in epiretinal membranes [14, 36, 39]. In macular pucker, only few macrophages are detected [10] and our finding of a non-significant increase of vitreal vitronectin correlates with the slow progression of this disease. To prove intraocular synthesis of vitronectin, however, further investigations such as in situ hybridization will have to be carried out.

The low level of total vitreal protein reflects the minimal breakdown of the blood-retina barrier in macular pucker. In traumatic and idiopathic PVR, blood-retina barrier breakdown is much more pronounced, as indicated by the high levels of intravitreal protein. Similary, vitronectin levels are elevated in these groups. Campochiaro and co-workers noted that vitreous aspirates from patients with proliferative vitreoretinal disorders stimulate retinal pigment epithelial cell migration [5]. Other authors demonstrated the chemoattractant activity of pathological vitreous to human scleral fibroblasts [43]. The detection of high vitronectin levels in our study and the findings of elevated fibronectin levels in PVR in another study [37, 38] could in part account for these stimulatory effects. However, only absolute levels of fibronectin were elevated in the latter study. In comparison with total vitreal protein relative fibronectin levels were not significally elevated, as was true in our study for vitronectin in most disease entities. However, in patients with type II diabetes and PDR the relative rise in vitronectin was significant ($P \leq 0.01$). Although fibronectin is a high-molecular-weight protein (220–440 kDa), a selective breakdown of the blood-retina barrier for the much smaller (75 kDa) vitronectin molecule only in patients suffering from PDR with type II diabetes and not in the other group seems rather unlikely, pointing to an additional source of damage. A possible explanation is given by the high plasma level of vitronectin in patients with type II diabetes (Table 3). Musso and co-workers reported a similar increase for fibronectin levels in the plasma of patients with diabetes mellitus; however, no distinction was made between type I and type II diabetes and the occurrence of PDR. The increase was explained as being due to increased vascular endothelial synthesis and release [24]. Since neovascularization is a dominant feature of PDR, the description of vitronectin synthesis and secretion by mesothelial and arterial smooth muscle cells [27] points to a vascular source of intravitreal vitronectin in addition to the breakdown of the blood-retina barrier as the most likely explanation for the increased levels of vitronectin in the vitreous.

Diabetes mellitus has long been associated with increased fibrinogen turnover and high risk of thromboembolic complications due not only to micro- or macroangiopathy but also to a shift in coagulation parameters [16]. The reason for this has not yet been determined. Alteration of coagulation parameters, espe-

cially increased levels of antithrombin III, in patients with diabetes mellitus have been reported by many authors [6, 20]. Our findings of elevated plasma levels of vitronectin in diabetic patients is important, since vitronectin has been shown to inhibit the heparin-catalyzed inhibition of thrombin by antithrombin III [29]. Vitronectin has also been shown to stabilize type 1 plasminogen activator inhibitor (PAI-1), a physiological inhibitor of both tissue-type plasminogen activator and urokinase-type plasminogen activator, key enzymes in the initiation of fibrinolysis [23]. Also, the binding of PAI-1 to the extracellular matrix is mediated by serum-derived vitronectin [32]. Acquired deficiencies of vitronectin have been found in patients with disseminated intravascular coagulation [7]. In vitro studies indicate increased fibrinogen turnover in diabetes and heparin infusion restoration of fibrinogen survival [16]. The difference between levels of vitronectin in patients with type II diabetes and those in type I diabetes may in part be explained by the sometimes highly significant correlation of coagulation factors with age [6]. Setiadi and co-workers have demonstrated the increase of endothelial cell growth by sera from diabetic patients with proliferative retinopathy [33]. A possible explanation of these findings may be found in the elevated vitronectin level, since vitronectin may regulate the organization of its respective Arg—Gly—Asp adhesion receptor in cultured human endothelial cells [8]. In conclusion, vitronectin may not only be involved in the regulation of epiretinal membrane formation at significantly higher levels in patients with type II diabetes; the increase of vitronectin in diabetic plasma may also help to explain the pathological alteration of the coagulation cascade in diabetes [3].

Acknowledgements This study was supported by the Retinovit Foundation and the Deutsche Forschungsgemeinschaft (Wi 880/3-2).

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