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Atrial natriuretic peptide in the vitreous humor and epiretinal membranes of patients with proliferative diabetic retinopathy

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Purpose: Atrial natriuretic peptide (ANP) has been recently described as an endogenous inhibitor of the synthesis and angiogenic action of vascular endothelial growth factor (VEGF). Given VEGF's key role in promoting neovascularization in proliferative diabetic retinopathy (PDR), this study was designed to evaluate the possibility that ANP could be involved in the neovascular and fibrotic complications of PDR.

Methods: We determined ANP by radioimmunoassay in plasma and vitreous humor samples collected from diabetic patients with and without PDR and from non-diabetic subjects. ANP was also immunohistochemically localized in the epiretinal membranes of patients with PDR.

Results: Vitreous ANP concentrations were significantly higher in patients with active PDR compared to patients with quiescent PDR, diabetes without PDR or controls <0.05. Significant differences were also observed between vitreous ANP levels in diabetic patients without PDR and control subjects. There was no significant correlation between serum and vitreous ANP levels in any of the patient groups. ANP was detected in the fibrovascular epiretinal tissue of patients with PDR.

Conclusions: Diabetic patients with active neovascularization have significantly higher levels of ANP in the vitreous humor than those without active PDR. Diabetic patients without PDR were also found to have significantly higher vitreous ANP levels than non-diabetic patients. Since plasma and vitreous ANP concentrations were found to be unrelated, we suggest intraocular ANP synthesis and/or an increase in the release of ANP into the vitreous, as opposed to diffusion from the blood, as the main factors contributing to the high vitreous ANP levels observed in diabetic patients. In the fibrovascular epiretinal tissue of these patients, ANP was found to be localized in vascular, glial, fibroblast-like and retinal pigment epithelium cells. Our findings suggest a role for ANP in PDR.

Diabetic retinopathy is among the leading causes of vision loss and blindness in industrialized countries. Proliferative diabetic retinopathy (PDR) is characterized by preretinal neovascularization induced by ocular ischemia and fibrosis, which ultimately leads to vitreous hemorrhage and tractional retinal detachment [1]. The formation of scarlike epiretinal membranes (ERMs) is a common finding during the progression of PDR, though the factors controlling their development are not yet understood. In PDR, growth factors, such as basic fibroblast growth factor (bFGF) [2,3] and vascular endothelial cell growth factor (VEGF) [4-6], have been found in high concentration in vitreous fluid and have been detected in the ERMs from these patients. VEGF has been identified as an endothelial cell-specific mitogen and angiogenic inducer in vivo. The expression of this growth factor is greatly increased by hypoxia in many retinal cell types [7]. Further, VEGF is thought to play an essential role in intraocular neovascularization in PDR and other ischemic retinal diseases. VEGF is also a potent stimulator of vascular permeability and has been implicated in the breakdown of the blood-retinal barrier in streptozotozin-induced diabetic rats [8,9]. Both hyperpermeability and breakdown of the blood-retinal barrier are major early functional disorders observed in diabetic retinopathy.

Pigment epithelium-derived factor (PEDF) [10] and atrial natriuretic peptide (ANP) [11] have been recently identified as natural inhibitors of angiogenesis. High concentrations of PEDF have been found within the retina as well as in the vitreous humor, where this factor is responsible for the antiangiogenic activity of this fluid [10]. ANP is a member of the natriuretic peptide (NP) family, a group of cardiovascular cyclic peptide hormones with diuretic, natriuretic, and vasodilatory properties [12]. Most biological effects of ANP are mediated by the guanylate cyclase coupled A-receptor (NPRA) [13]. However, there is increasing evidence to suggest that several of ANP's effects are mediated via a so-called "clearance receptor" (NPRC), which does not possess GC activity, but that reportedly signals through inhibition of adenylate cyclase in some cells [14]. Natriuretic peptides inhibit cardiomyocyte, vascular endothelial cell (EC) and/or astrocyte proliferation, in part, via the NPRC receptor [15,16].

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We previously demonstrated a link between ANP and the eye. In this context, we demonstrated the presence of immunoreactive ANP in rat and rabbit retinas by radioimmunoassay (RIA) [17]. Moreover, ANP binding sites in rat retinas were detected by binding assays and autoradiography [18]. The presence of mRNA transcripts encoding the three natriuretic peptide receptors (NPRA, NPRB and NPRC) has been demonstrated in the retina of the rat and rabbit eye by polymerase chain reaction (PCR) [19]. In addition, we reported the expression of NPR transcripts along with ANP, BNP, and CNP mRNA in the human retina and were able to detect NPs in the neural retina, as well as in the glial and vascular elements of the normal adult retina [20]. From a physiological perspective, there is much evidence linking ANP to the control of intraocular pressure [21-23].

More recently, it has been demonstrated that ANP inhibits VEGF transcription and protein production in cultured human vascular endothelial cells via the NPRC receptor [24]. Moreover, ANP has been found to inhibit the activation of several key signaling molecules (these include ERK, JNK, and p38 members of the MAP kinase family) with a role in VEGF-induced angiogenesis, mediating their actions through both NPRA and NPRC receptors [11]. Thus, ANP is one of the first described endogenous inhibitors of both the synthesis and angiogenic function of VEGF.

VEGF was also originally described as a potent vascular permeability factor (VPF). VEGF enhances permeability in aortic endothelial cells via a linked signaling pathway, sequentially involving Src, ERK, JNK, and phosphatidylinositol 3-kinase/AKT. This leads to the Ser/Thr phosphorylation and architectural disruption of protein components of the EC tight junction [25]. In this context, it has been described that ANP inhibits vascular permeability factor signaling, tight junction protein phosphorylation and localization, and vascular permeability factor-induced permeability [25].

Collectively, these data suggest that ANP might have a role in diabetic retinopathy and this led us to speculate that ANP could contribute to the fibrovascular complications of diabetic retinopathy by possible direct antiangiogenic effects in the eye. As an initial step towards evaluating this hypothesis, the present study was designed to assess the presence of ANP in the vitreous humor and to establish a possible link between ocular ANP levels and the angiogenic and fibrotic complications of diabetic retinopathy.

METHODS

Subjects: All research procedures involving human subjects were conducted according to institutional guidelines on the Declaration of Helsinki. The study protocol was reviewed and approved by our institutional board, and informed consent was obtained from all the subjects enrolled in the study.

Diabetic patients between 20 and 70 years of age, referred for vitrectomy and/or membrane peeling to Hospital Clínico San Carlos and Hospital Gregorio Marañón, Madrid (Spain) between October 2000 and November 2003 were initially considered. For inclusion in this study, patients must have been undergoing their first vitrectomy. Patients with previous intraocular surgery, glaucoma, systemic hypertension, heart failure, renal failure or HbA1c higher 10.5 were excluded from the study. Patients were excluded if they had previously experienced a tractional retinal detatchment. To avoid massive influx of plasma ANP into the vitreous humor, patients who had recently (less than 6 months) suffered vitreous hemorrhage were excluded. All patients with PDR had retinal photocoagulation before vitrectomy. Retinopathy was graded intraoperatively in all selected eyes (n=50) using a previously reported method [26]. In brief, neovascularization was considered to be active (n=19) in the presence of perfused preretinal capillaries, and to be quiescent (n=3) if only non-perfused, gliotic vessel or fibrosis were detected.

Finally, 50 diabetic patients were considered, 22 of them with PDR (12 with type 1 diabetes and 10 with type 2 diabetes), ranging in age from 20 to 70 (mean 56.2), and 28 of them without PDR (15 with type 1 diabetes and 13 with type 2 diabetes), 14 females and 14 males, ranging in age from 25 to 70 (mean 60.2). In the diabetic without PDR group, 4 patients underwent vitrectomy for asteroid hyalosis, 17 for vitreous organization, and 5 for macular oedema.

During the same time frame, a group of non-diabetic patients (fasting blood glucose levels below 5.3 mmol/l) with ages similar to diabetic group (range between 20 and 70 years; mean 50.5 years), were consecutively selected as “controls”. Inclusion among this control group required that the retina was not affected by neovascularization. For inclusion in this study, patients must have been undergoing their first vitrectomy. Patients with previous intraocular surgery, glaucoma, systemic hypertension, heart failure, or renal failure were excluded from the study. In this control group (n=29), 10 patients underwent vitrectomy for primary or recurrent retinal detachment, 9 for dislocated crystalline lens, and 10 for macular holes.

Vitreous humor and blood samples: Undiluted vitreous humor samples were collected at the time of vitreoretinal surgery by using a syringe attached to an automated vitrector before starting the intravitreal infusion of balanced salt solution. Blood samples were obtained at the time of surgery. Both blood and vitreous samples were collected in chilled tubes containing proteases inhibitors at the following final concentrations: 10 μM phenyl methyl sulphonyl fluoride (PMSF), 5 μM pepstatin A, and 10 μM trasylol. Vitreous samples were centrifuged at 11,000x g for 15 min at 4 °C, and blood samples were centrifuged at 1500x g for 20 min at 4 °C. The supernatants were stored at -70 °C less than 3 months before testing.

Epiretinal membranes: Ten ERMs were surgically excised from 10 diabetic patients with PDR during pars plana vitrectomy. The ERMs were carefully peeled off the retinal surface, excised with microscissors and taken out of the vitreous cavity by a microforceps. Immediately after excision, the membrane specimens were placed in buffered formalin for 24 h, embedded in paraffin, sectioned at 4 μm and dried on Snowcoat X-tra slides (Surgipath, Winnipeg, Manitoba, Canada) for immunohistochemistry.

Immunoreactive-ANP in vitreous and plasma samples: ANP was extracted from supernatants of vitreous and plasma

samples according to a previously described procedure [17]. Before extraction, 0.1% bovine serum albumin (BSA), 0.1% sodium chloride in 0.1 ml phosphate buffer 0.1 M, pH 7.4 were added to the tube containing the vitreous fluid to improve ANP recovery from Sep-Pack C-18 cartridges and to prevent ANP adhering to the tube wall. In brief, the Sep-Pack C-18 cartridges (Waters Associates, Milford, MA) were activated by washing with 10 ml acetonitrile (pure) and then washed with 10 ml ammonium acetate (0.2%, pH 4.0). The samples were applied to the cartridges, washed with 5 ml ammonium acetate (0.2%, pH 4.0), and the adsorbed ANP was eluted with 3 ml acetonitrile (60%) in ammonium acetate (0.2% pH 4.0). The organic solvent was evaporated under nitrogen stream followed by lyophilization. The resultant residue was taken up in phosphate buffer 0.1 M, pH 7.4 containing trifluoroacetic acid (0.1%). ANP was determined by a specific and sensitive radioimmunoassay (RIA), as described by the present authors [27] using an antibody (Peninsula Laboratories, Belmont, California) against synthetic ANP at a final dilution of 1:200,000. The antibody reacts 100% with α-ANP (human, canine) and β-ANP 1-28 (human). It did not show any cross-reactivity with human ET-1, BNP-45, and γ-ANP. ^{125}I was used as a tracer. Synthetic α-ANP 1-28 (human, canine) was used as standard. The 50% intercept was 23 pg/ml. The sensitivity of the RIA was 1.9 pg/ml. Recovery of 2 and 3 pg of ANP added to pooled human vitreous was 80±2% (n=10). The interassay variation was 10% and intra-assay variation was 12%.

Protein assay: The protein content of the vitreous samples was determined by the method of Lowry [28] using albumin as the standard.

Statistical analysis: ANP concentrations in plasma and vitreous humor were displayed as median and range, in view of the skewed distribution. Data were statistically analysed with the Mann-Whitney U tests for comparison of the two groups and with the Kruskal-Wallis test for multiple groups in the vitreous and plasma concentrations of ANP and protein. Acceptable significance was corrected by Bonferroni's method. Linear correlation between vitreous and plasma ANP concentrations was assessed by Spearman correlation. SPSS version 11.0 software (SPSS Inc., Chicago, IL) was used for the statistical analysis.

The differences in ANP protein concentration in patients with PDR could be an unspecific event that reflects the breakdown of the blood-retinal barrier and subsequent influx of plasma proteins into the vitreous humor. Thus, we tested both, the concentration of ANP in the vitreous fluid (IR-ANP pg/ml) and normalized ANP (IR-ANP pg/mg protein) after adjusting for intravitreous protein concentration.

Immunohistochemistry for ANP, GFAP, and cytokeratin: Immunohistochemical staining was performed as previously described [20]. Mouse monoclonal antibody to human ANP (Cymbus Biotechnology Ltd., Hampshire, UK) with specificity for whole molecules and residues 4-28, mouse monoclonal antibody to glial fibrillary acidic protein GFAP, clone 6F2 (DAKO Corp., Carpinteria, CA) and mouse monoclonal anti-human cytokeratin AE1/AE3 antibody (DAKO Corp.) were used. Briefly, deparaffinized and hydrated sections were incubated in blocking solution TBT (Tris Base Saline (TBS) 0.5 M pH 7.4 containing 3% (w/v) BSA and 0.05% (v/v) Triton X-100) for 30 min at room temperature to reduce non-specific binding. A previous step of heat-induced antigen retrieval technique [20] was used for both antibodies ANP and cytokeratin but not for GFAP. This took the form of pressure cooker heating for 5 min in a solution of 0.01 M sodium citrate prior to incubation with the primary antibodies. The sections were incubated overnight at 4 °C in a humidified chamber, with anti-ANP monoclonal antibody at 1:50 dilution. The slides were washed for 5 min in TBS. Immunodetection was performed with biotinylated antimouse immunoglobulins followed by streptavidin conjugated with alkaline phosphatase (using an LSAB2 kit from DAKO) and with naphthol phosphate and Fast Red chromogen (Sigma-Aldrich; St. Louis, MO), which resulted in red staining. The sections were lightly counterstained with Mayer's hematoxylin. Final mounting was done in the water soluble media Glicergel (DAKO).

As negative controls, tissue sections were incubated either with primary antibody preabsorbed with 10 nM ANP or with normal rabbit serum instead of the primary antibody. Human auricle was used as a positive control to assess the immunohistochemical detection of ANP.

In addition, antibody to GFAP and antibody to cytokeratin, both at a 1:50 dilution overnight at 4 °C, were used to detect glial and RPE cells, respectively.

Image analysis: Images were captured using Leica Qwin image processing and analysis software (Leica Microsystems, Heerbrugg, Switzerland) on a personal computer linked to a high resolution video camera (Leica DC100) mounted on a microscope (Zeiss, Oberkochen, Germany). Results were derived from examination of at least three sections from all the studied ERMs.

RESULTS

ANP concentration in human plasma and vitreous humor: ANP was detected in the extracts of vitreous samples. A clear parallelism was observed between various dilutions of the extracts from two different pools of vitreous and the standard curve, indicating that ANP present in vitreous is indistinguishable from the peptide used in the standard curve.

Vitreous ANP levels were highest in diabetic patients with active PDR (median 60.5 pg/ml, range 57.60-68.5 pg/ml; n=19), followed by diabetic patients with quiescent PDR (median 43.20 pg/ml, range 41.50-45.00 pg/ml; n=3) and those with diabetes without PDR (median 23.1 pg/ml, range 22.10-25.05 pg/ml; n=28) and controls (median, 15.6 pg/ml, range 14.75-16.8 pg/ml; n=29). Significant differences were found among these four groups <0.0001, (Kruskal-Wallis test). The vitreous concentration of ANP was significantly higher in patients with active PDR than in patients with quiescent PDR (p=0.006, Mann-Whitney U test). Significant differences in vitreous ANP were also recorded between the diabetic patients without PDR and the control group p<0.0001, (Mann-Whitney U test; Figure 1A). Higher vitreous protein concentrations were also detected in the diabetes with active PDR group (median

2.03 mg/ml, range 1.65-2.15 mg/ml; n=22) than in the diabetes without PDR (median 0.80 mg/ml, range 0.68-0.92 mg/ml; n=28) <0.0001, (Mann-Whitney U test), or control group (median 0.6, range 0.40-0.80 mg/ml; n=29) p<0.0001, (Mann-Whitney U test). Moreover, these significant differences p<0.0001, (Mann-Whitney U test) persisted when we compared ANP/protein ratios among the groups (diabetes with active PDR 59.46±3.42 pg/mg protein, diabetes with quiescent PDR 46.2±1.50 pg/mg protein, diabetes without PDR 25.3±1.04 pg/mg protein, and control 13.9±1.49 pg/mg protein; Figure 1B).

As shown in Table 1, there were no significant differences in plasma ANP among the four study groups. Further, no significant correlation was observed between plasma and vitreous ANP concentrations in the diabetes with active PDR, diabetes with quiescent PDR, diabetes without PDR, and control groups (Spearman correlation: r=0.32, p>0.05; r=0.48, p>0.05; r=0.29, p>0.05; and r=0.20, p>0.05, respectively). We did not observe significant differences in ANP levels between type 1 and type 2 diabetic patients either in plasma or the vitreous humor.

ANP in epiretinal membranes from diabetic patients with PDR: Serial sections were cut through the ERMs and immunostained sequentially with a monoclonal antibody against ANP and a panel of monoclonal antibodies to the following cellular intermediate filaments: glial fibrillary acidic protein (GFAP), a glial cell marker, and cytokeratin (an epithelial marker). Histological examination showed that all the ERMs contained heterogeneous cell populations with diverse morphologic characteristics: macrophages, RPE cells, glial cells, fibroblast-like cells, and some blood vessels.

All 10 epiretinal membranes from diabetic patients with PDR were positive for ANP. Two representative cases are presented. Figure 2 shows a section of a vascularized epiretinal membrane from a 52 year old man with type 1 diabetes of 20 years duration. Most of the vascular EC in this membrane were immunopositive for ANP, including vessels of larger caliber and those that appear to be of capillary size. ANP labelling, however, is not limited to the vascular endothelium, since vascular smooth muscle cells (VSMC) also stain positively (Figure 2B). Moreover, ANP was detected in most fibroblast-like cells, despite some being immunonegative for ANP, as shown in Figure 2D.

The image in Figure 3 is a section through a fibroinflammatory, glial epiretinal membrane from a 20 year old man with PDR. Macrophages showed intense immunoreactivity for ANP (Figure 3B). Serial sections from this specimen were immunostained for GFAP (Figure 3E) and cytokeratin (Figure 3G). In many cases, the same structures showed positive staining for both ANP (Figure 3D) and GFAP (Figure 3E). Similar observations were made when the sections were immunostained for cytokeratin. RPE cells were also immunopositive for both ANP (Figure 3F) and cytokeratin (Figure 3G) at apparently identical locations.

Positive ANP immunostaining was abolished when the sections were incubated with either antigen-absorbed antibodies or normal rabbit serum instead of primary antibody (Figure 2C, Figure 2E, Figure 3C).

Thus, ANP appears to be located at vascular EC, VSMC, RPE, glial, and fibroblast-like cells in all ERMs, yet seems to be absent from the extracellular matrix.

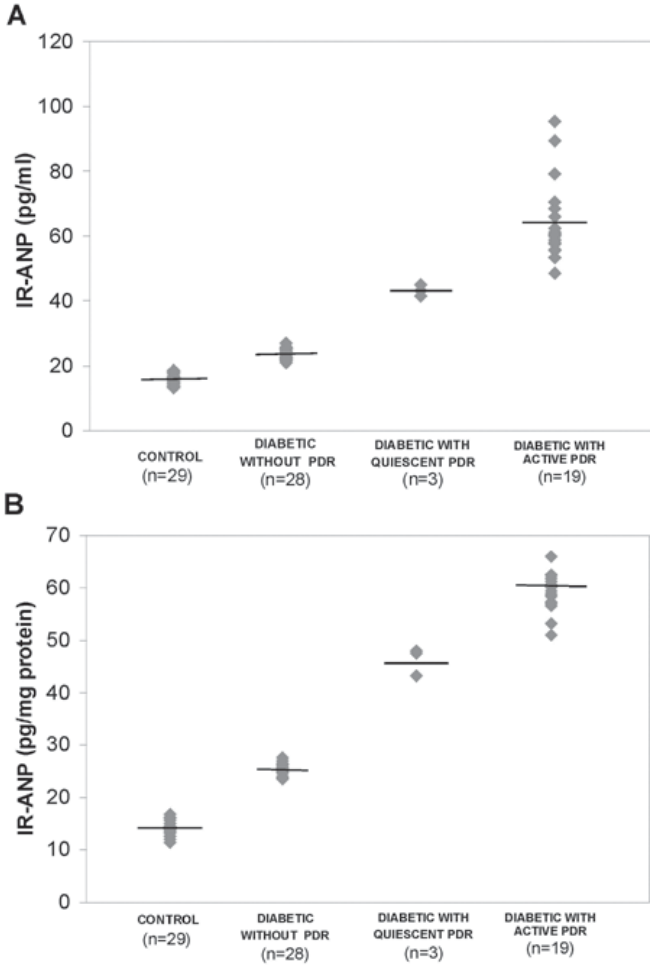

Figure 1. Vitreous humor ANP levels in diabetic patients with and without PDR and in non-diabetic patients. Vitreous humor ANP levels in diabetic patients with and without PDR and in non-diabetic patients. **A**: ANP levels are expressed as IR-ANP pg/ml. **B**: ANP levels are expressed as IR-ANP pg/mg intravitreous protein. The concentration of ANP in the humor vitreous remained high in diabetic patients after adjusting for intravitreous protein concentration. The horizontal bars mark the mean of each group. The table below summarizes all possible statistical comparisons (Mann-Whitney U test, Bonferroni correction) between the groups. All six p-values in the Table represent a significant difference after the Bonferroni correction.

	Diabetic		
	No PDR	Quiescent	Active PDR
Non-diabetic Controls	<0.0001	0.005	<0.0001
Diabetic No PDR	-	0.005	<0.0001
Quiescent	-	-	0.006

DISCUSSION

The development of PDR is a complicated process, involving several types of growth factors [2,4] and autoimmune responses [29]. Many criteria implicate growth factors in retinal neovascularization including their presence or up-regulation in the vitreous humor of patients with PDR and their detection in ERMs from these patients [2-6]. Given that ANP is an antiangiogenic [11] and vascular antipermeability factor [25], we suggest that this peptide may have a role in the vascular pathobiology of diabetic retinopathy.

The present report is the first demonstration of significantly increased vitreous ANP levels in patients showing active neovascularization. Diabetic patients without PDR were also found to have significantly higher vitreous ANP levels than non-diabetic patients. The breakdown of the blood-retinal barrier and increased vessel permeability that occur in diabetic retinopathy could allow the passage proteins from the blood to the eye chambers. Indeed, patients with PDR had a vitreous protein concentration that was about 3 times the normal level, though vitreous ANP levels were still higher in PDR patients after adjusting for vitreous protein concentration. We could speculate that raised ANP levels in patients with PDR is an unspecific event that reflects the breakdown of the blood-retinal barrier and subsequent influx of plasma proteins into the vitreous humor. However, our results argue against this hypothesis since the concentration of ANP in the vitreous fluid remained high in PDR patients after adjusting for protein concentration. ANP diffusion from plasma can be ruled out since we observed no difference in plasma ANP among the four patient groups, and there was no correlation between plasma and vitreous ANP concentrations in diabetic or non-diabetic patients. It would therefore appear that the main contributing factors to the high vitreous ANP concentration observed in patients with PDR are the intraocular ANP synthesis and/or an increase in the release of ANP into the vitreous, rather than diffusion from the blood. Moreover, the increase in vitreous ANP levels in patients with PDR may result from less degradation of ANP probably due to a decrease of the neutral endopeptidase (NEP; EC 3.4.24.11), which is involved in the

TABLE 1. PLASMA CONCENTRATIONS OF ANP

Group	n	Age	Plasma ANP (pg/ml)
Control Subject	29	50.5 (20-70)	36.40 (10.50-62.30)
Diabetic Subject without PDR	28	60.2 (25-70)	37.60 (24.49-50.27)
Diabetic Subject with quiescent PDR	3	63.8 (20-70)	47.80 (32.60-63.00)
Diabetic Subject with active PDR	19	56.2 (20-70)	49.50 (19.50-79.10)

The levels of ANP in plasma were not significantly different between any two of the the four groups included in this study. Plasma ANP levels and age are expressed as medians and range.

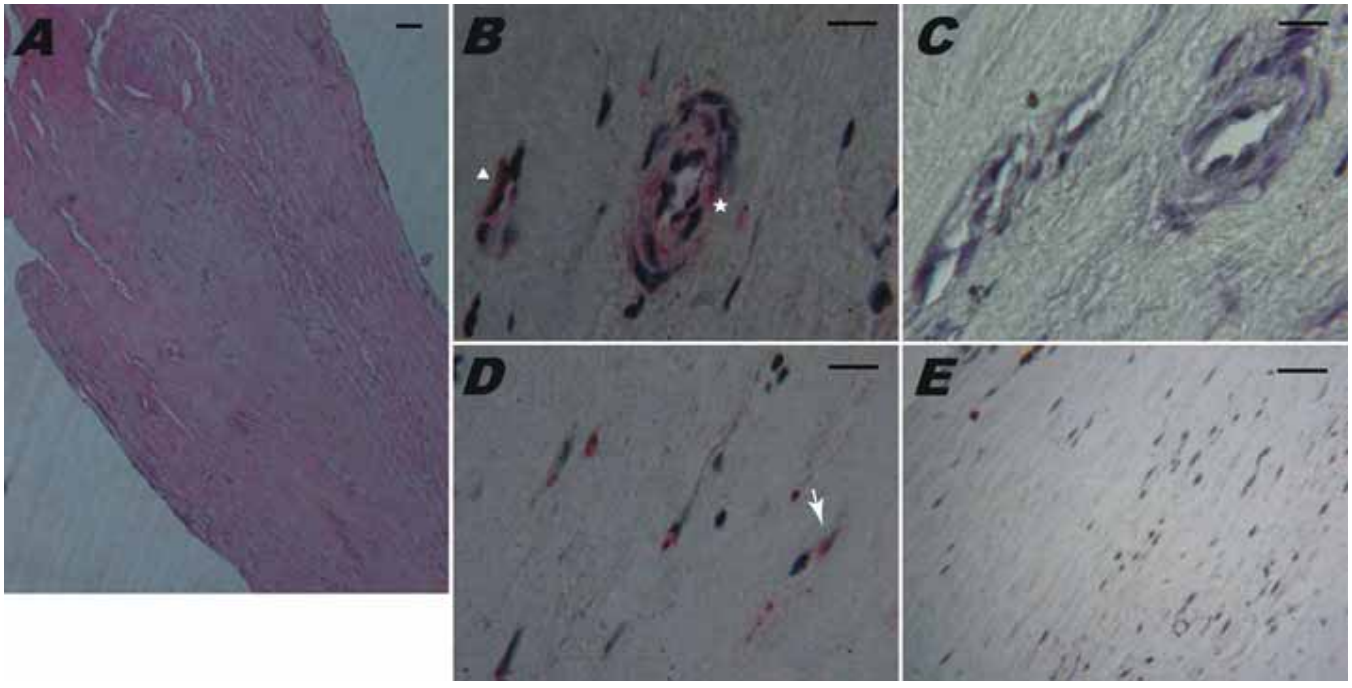

Figure 2. Localization of ANP-Immunoreactivity in epiretinal membranes. **A** Hematoxylin and eosin stain. Scale bar represents 100 μm. **B** Positive staining for ANP was observed in both vascular endothelial cells (EC; white triangles) and vascular smooth muscle cells (VSMC; white stars) **D**: ANP staining shown by fibroblast-like cells (arrows). **C, E** Positive immunostaining was abolished when the adjacent sections of the same membrane were incubated either with anti-ANP antibody adsorbed with 10 nM ANP or with normal rabbit serum instead of primary antibody. Scale bar in **B-E** represents 50 μm. Sections counterstained with Mayer's Hematoxylin.

degradation of natriuretic peptides [12] and from a decrease of NPRC, which regulates the clearance of ANP in the eye [23].

Notably, the mean plasma ANP level of our PDR patients was similar to that of control group. In agreement, it has been reported that plasma ANP levels are in the range of normality in normotensive type 1 and type 2 diabetic patients [30].

Interestingly, the narrow range of ANP levels in the vitreous samples contrasts with the wide range that we observed in plasma samples. This could be explained by the fact that the ANP levels detected in plasma comes mainly through the heart and its secretion is influenced by many systemic factors such as age, posture, dietary sodium loading and the angiotensin-renin system [12]. However, the vitreous ANP levels could be regulated by other local ocular factors that probably are less numerous that the systemic ones.

Very recently, we reported the expression of NPR transcripts along with ANP, BNP, and CNP mRNA in the human retina and were able to detect NPs in the neural retina, as well as in the glial and vascular elements of the normal adult retina [20]. Thus, locally synthesized intraocular ANP and its receptors could directly participate in neovascular ocular disease. However, the vitreal concentration of ANP recorded here in PDR eyes (20 pM) is not comparable to levels effective at promoting antiangiogenesis in in vitro assays (100 nM) [11]. This might indicate that the increased ANP levels in the vitreous humor of patients with PDR would not be sufficient to compensate the angiogenic effect of growth factors such as VEGF.

Hyperglycemia needs to be considered as factor possibly leading to increase vitreous ANP in patients with diabetes. Hyperglycemia, the most crucial risk factor for microvascular diabetic complications, leads to an increased plasma ANP concentration in Type 1 diabetes mellitus [31]. Further, rats with hypoxia-induced pulmonary hypertension show increased ANP mRNA expression in the right ventricle [32]. It has also been

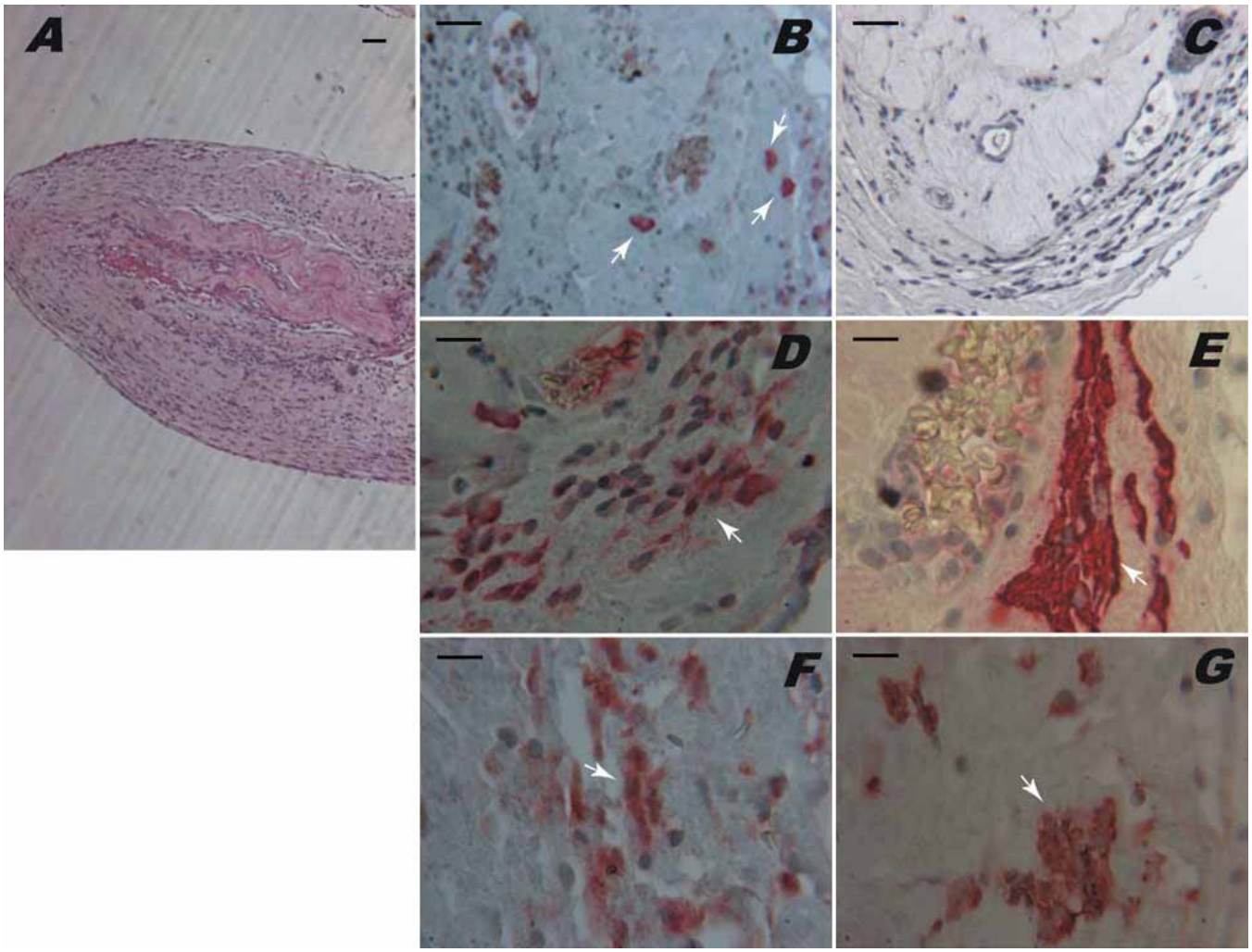

Figure 3. ANP, GFAP, and cytokeratin immunoreactivities in epiretinal membranes. **A**: Hematoxylin and eosin. Scale bar represents 100 μm. **B**: Positive staining for ANP shown by macrophages (arrows). **D, E**: Positive staining for both ANP and GFAP shown by glial cells. **F, G**: Positive staining for both ANP and cytokeratin shown by RPE cells. **C**: Positive immunostaining was abolished when the adjacent sections of the same membrane were incubated either with anti-ANP antibody adsorbed with 10 nM ANP or with normal rabbit serum instead of primary antibody. Scale bar in **B-G** represents 50 μm. Sections counterstained with Mayer's Hematoxylin.

established that ANP protects against hypoxic injury in freshly isolated rat hepatocytes by maintaining Na^+ homeostasis through the involvement of both NPRA and NPRC receptors [33]. It is therefore possible that the increased ANP levels we observed in the vitreous humor of our patients with PDR were triggered by a series of events in which hyperglycemia is followed by hypoxia. Retinal photocoagulation could also contribute to those high ANP levels.

Diabetic epiretinal membranes are the main pathological changes that occur in PDR, which may provoke severe complications resulting in blindness. The mechanism of ERM formation in PDR is still unknown, but the first step is thought to be neovascularization that takes place when retinal vessels interact with the vitreous humor. Several angiogenic factors play a key role in this process [34,35].

Histopathology and immunohistochemistry studies have demonstrated that these membranes consist of different types of cells such as fibroblasts, glial cells, RPE cells, macrophages, and different types of extracellular matrices [36]. As far as we are aware, ours is the first demonstration that ANP occurs in most of the cells that make up ERMs.

We were able to detect the presence of ANP in most of the vascular EC and VSMC of the ERMs. We also detected ANP in fibroblast-like cells and in macrophages of the ERMs. We also found that RPE cells express ANP in ERMs from patients with PDR, and glial cells also showed positive staining for ANP. Thus, reports that ANP inhibits astrocyte proliferation [16] suggests the possibility that ANP is involved in the abnormal glial proliferation that leads to the formation and development of PDR membranes.

In conclusion, the presence of ANP was detected in human vitreous humor, significantly raised levels being noted in patients with active PDR. Diabetic patients without PDR were also found to have significantly higher vitreous ANP levels than non-diabetic patients. Our findings indicate that the main contributing factors to this higher ANP concentration in diabetic patients are the intraocular ANP synthesis and/or an increase in the release of ANP into the vitreous, rather than diffusion from the blood. ANP localizes within epiretinal proliferative tissue in diabetics. Most cells of this ERM tissue, that is, vascular EC, VSMC, RPE cells, glial cells, macrophages and fibroblast-like cells, express ANP, which may have a role in regulating membrane growth in PDR. Further studies are needed to define ANP's full pathophysiological role in the PDR.

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