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Differences between type 1 and type 2 diabetics in retinal neovascular tissue and vitreous humour

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ABSTRACT

Aims

Evaluate the histopathology of neovascular tufts and vitreous samples collected from diabetic patients.

Methods

Vitreous samples and neovascular tufts were collected from type 1 (n=13) and type 2 diabetics (n=17) with proliferative retinopathy and controls with macular hole (n=5). Neovessels were analyzed using immunohistochemistry and vitreous samples with Enzyme-linked immunosorbent assay (ELISA). Main outcome measure was to find differences in growth factors in proliferative retinopathy between type 1 and type 2 diabetics.

Results

VEGF-A was most strongly present in the samples in type 1 diabetics. In type 2 diabetics, VEGF-D was more abundantly present than in type 1 diabetics. Also ANG-2 was abundantly present. Macrophages and nuclear factor kappa B (NFκB) were found indicating the presence of an inflammatory process in the neovascular tissues.

Conclusions

VEGF-A and ANG-2 are equally important in neovascular process in both type 1 and type 2 diabetics. In type 2 diabetics, VEGF-D is abundantly present. To achieve better control of diabetic retinopathy, it might be beneficial to target also against the actions of ANG-2 and VEGF-D.

Introduction

The pathogenesis of diabetic retinopathy is characterized by alterations in retinal microvasculature. First sign of the diabetic retinopathy is a reduction of pericytes, leading to microvascular hyperpermeability, occlusion and hypoxia. When over 50% of the pericytes have been damaged, vascular cell proliferation begins.[1] Patients with retinal neovascularization have elevated levels of VEGF-A in their vitreous humour.[2] Vascular endothelial growth factors (VEGFs) refer to a family of polypeptides with six members: VEGF-A, -B, -C, -D, placenta growth factor (PlGF) and a viral homologue VEGF-E. Their signalling is mediated via tyrosine kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3 (Flt-4).[3] No information is available about other members of the VEGF family than VEGF-A in diabetic eyes. VEGF-B seems not to play an important role in retinal neovascularization.[4] VEGF-D is a close homologue to VEGF-C, produced as a prepropeptide and further processed to a biologically active form that binds to VEGFR-2 and -3 present on endothelial cells.[5,6] VEGF-D induces angiogenesis and lymphangiogenesis.[7,8] Additionally, VEGF-D has been found in smooth muscle cells of arteries and arterioles.[9,10] In the eye, the role of VEGF-D remains unclear. Similarly, the role of angiopoietin (ANG)-1 and ANG-2 remains unclear in diabetic retinopathy.

The aim of this study was to evaluate the presence of angiogenic factors in the proliferative neovascular tissues and vitreous humour of diabetic patients and to find out their possible roles in type 1 and type 2 diabetes.

Methods

Patient data

Informed consent was obtained from the subjects. The study protocol was approved by the Ethical Committee of Kuopio University Hospital and the study conforms with the principles outlined in the Declaration of Helsinki.

Vitreous sample of 13 type 1 diabetics and 17 type 2 diabetics was studied. Additionally, neovascular tissue of 8 type 1 and 10 type 2 diabetic eyes was analyzed. Mean age of the patients in type 1 diabetics was 34.69 ± 8.75 years (mean \pm standard deviation (SD)) and in type 2 diabetics 60.41 ± 8.43 . The average duration of diabetes was 24.15 ± 5.94 years in type 1 and 17.50 ± 8.39 years in type 2 diabetics. Inclusion criterias were proliferative diabetic retinopathy and clinically observed retinal neovascularisation. Vitrectomy was indicated for all patients. All patients had had panretinal laserphotocoagulation before the surgical operation. Over all, the diabetic control was poor with the patients participating this study, average glucosylated haemoglobin (GHbA1c) being $10.16 \pm 1.9\%$ in type 1 and $9.15 \pm 1.9\%$ in type 2 diabetics. Exclusion criteria were previous vitreoretinal surgery, opening of the posterior lens capsule and less than 1 month old vitreous hemorrhage. Patient's age, gender, type of diabetes, duration of diabetes and the last value of GHbA1c were recorded. Vitreous control samples were collected from five patients undergoing pars plana vitrectomy and membranectomy due to idiopathic macular hole. Control tissue samples were analysed from the retinas of two non-diabetic normal eyes removed from trauma patients.

Vitreous body and neovascular samples

Vitreous humour samples were collected at the beginning of the pars plana vitrectomy into a 2 ml aspiration syringe. The sample was stored at -70°C until analysed. A fibrovascular membrane was isolated and grasped with vitreous forceps to be pulled out from a sclerotomy. The

sample was immediately covered with 4% PFA-sucrose, embedded in paraffin and processed for immunohistochemistry.[11]

Immunohistochemical analyses

Samples were immunostained with antibodies against endothelial cells (CD31, clone JC70A, 1:50, Dako, Carpinteria, CA) macrophages (CD68, clone KP1, 1:50, Dako, Carpinteria, CA), smooth muscle cells (α -SMA, clone 1A4, 1:200, Sigma, St Louis, MO), VEGF (VEGF-A, clone sc-7269, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), VEGF-B (VEGF-B, clone sc-1878, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), VEGF-C (VEGF-C, clone sc-1881, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), VEGF-D (VEGF-D, clone 78923.11, 1:500, R&D Systems, Minneapolis, MN), placenta growth factor (PlGF, clone sc-1880, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), platelet derived factor B (PDGF-B, 1:100, R&D Systems, Minneapolis, MN), ANG-1 (ANG-1, clone sc-6319, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), ANG-2 (ANG-2, clone sc-7015, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), Tie-1 (Tie-1, clone 88016, 1:25, R&D Systems, Minneapolis, MN), Tie-2 (Tie-2, clone 83715, 1:25, R&D Systems, Minneapolis, MN), hypoxia inducible factor (HIF-1 α , clone H1 α 67, 1:100, Neomarkers, Fremont, CA), vascular endothelial growth factor receptors -1 (Flt-1, clone sc-316, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), -2 (KDR, clone sc-6251, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and -3 (Flt-4, clone sc-321, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), nuclear factor κ B (NF- κ B, clone 20, 1:250, BD Biosciences, San Jose, CA) and proliferating cell antigen (anti-Ki-67, clone 7B11, 1:100, Zymed Laboratories, Carlsbad, CA).[12]

Controls for immunostainings included stainings where the first antibody was omitted and staining with species and class-matched irrelevant immunoglobulins.[11] Photographs of the histological sections were taken as described.[13]

ELISA assays

VEGF-A, VEGF-D, ANG-1 and ANG-2 were analyzed with Enzyme-linked immunosorbent assay (ELISA) from vitreous samples as described.[13]

Statistical Analyses

Statistical significances were evaluated using Kruskal-Wallis test followed by Mann-Whitney U-test where appropriate. Correlation analyses were performed using Spearman's correlation analysis. A value of $P < 0.05$ was considered statistically significant.

Results

Type 1 diabetics

There were detectable amounts of VEGF-A, ANG-1 and ANG-2 in the vitreous samples but there was no difference between type 1 and type 2 diabetics (Figure 1). VEGF-D was not found from the vitreous samples. ANG-2 was found in low amounts also in the control eyes. However, the difference between the control samples and the diabetic samples was significant ($p < 0.05$). The amount of ANG-2 in the vitreous humour had a positive correlation with the number of endothelial cells ($p < 0.05$) (Figure 2). The amount of VEGF-A in the vitreous humour had no correlation with other variables. GHbA1c levels did not correlate with the growth factor levels in the vitreous humour or neovascular samples.

In the neovascular samples, numerous CD31-positive endothelial cells and several CD68 positive macrophages were present (Figure 3). In addition, transcription factor NF κ B was also present in the nuclei in the samples indicating an ongoing inflammatory process in the neovascular tissues (Figure 3). There were no NF κ B positive cells in the control eyes. A positive staining for Ki-67 indicates a proliferative process in the tufts in all patients. Almost every sample had HIF-1 α stained nuclei reflecting hypoxic conditions in the eye. There were no Ki-67 or HIF-1 α positive cells in the control retinas (Figure 3). α -SMA positive pericytes were found in the neovascular tufts in the same areas as endothelial cells. There were more endothelial cells than activated pericytes in the tissues. Endothelial cells and α -SMA positive pericytes were also present in non-diabetic eyes (Figure 3).

Number of endothelial cells and age had a significant negative correlation ($p < 0.05$). GHbA1c levels ($p < 0.01$) had a positive correlation with the number of endothelial cells (Figure 4).

VEGF-A was most strongly present in the samples. Also VEGF-D and ANG-2 were abundantly present (Table 1).

ID	Age, years	Sex	Type of DM	Duration of DM, years	GHbA1c, %	CD-31 sma	CD-68	VEGF-A ng/ml	ANG-2 ng/ml	VEGF-A	VEGF-B	VEGF-C	VEGF-D	PIGF	PDGF-B	VEGFR-1	VEGFR-2	VEGFR-3	HIF-1 α	Ki-67	M α B	ANG-1	ANG-2	Tie-1	Tie-2
19	37	F	1	31	12.3	++	+++	3.33	2.72	++	0	0	+	0	0	+++	+++	+++	++	+++	+++	+++	+++	0	0
33	25	F	1	17	14.0	+++	+++	3.15	5.97	+++	++	++	+	+	+	+++	+	+++	++	+	+++	++	+++	++	++
34	58	F	1	27	9.1	++	+	0.38	0.08	+++	-	-	++	-	+	+++	+++	+++	+	+	+++	+	++	+	+
44	39	M	1	27	9.0	+++	++	14.10	1.31	+++	0	0	+	0	0	+++	+++	+++	0	0	0	+	0	0	0
50	28	M	1	15	10.0	+++	+++	9.67	3.34	+++	+	+++	++	+	-	+++	+++	++	+	+	+++	++	+++	+++	+++
51	26	M	1	22	8.2	++	++	3.33	2.72	0	0	+	++	+	-	+++	++	++	+	+	+++	++	++	++	++
52	38	M	1	22	11.2	+++	+++	2.12	2.20	+++	+	+	+	+	-	+++	+++	+++	++	++	+++	+	+++	+++	+++
56	38	M	1	23	10.8	++	+++	3.33	2.72	++	+	+	+++	+	-	+++	++	+++	+	+	+++	+	+++	+++	+++
23	58	M	2	10	7.9	++	+++	0.96	5.20	++	++	++	++	+	++	+++	++	+	+++	+++	+++	+++	++	++	+++
27	48	M	2	9	10.3	++	++	2.93	2.30	+	+	-	++	++	++	++	++	+++	++	++	+++	++	++	+	+
35	51	F	2	10	10.8	++	+	1.17	2.71	+	-	-	++	-	-	++	++	++	+	-	+++	+	+	+	-
39	62	M	2	23	12.8	+++	+++	0.86	1.75	++	+	+	+++	+	+	++	+	++	+	+	+++	++	++	++	+
40	69	F	2	14	8.9	++	++	3.85	3.14	++	++	+	+++	++	++	++	++	++	++	++	++	++	++	+++	++
43	67	F	2	27	11.2	+	++	0.72	0.70	++	+	0	+	+	-	+	-	+	-	+	+	+	0	++	+
45	49	M	2	17	10.0	++	+	14.36	11.78	+++	-	+	+	+	-	+	+++	++	+	+	+++	++	++	+	++
48	72	M	2	29	8.2	+	++	6.32	0.78	+++	-	-	0	0	0	++	0	+	0	+	++	0	0	+	0
53	48	M	2	16	7.7	+++	+++	2.62	2.30	+++	++	++	+++	++	-	+++	++	++	+++	+++	+++	0	+++	++	+++
54	68	F	2	28	11.2	+++	++	2.19	1.95	0	0	+	+	+	-	+++	+	+++	+	+	+++	++	+++	++	+
c	60	F	-	-	-	+	-	-	-	+	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-

Table 1. Patient data. c=control, M=male, F=female; DM=diabetes mellitus; GHbA1c=glucosylated haemoglobin; CD31=endothelial cells; α -sma=active pericytes; CD-68=macrophages; VEGF-A=vascular endothelial growth factor A; VEGF-B=vascular endothelial growth factor B; VEGF-C=vascular endothelial growth factor C; VEGF-D=vascular endothelial growth factor D; PIGF=placenta growth factor; PDGF-B=platelet derived growth factor B; VEGFR-1=vascular endothelial growth factor receptor 1; VEGFR-2=vascular endothelial growth factor receptor 2; VEGFR-3=vascular endothelial growth factor receptor 3; HIF-1 α =hypoxia inducible factor; Ki67=proliferating cell antigen; Nf κ B=nuclear factor kappa B; ANG-1=angiopoietin 1; ANG-2=angiopoietin 2; Tie-1=tyrosine kinase receptor 1; Tie-2=tyrosine kinase receptor 2. 0 = no sample; - = not detectable staining; + = weak staining; ++ = moderate staining; +++ = strong staining.

Overall order of the growth factors was VEGF-A > ANG-2 > VEGF-D, ANG-1 > VEGF-C > VEGF-B, PIGF > PDGF-B. ANG-1 and ANG-2 were mostly present in the same areas as pericytes. Immunostaining for PDGF-B was weak (Figure 5).

VEGFR-2 mediates most of the angiogenic effects of VEGFs in blood vessels. Accordingly, several VEGFR-2 positive cells were found in the neovascular tufts. Strong stainings were also found for VEGFR-1 and VEGFR-3. The presence of VEGFR-1 and VEGFR-3 was significantly higher in type 1 diabetics than in type 2 diabetics ($p < 0.05$). Tie-2 receptor was present in the samples and Tie-1 was found at the same level as Tie-2. Both of these receptors located in the same areas as ANG-1 and ANG-2 (Figure 5). VEGF-A and VEGFR-1 were also present in the control samples but much less abundantly than in the diabetic tissues.

Type 2 diabetics

The amounts of VEGF-A, VEGF-D, ANG-1 and ANG-2 in the vitreous samples did not differ between type 1 and type 2 diabetics in ELISA arrays (Figure 1). Unlike type 1 diabetics, amount of ANG-2 in the vitreous humour did not correlate with the number of endothelial cells. VEGF-A and ANG-2 in the vitreous humour correlated positively ($p < 0.01$) (Figure 2). GHbA1c levels, age or duration of diabetes did not correlate with the presence of VEGF-A in the vitreous humour. ANG-1 was found in the vitreous humour in much lower levels than other measured growth factors and it did not correlate with any other variables tested.

The number of endothelial cells in fibrovascular tufts was significantly lower than in type 1 diabetics ($p < 0.05$). Despite of this, there were no differences in the presence of NF κ B, CD68 positive macrophages, number of proliferating cells, immunostaining for HIF-1 α or α -SMA stainings between type 1 and type 2 diabetics (Figure 3). Age of patient and duration of disease had a positive correlation ($p < 0.01$) but neither had effect on the number of endothelial cells. Unlike in type 1 diabetics, GHbA1c levels did not correlate with the number of endothelial cells (Figure 4).

In type 1 diabetics, VEGF-A was most strongly present growth factor. Instead, VEGF-D was more abundantly present in the neovascular tissues of the type 2 diabetic patients than in those of the type 1 diabetic patients. Similarly also the amount of VEGF-B was increased in type 2 diabetics compared to type 1 diabetics in the neovascular tissues and the presence of VEGF-C was higher in type 1 diabetics than in type 2 diabetics (Table 1). All VEGF receptors were abundantly present but in lower levels than in type 1 diabetics. Presence and location of PDGF-B, ANG-1, ANG-2, Tie-1 and Tie-2 in type 2 diabetics were similar to type 1 diabetics (Figure 5). In type 2 diabetics, overall order of the growth factors was VEGF-D, VEGF-A > ANG-2 > ANG-1 > PLGF > VEGF-B > VEGF-C > PDGF-B.

Discussion

Type 1 diabetics have much higher risk to develop proliferative retinopathy than type 2 diabetics[14] and in type 2 diabetics, diabetic maculopathy is the most prevalent sight-threatening manifestation of retinopathy.[15] In this study, VEGF-A was most abundantly present in type 1 diabetics. However, VEGF-D was more abundantly present in the neovascular tissues of the type 2 diabetic patients than in type 1 diabetic patients. Both VEGF-A and VEGF-D angiogenic effects are mediated by VEGFR-2. Signalling of VEGF-A by VEGFR-2 is fast and transient whereas signalling of VEGF-D is slower and more long-lasting.[16] In addition, Ikeda et al. have shown that in the human retinal pigment epithelium (RPE), cell-cell adhesion and attachment regulate the VEGF-D expression, suggesting that the breakdown of these interactions causes the overexpression of VEGF-D.[17] In the pathogenesis of vascular diseases in type 2 diabetes, the damage and dysfunction of endothelial cells have shown to play a crucial role. [18] Similarly to studies in RPE cells, VEGF-D may contribute to the breakdown of the inner blood retina barrier in diabetic patients leading to maculopathy and oedema in type 2 diabetics. Macrophages secrete many angiogenic factors, including VEGF-D.[19] Also in our patients, the cells with positive staining for VEGF-D, CD68 and CD31 were located in the same areas indicating that VEGF-D is produced by several different cell types in the neovascular tissues. Although the precise role of inflammation in the development of diabetic retinopathy is still unclear, it is possible that inflammatory mechanisms accelerate proliferative retinopathy in type 2 diabetics by VEGF-D dependent pathways. On the other hand, the fact that type 1 diabetics had more VEGF-A in the neovascular tissues than VEGF-D, suggests that with type 1 diabetics, hypoxia is more important in the development of proliferative retinopathy than inflammation. In order to stop the proliferative process, it might be beneficial also to prevent inflammation in the eye especially in type 2 diabetics. The difference in growth factor expression between type 1 and type 2 diabetic retinopathy might contribute to the pathogenesis of these diseases and also to differences in their clinical course. In both types of

diabetics the immunoreactive VEGFR-1 and VEGFR-3 were abundantly present in the neovascular tufts. Expression of VEGFR-3 has also been found particularly in areas with leaky retinal microvessels in a previous study.[20] This might suggest the important role of VEGFR-1 and VEGFR-3 in the formation and maintenance of microvessels such as retinal capillaries.

VEGF-A and ANG-2 were equally abundant in the vitreous humour. The amount of VEGF-A in the vitreous humour was at the same level as in previous studies,[2] and also increased ANG-2 levels have been found in the vitreous humour of diabetic patients.[21] However, VEGF-D was not found in the vitreous humour in any patient. We cannot fully explain this phenomenon. However, it is known that VEGF-D binds to extracellular matrix components with high affinity.[6] The finding that VEGF-D is absent in the vitreous humour suggests that VEGF-D binds to retinal tissues more efficiently than the other growth factors in diabetic patients.

HIF1- α has been found in the epiretinal membranes of diabetic patients without previous laser photocoagulation,[22] and it was also present in this study despite of the previously performed panretinal laser photocoagulation. Although the retinal vessels are autoregulated in hypoxic conditions, it seems that in diabetic retinopathy, the normal attenuation mechanism of the eye fails leading to active angiogenic process and proliferation.

In conclusion, there were differences in the measured angiogenic factors between type 1 and type 2 diabetic retinopathy. In type 2 diabetics, VEGF-D was more abundantly present than in those of the type 1 diabetics. Also, ANG-2 seems to be an important factor in neovessel growth in both types of diabetes. These differences should be considered when developing new treatment options for patients with proliferative diabetic retinopathy. In type 1 diabetics, it is most likely that blocking VEGF-A, VEGFR-2 and ANG-2 can cause therapeutic effects but in type 2 diabetics, also preventing the action of VEGF-D and other VEGFRs could be beneficial.

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Competing Interest: None declared.

FIGURE LEGENDS

Figure 1. Elisa analyses of vitreous humour in type 1 diabetic (n=13), type 2 diabetics (n=17) and controls (n=5). VEGF-A, VEGF-D, ANG-2 and ANG-1 were analysed. $P < 0.05$ is considered statistically significant.

Figure 2. Scatter Plot analysis of type 1 and type 2 diabetic patients with proliferative retinopathy. A) Correlation between the number of CD-31 positive endothelial cells in the tissue/ mm^2 and ANG-2 in vitreous humour ($p < 0.05$). B) Correlation between ANG-2 and VEGF-A in vitreous humour ($p < 0.01$). $P < 0.05$ is considered statistically significant.

Figure 3. Neovascular tissues of diabetic patients with proliferative retinopathy and the retina of control patients. CD31 (A,B) shows lots of endothelial cells in the samples. Inflammatory response is strong in the diabetic tissues (macrophages; CD68 (C) and NF κ -B; (E)). Ki67 indicates active proliferation in the diabetic tissues (G). The tissues are also hypoxic (HIF1- α (I)). There are few active pericytes (α -SMA (K)) present in the diabetic retinas but none in the control retinas (L). There are no CD68 or NF κ -B positive cells in the control retinas (D,F). Furthermore, proliferative process or hypoxia is absent in the control tissues (H,J). Brown colour indicates positive staining. Scale bar = 0.3mm (applies to all panels).

Figure 4. Scatter Plot analysis of type 1 and type 2 diabetic patients with proliferative retinopathy. A) Correlation between duration of diabetes and the age of patients ($p < 0.05$). B) Correlation between the number of CD-31 positive endothelial cells in the tissue/ mm^2 and age of patients ($p < 0.05$). C) Correlation between the number of CD-31 positive endothelial cells in the tissue/ mm^2 and glycosylated haemoglobin (GHbA1c, %)($p < 0.01$). $P < 0.05$ is considered statistically significant.

Figure 5. Growth factors and their receptors in the neovascular tissues of diabetic patients with proliferative retinopathy. Expression of vascular endothelial growth factor (VEGF) -A is strong (A,B) in both types of diabetes. VEGF-B is more abundant in type 2 diabetes (C,D) and VEGF-C in type 1 diabetes (E,F). In type 2 diabetics, the expression of VEGF-D (G,H) is more abundant than in type 1 diabetics. Angiopoietin (ANG) -1 is equally abundant (I,J) and there was a strong staining for ANG-2 (K,L) in both types of diabetes. Placenta derived growth factor (PlGF) (M,N) and platelet derived growth factor (PDGF-B) (O,P) were less abundant in the tissues. Both Tie-1 (Q,R) and Tie-2 (S,T) were equally abundant in the tissues. The presence of VEGF receptor 1 (VEGFR-1)(Flt-1;U,V) was significantly higher in type 1 than in type 2 diabetics. In addition to VEGF receptor 2 (VEGFR-2)(KDR;W,X), also VEGFR-1 and VEGF receptor 3 (Flt-4;Y,Z) were abundantly present in the neovascular tissues. Brown colour indicates positive staining. Haematoxyline-eosin stained control sample (A). Scale bar = 0.3mm (applies to all panels).

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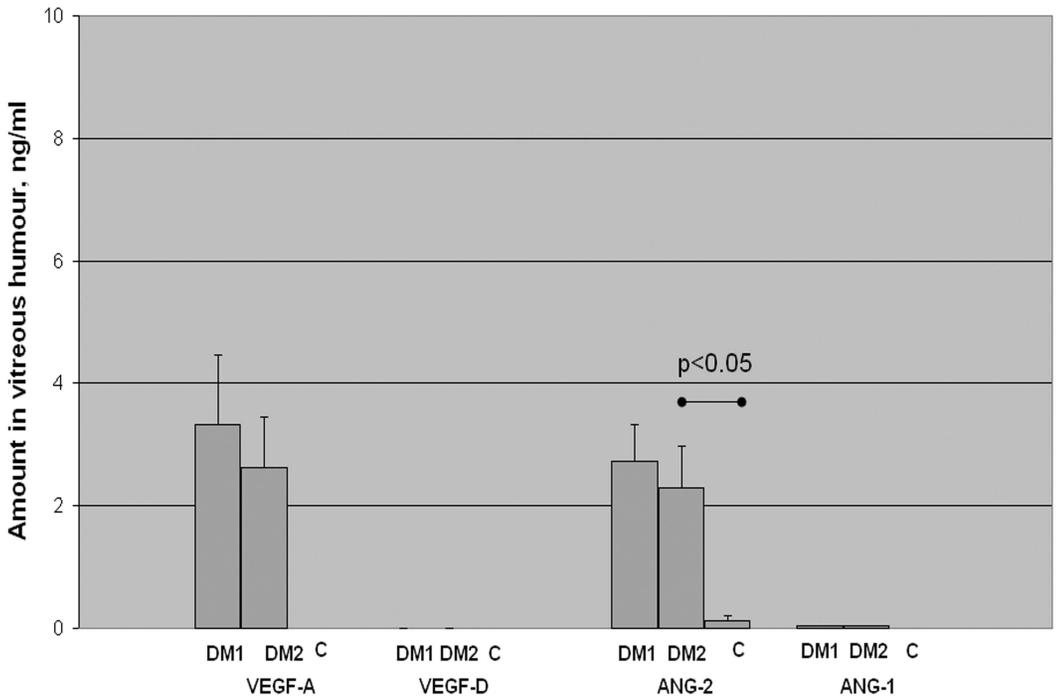


Figure 1.

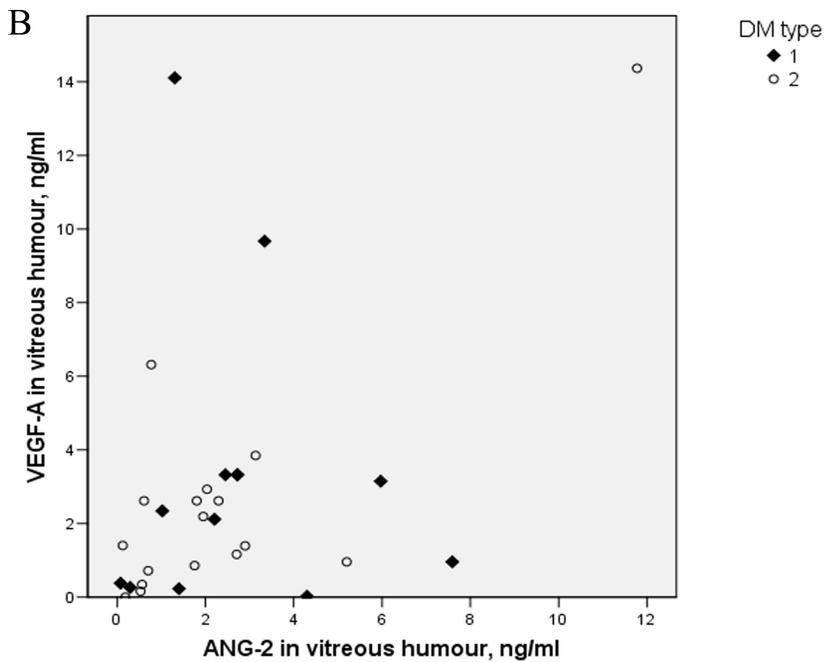
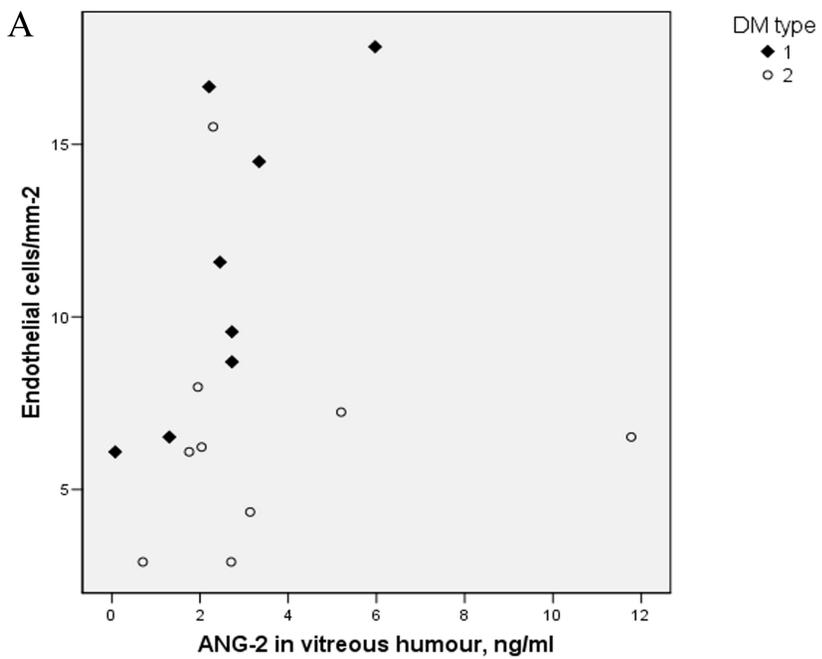


Figure 2.

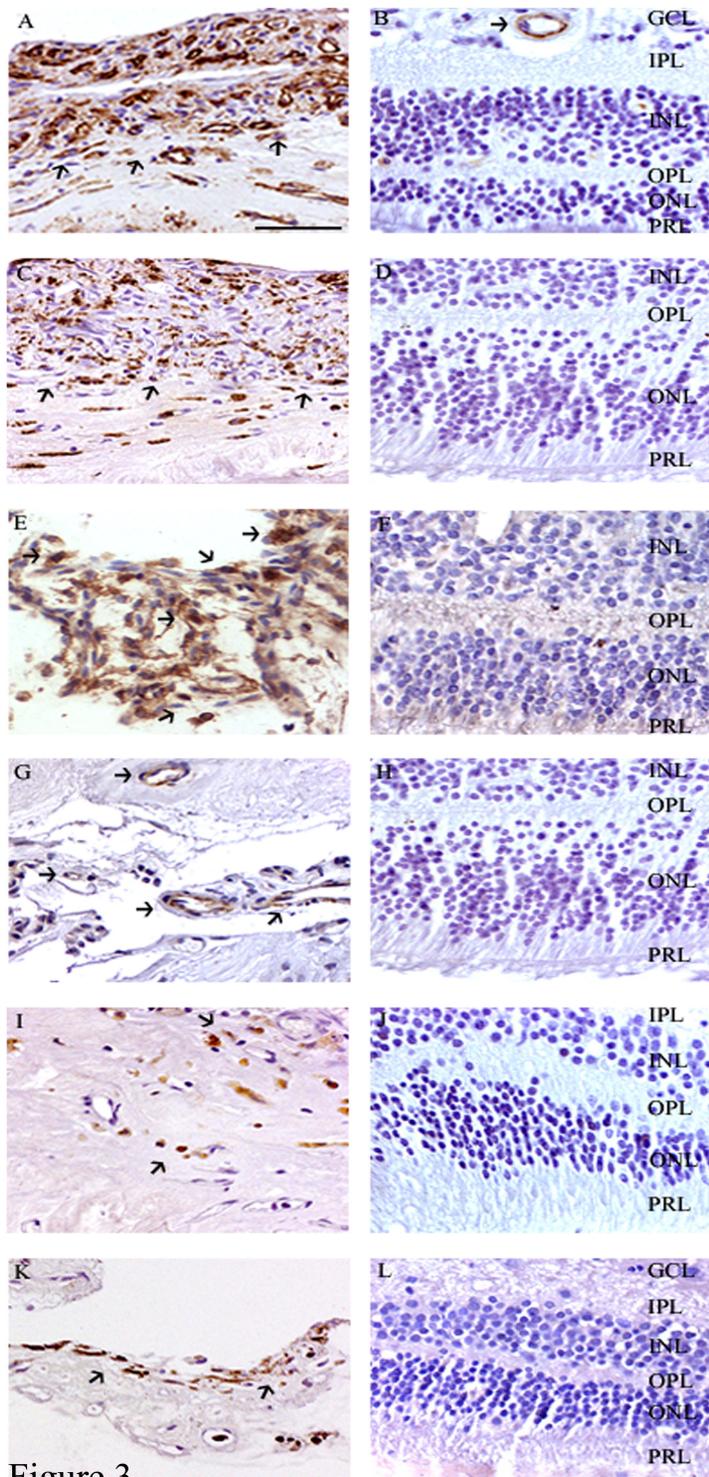


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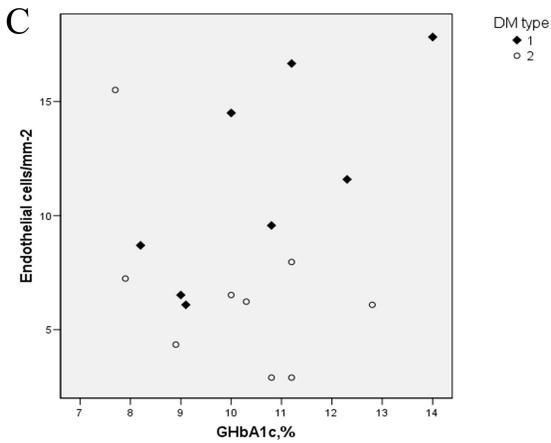
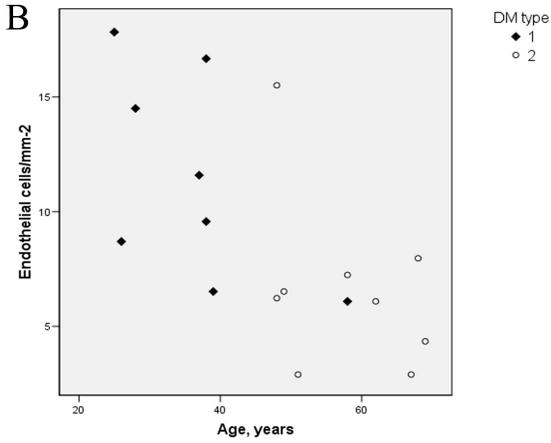
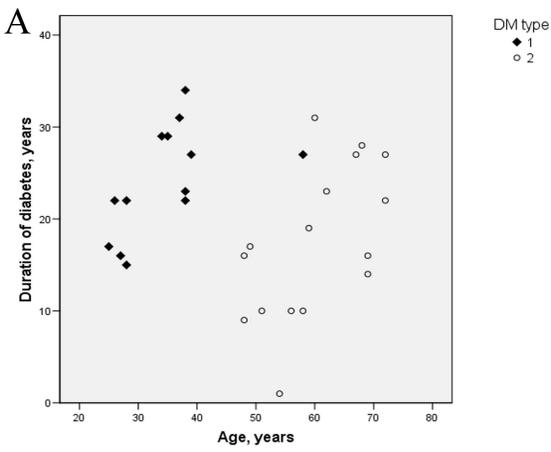


Figure 4.

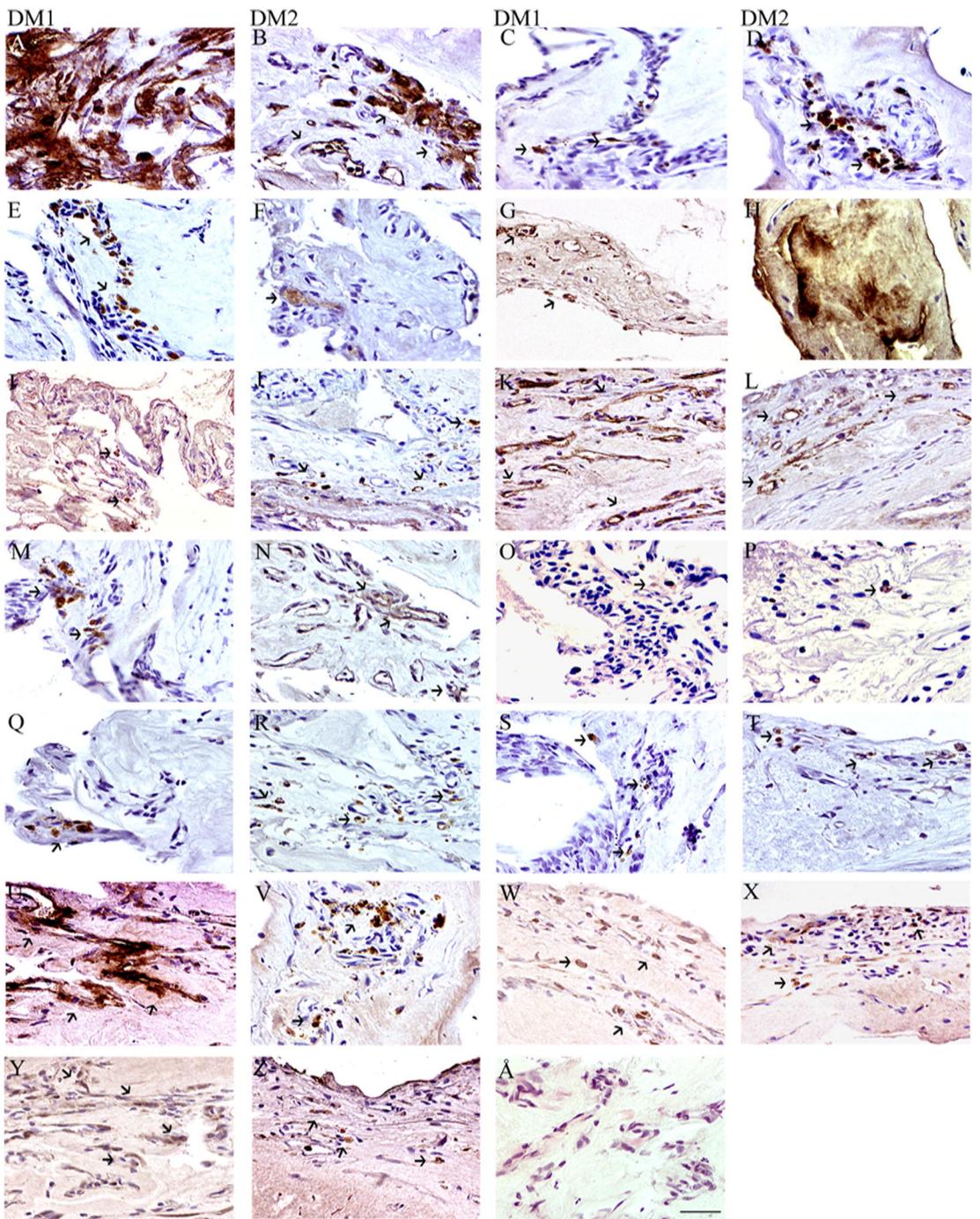


Figure 5.