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Immunohistochemical localization of low density lipoprotein receptor-related protein 1 and α_2-Macroglobulin in retinal and choroidal tissue of proliferative retinopathies☆

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ABSTRACT

The immunolocalization of the low density lipoprotein receptor-related protein 1 (LRP1) and its ligand α 2-Macroglobulin (α_2M) was examined in tissues from human donor eyes of normal, diabetic and sickle cell disease subjects. Streptavidin alkaline phosphatase immunohistochemistry was performed with a mouse anti-human LRP1 and rabbit anti-human α_2M antibodies. Retinal and choroidal blood vessels were labeled with mouse anti-human CD34 antibody in adjacent tissue sections. Mean scores for immunostaining from the pathological and control eyes were statistically compared.

LRP1 immunoreactivity was very weak to negative in the neural retina of normal subjects except in scattered astrocytes. LRP1 expression in diabetic eyes was detected in the internal limiting membrane (ILM), astrocytes, inner photoreceptor matrix, choriocapillaris and choroidal stroma. The ligand α_2M, however, was limited mainly to blood vessel walls, some areas of the inner nuclear layer (INL), photoreceptors, RPE–Bruch's membrane–choriocapillaris complex, intercapillary septa, and choroidal stroma. In sickle cell eyes, avascular and vascular retina as well as choroidal neovascularization (CNV) were analyzed. In avascular areas, LRP1 immunoreactivity was in innermost retina (presumably ILM, astrocytes, and Muller cells) and INL as well as RPE–Bruch's membrane–choriocapillaris complex and choroidal stroma. α_2M was very weak in avascular peripheral retina compared to vascularized areas and limited to stroma in choroid. In contrast, in areas with CNV, LRP1 immunoreactivity was significantly decreased in overlying retina and in RPE–Bruch's membrane and choroidal stroma compared to the controls, while α_2M was elevated in RPE–Bruch's membrane near CNV compared to normal areas in sickle cell choroid. The mean scores revealed that LRP1 and α_2M in neural retina were significantly elevated in astrocytes and ILM in diabetic eyes ($p \leq 0.05$), whereas in sickle cell eyes scores were elevated in ILM and INL ($p \leq 0.05$). In addition, α_2M immunoreactivity was in photoreceptors in both ischemic retinopathies. In choroid, the patterns of LRP1 and α_2M expression were different and not coincident.

This is the first demonstration of the presence of LRP1 and α_2M in human proliferative retinopathies. Elevated LRP1 expression in sickle cell neural retina and diabetic inner retina and choroid suggests that LRP1 plays an important role in ischemic neovascular diseases.

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1. Introduction

Low density lipoprotein receptor-related protein 1 (LRP1 also called CD91) is a high-molecular weight receptor that is a member of the low density lipoprotein (LDL) receptor gene family. It is synthesized as a single polypeptide precursor of approximately 600-kDa, and is cleaved by furin in the trans-Golgi network to 515-kDa α subunit and 85-kDa transmembrane β subunit (Herz et al., 1990). The 515-kDa α subunit of LRP1 contains binding sites for ligands that are functionally diverse, including proteinases and proteinase inhibitors such as activated α_2-Macroglobulin (α_2M). It has been demonstrated that LRP1 is an important regulator of extracellular proteolytic activity (Herz and Strickland, 2001; Howell and Herz, 2001; Sanchez et al., 2001; Chiabrando et al., 2002; Strickland et al., 2002). In addition, this receptor is abundantly present in numerous tissues and organs such as liver, lung, placenta and brain. In brain it is expressed in neurons but not in normal microglia (Marzolo et al., 2003).

Recently, we have demonstrated that LRP1 and its ligand α_2M are highly expressed in retinas of rats with oxygen-induced retinal neovascularization (NV) (Sanchez et al., 2006). By immunofluorescence microscopy we detected LRP1 on cellular retinaldehyde binding protein (CRALBP)-positive cells in rat retinal NV, suggesting that LRP1 expression in Müller cells under hypoxic conditions plays a role in modulating retinal NV (Sanchez et al., 2006). It has been suggested that the LRP1 expression in these cells is involved in neovascular processes (Sanchez et al., 2006) because Müller cells are important producers of angiogenic factors in the neural retina during hypoxic conditions and they have contact with endothelial cells (Stone et al., 1995). Although generally considered to be an endocytic receptor, it has been reported that LRP1 also promotes intracellular signaling, which downstream may mediate cellular proliferation, migration and differentiation in macrophages, vascular smooth muscle cells and neurons (Barnes et al., 2003; Strickland and Ranganathan, 2003; Hu et al., 2006; Bonacci et al., 2007). In support of our results, other authors have demonstrated that LRP1 is a hypoxia-inducible gene product (Koong et al., 2000; Wykoff et al., 2000), indicating that this receptor may be a critical factor in retinal NV associated with ischemic diseases such as diabetes mellitus and sickle cell disease, among other ocular ischemic pathologies.

Earlier studies on the functions of α_2M indicate that it is a major proteinase inhibitor and a cargo protein of growth factors and cytokines in the blood and in other extracellular spaces (LaMarre et al., 1990). Recent studies in cultured hippocampal neurons (Bacskai et al., 2000; Qiu et al., 2002) and macrophages (Bonacci et al., 2007) have demonstrated that α_2M can also regulate cell signal transduction mediated by LRP1. In addition, we have previously demonstrated increased α_2M in human vitreous samples from proliferative diabetic retinopathy (PDR) subjects with and without previous panretinal photocoagulation (Sanchez et al., 2007). However, α_2M in these PDR patients was activated suggesting a different proteolytic state in PDR subjects.

In the present study, we examined the distribution and relative levels of LRP1 and its ligand α_2M, in human retinal and choroidal tissue from patients with preproliferative diabetic retinopathy and proliferative sickle cell retinopathy. Considering that ischemia occurs in both of these retinopathies, we hypothesized that LRP1 would be upregulated in these pathological retinas.

2. Materials and methods

Eight normal aged human donor eyes, eight eyes with preproliferative diabetic retinopathy and two eyes with proliferative sickle cell retinopathy [one with sickle cell anemia (SS-genotype) and one with SC disease (SCD; SC-genotype)] were evaluated. The characteristics of each donor are summarized in Table 1. The diagnosis of diabetic retinopathy was made by reviewing the systemic and ocular medical history from the eye bank and the postmortem gross examination of posterior eyecup using a Zeiss dissecting microscope (Zeiss Stemi 2000, Carl Zeiss, Inc., Thornwood, NY, USA). For most subjects the fellow retinas were incubated for adenosine diphosphatase activity, which stains viable vasculature, allowing exact determination of retinopathy and its severity according to the Airlie House System (Kunz Mathews et al., 1997). Our criterion for proliferative sickle retinopathy was that the eye had intravitreal neovascularization at the border of the non-perfused and perfused regions. The mean age of the normal, diabetic and sickle cell subjects were 66.4 ± 14.1, 66 ± 16.1 and 47 ± 9.8 years, respectively.

2.1. Immunohistochemistry

Human donor eyes were cryopreserved as described previously and serially sectioned (Lutty et al., 1993). Streptavidin alkaline phosphatase (APase) immunohistochemistry was performed by the method of Bhutto et al. (Bhutto et al., 2004). Briefly, 8 μm-thick cryosections were permeabilized with absolute methanol, and blocked with 2% goat serum and avidin–biotin complex (ABC) blocking kit (Vector Labs, Inc., Burlingame, CA, USA). After washing in Tris-buffered saline (TBS), the sections were incubated overnight at 4 °C with one of the following primary antibodies: mouse anti-CD34 (1:200, Covance Research Products, Princeton, NJ, USA) to identify viable blood vessels; mouse anti-human LRP1 (1:50, Invitrogen, Carlsbad, CA, USA); and rabbit anti-human α_2M (1:50,000, Abcam, Cambridge, MA, USA). All antibodies were diluted in TBS with 1% bovine serum albumin (BSA). After washing in TBS, sections were incubated for 30 min at room temperature with appropriate biotinylated secondary antibodies diluted 1:500 (Kirkegaard and Perry, Gaithersburg, MD, USA). Finally, sections were incubated

Table 1
Brief history of subjects.

Case	Time (h)		Age/race sex	Condition	Cause of death
	DET	PMT			
1	4	22	53/CF	Normal	Breast Cancer
2	3.5	29	73/CF	Normal	Colon CA
3	2.5	33	75/CF	Normal	Heart Disease
4	2	16	61/CF	Normal	Breast Cancer
5	4	28	39/BM	Normal	SC - Trait
6	4	27	78/CM	Normal	End stage Renal Failure
7	3	27	74/CM	Normal	Cancer with Mets
8	4	27	78/CM	Normal	Prostate Cancer
9	4.5	15.5	62/BM	Hypoxic encephalophathy/ IDDM	Respiratory Failure
10	4	15	63/CM	MI, HTN, IDDM/28 yrs. ID	Cardiac arrest
11	2.1	26	88/CM	DM II	Acute Myocardial Infarction
12	2	30	42/CM	IDDM/30 yrs	Myocardial Infarction
13	6	30	51/CM	IDDM/Diabetic retinopathy	CPA
14	2	19.5	58/CF	IDDM/12 yrs DM/9 yrs. ID	Multi system failure
15	3	12	83/CM	HTN, IDDM/AMD, early	Prostate CA
16	3.5	13	81/CM	IDDM/16 yrs duration	Myocardial Infraction
17	2	24	40/BF	SC Anemia (SS-genotype)/ Proliferative retinopathy	ASCVD
18	3	23	54/BF	SCD (SC-genotype), NIDDM/Proliferative retinopathy	Cardiovascular Accident

(DET, death to enucleation time; PMT, postmortem time (from death to fixation); SS, homozygous for the hemoglobin S mutation; SC, heterozygous for S and C mutation; B, black; C, caucasian; M, male; F, female; CA, cancer; mets, metastatic sites; IDDM, Insulin-Dependent Diabetes Mellitus; CPA, cardiopulmonary accident; ASCVD, arteriosclerotic cardiovascular disease.).

with streptavidin APase diluted 1:500 (Kirkegaard and Perry, Gaithersburg, MD, USA) and then APase activity was developed with a BCIP-NBT kit (Vector Laboratories, Inc., Burlingame, CA) with the addition of 1 mM (−)-Tetramisole HCl (Sigma–Aldrich, St. Louis, MO, USA), yielding a blue reaction product at sites of antibody binding. After immunohistochemistry, sections were post-fixed and bleached as reported previously (Bhutto et al., 2004).

2.2. Grading system

Four independent masked observers scored blindly the relative intensity of the immunoreactivity for each antibody in retinal and choroidal structures using a modified grading system of Page and coworkers (Page et al., 1992; McLeod et al., 1995). The grades in the system were: 7, uniformly intense immunoreactivity; 6, uniform and moderate; 5, patchy and moderate; 4, uniform and weak; 3, patchy and weak; 2, uniform and very weak; 1, patchy and very weak; and 0, comparable to non-immune IgG -incubated negative control section. The mean values from the graders +/− the standard deviation for each structure in each group is given in Table 2. Unfortunately, only two proliferative sickle cell retinopathy subjects were available so two areas in each of the two retinas were evaluated yielding data from three observers on four areas. The p values were determined by comparing mean scores from the control subjects with scores from subjects with diabetic and sickle cell retinopathy using the Student's t-test and assuming unequal variance and two tails; $p \leq 0.05$ was considered significant.

3. Results

3.1. Immunolocalization of LRP1 and α_2M in normal versus diabetic retina

The localization of both LRP1 and α_2M was often heterogeneous even within the control subjects. Fig. 1 shows representative sections from a normal control eye (Subject# 2) and a diabetic eye (Subject #10). In controls, the LRP1 staining pattern was generally weak and diffuse at the level of the internal limiting membrane (ILM) and in the vicinity of large retinal blood vessels. LRP1 staining in blood vessels was always abluminal not luminal, suggesting it was in pericytes and smooth muscle cells and not endothelial cells (Fig. 1E, F and 2C). There was some weak staining for α_2M in and around blood vessels and diffuse patchy staining in the perivascular neural retina. The staining for LRP1 in diabetic retina was generally much more intense than in controls especially in the innermost retina (presumed astrocytes). In serial sections from another diabetic subject (Subject #9, Fig. 2), it was apparent that GFAP, a marker for astrocytes, has very similar localization to LRP1. GFAP in diabetic retina is often expressed in Müller cells as well, which is apparent in Fig. 2D. α2M immunoreactivity was also increased in diabetic subjects where diffuse labeling was seen in blood vessel walls and around blood vessels and throughout the neural tissue (Fig. 1H). Immunoreactivity for LRP1 and α_2M was also associated with the photoreceptor outer segments, which was more intense in diabetics compared to controls (Fig. 1). Overall, the most prominent LRP1 immunoreactivity in retina was innermost retina, presumably astrocytes, and it was significantly upregulated in diabetics. The most intense α_2M labeling was associated with retinal blood vessels.

3.2. Immunolocalization of LRP1 and α_2M in normal versus diabetic RPE/choroid

In normal control eyes, LRP1 was prominently localized to RPE cells and throughout choroid but was most intense in choroidal stroma (Fig. 3E). When we analyzed choroids of diabetic eyes, the immunoreactivity for LRP1 was present in RPE-BrM and choroidal stroma but it was increased compared to controls in choriocapillaris ($p < 0.05$)(Fig. 3E, F). The immunoreactivity for α_2M was also more intense in RPE-BrM and choroidal stroma in diabetic choroid than in controls (Fig. 3G, H). More α_2M was present around choroidal arteries in diabetic subjects than in nondiabetic subjects.

3.3. Immunolocalization of LRP1 in sickle cell retina and choroid

In sickle cell eyes, we analyzed avascular (presumed non-perfused) and vascular (presumed perfused) areas as well as the border between these two areas where neovascularization often forms. LRP1 was present in innermost retina in all three areas of sickle cell eyes (Fig. 4). However, the greatest immunoreactivity was in the non-perfused retina, where it was diffusely throughout retina (Fig. 4D). Preretinal neovascularization, called sea fan formations, was observed at the border of perfused and non-perfused retina and LRP1 was prominently localized to these new vessels (Fig. 5C). Localization of both LRP1 and α_2M in sickle cell choroid was comparable to the diabetic choroid (Fig. 6 C, E).

Choroidal neovascularization (CNV) was observed in one sickle subject (Fig. 6). LRP1 immunoreactivity was decreased in RPE-BrM-CC complex and choroidal stroma in areas with CNV (Fig. 6D). On the other hand, α_2M staining was diffuse and intense and increased in RPE-BrM and choroidal stroma in areas with CNV (Fig. 6F). This same scenario was observed in a diabetic subject with CNV (data not shown).

3.4. Scoring immunohistochemistry in ischemic retinopathies

There was a significant difference in staining intensity with anti-LRP1 and anti- α_2M antibodies in the neural retina in diabetic eyes compared to normal eyes ($p \leq 0.05$). In neural retina, the immunostaining of both LRP1 and α_2M proteins was most intense in astrocytes and ILM (Table 2a). In addition, α_2M also showed significant higher immunoreactivity in diabetic photoreceptors

Table 2a
Mean scores for retina.

	Subjects	Astrocytes	Capillaries	I LM	Muller	Photoreceptors	Ret Blood Vessel		Sensory retina
							A	V	
LRP1	Normal	2.6 ± 1.4	0.5 ± 0.6	0.6 ± 1.2	0.0 ± 0.0	2.7 ± 2.2	2.5 ± 1.2	2.1 ± 1.2	1.5 ± 1.2
	Diabetic	4 ± 1.2[a]	0.5 ± 0.8	1.2 ± 1.5 [b]	0.0 ± 0.2	2.4 ± 1.4	2.1 ± 1.2	1.8 ± 1.1	1.7 ± 1.6
	Sickle Cell	3.3 ± 1.4	0.9 ± 1.3	0.8 ± 1.0	2 ± 2.2 [c]	3.1 ± 0.6	3 ± 0.9	2.8 ± 1.2	2.5 ± 1.4
α_2M	Normal	0.8 ± 0.8	5.3 ± 1.8	1.1 ± 1.4	0.0 ± 0.2	1.3 ± 1.3	2.6 ± 1.4	4.8 ± 1.6	2.2 ± 1.8
	Diabetic	1.8 ± 1.2 [d]	4.4 ± 1.4	2.6 ± 2.0 [e]	0.8 ± 1.2	3.3 ± 2.3 [f]	4 ± 1.2	4.5 ± 1.1	2.8 ± 2.1
	Sickle Cell	3.3 ± 0.9	4.3 ± 0.9	3.3 ± 2.2	2.8 ± 1.3 [g]	2.8 ± 0.5 [h]	3.3 ± 1.4	3.5 ± 1.3	2.8 ± 0.5

ILM, Internal Limiting Membrane; **A**, Arterials; **V**, Venous.[a,b,d,e,f] Statistically significant ($p \leq 0.05$) between retinal cells and structures in normal and proliferative diabetic subjects [c,g,h] Statistically significant ($p \leq 0.05$) between retinal cells in normal and proliferative sickle cell subjects.

Table 2b
Mean scores for choroid.

	Subjects	RPE		Bruch's membrane	Inter capillaries Septa	Choriocapillaris	Choroid blood vessel		Stroma
		Apical	Basal				A	V	
LRP1	Normal	3.8 ± 1.9	2.8 ± 1.7	0.7 ± 1.1	2.2 ± 1.1	2.7 ± 1.3	2.9 ± 1.1	2.7 ± 1.0	4 ± 1.1
	Diabetic	3.9 ± 1.7	3.3 ± 1.9	1.2 ± 1.0[a]	2.7 ± 1.5	3.6 ± 1.5[b]	3.1 ± 1.0	2.9 ± 1.0	5 ± 1.5[c]
	Sickle Cell	4.7 ± 1.7	3.8 ± 2.3	1.2 ± 1.2	3 ± 0.7	3.3 ± 1.4	3.5 ± 1.0	3.6 ± 1.6[d]	4.5 ± 1.0
α_2M	Normal	1.6 ± 1.6	1.7 ± 1.5	2.9 ± 1.9	6.1 ± 1.4	5.3 ± 1.9	3.6 ± 1.5	3.6 ± 1.6	3.5 ± 1.8
	Diabetic	4.1 ± 1.0[e]	3.6 ± 1.4[f]	2.9 ± 1.8	5.8 ± 0.9	4.4 ± 1.4	4.6 ± 1.2[g]	4.3 ± 1.3	4.6 ± 1.5[h]
	Sickle Cell	3.5 ± 1.0	2.3 ± 1.5	2 ± 0.8	5 ± 0.4	4.2 ± 0.9	4.5 ± 1.3	4 ± 1.4	3.7 ± 0.9

RPE, retinal pigment epithelium. [a,b,c,e,f,g,h] Statistically significant ($p \leq 0.05$) between choroid structures and cells in normal and proliferative diabetic subjects. [d] Statistically significant ($p \leq 0.05$) between choroid structures in normal and proliferative sickle cell subjects.

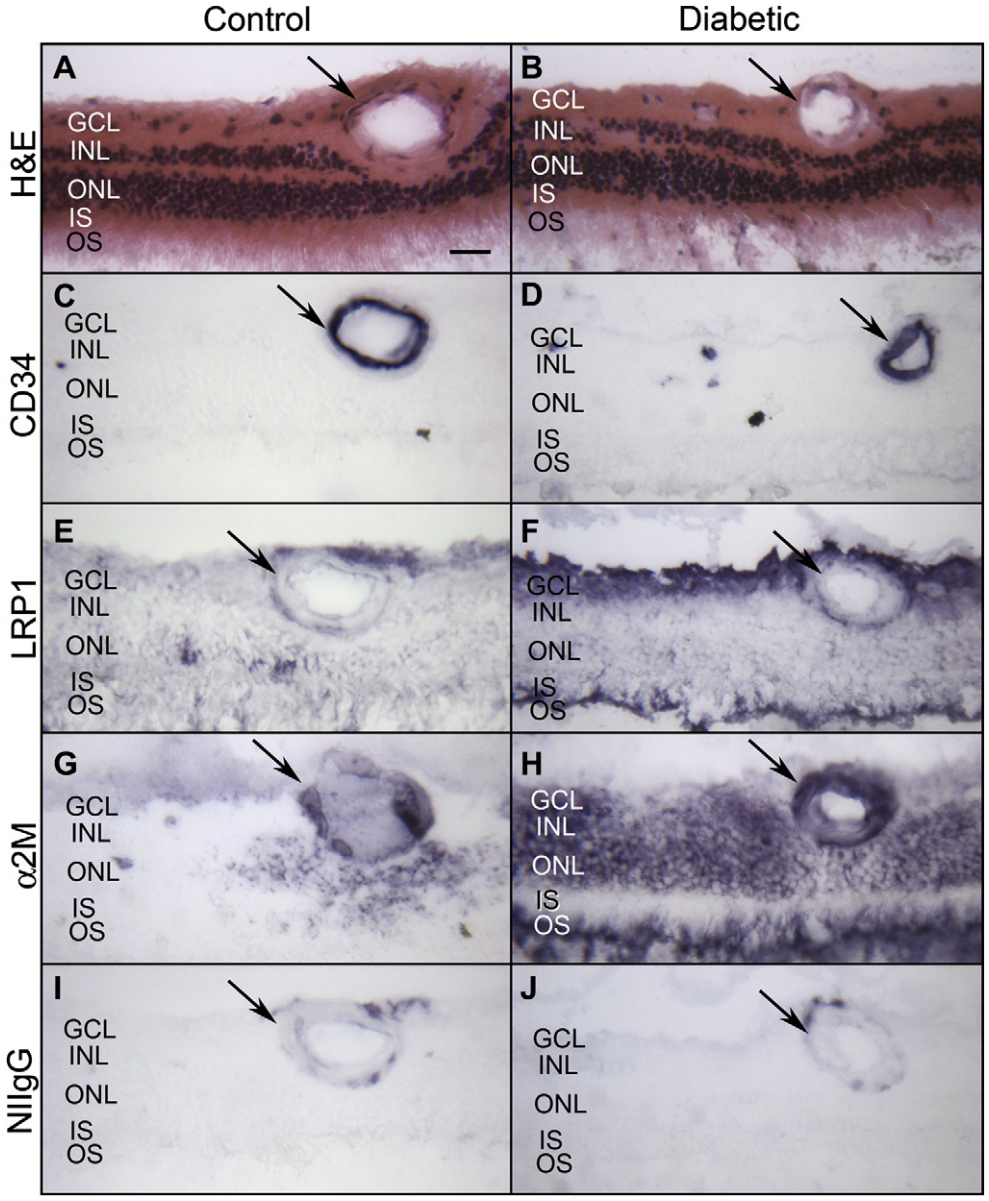

Fig. 1. Comparison of a control subject (Subject #2) (A, C, E, G, I) and a subject with diabetic retinopathy (Subject #10)(B, D, F, H, J). (A,B) Hematoxylin and eosin staining demonstrates that areas in the two subjects are comparable and morphologically normal. (C, D) CD34 labeling demonstrates that there is a major viable artery (arrow) and several capillaries in the two areas shown. LRP1 in the control retina is limited to some perivascular tissue (E) while it is throughout inner retina and associated with photoreceptor outer segments (OS) in the diabetic subject (F). Some α_2M immunoreactivity is present the blood vessel wall and around the blood vessel in the inner nuclear layer in the control subject (G). α_2M is prominent throughout the diabetic neural retina and around the photoreceptor OS in the diabetic eye (H). There was some nonspecific binding of the non-immune IgG in the control (I) and diabetic retina (J). (GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segments of photoreceptors; OS, outer segments of photoreceptors) (A–B hematoxylin and eosin; C–J APase; Bar = 30 μm).

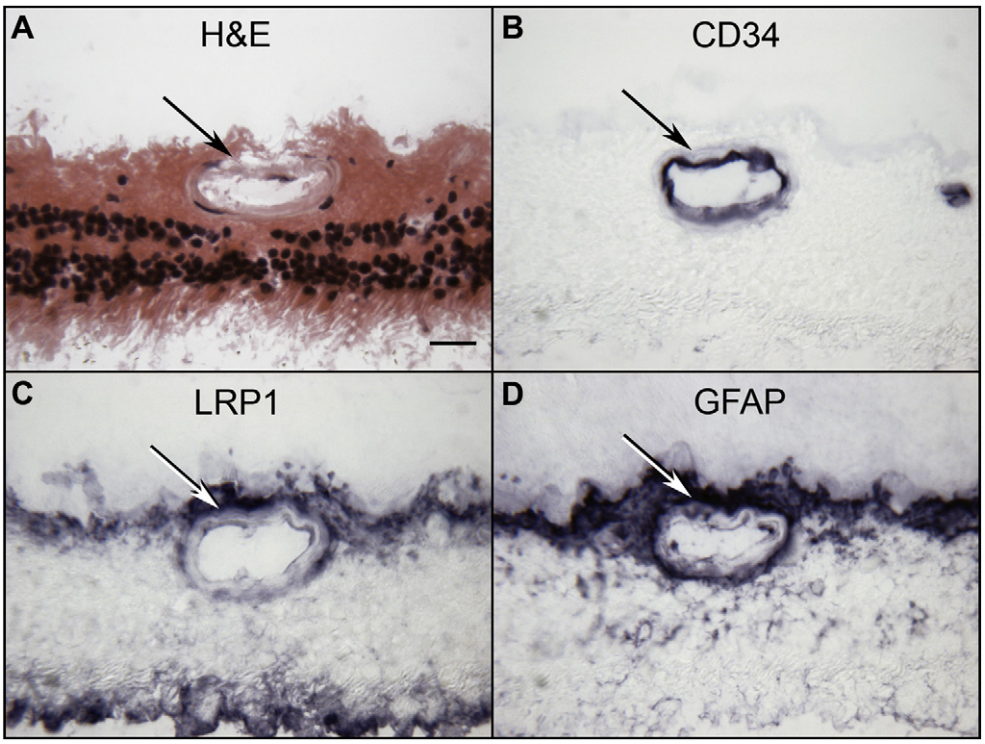

Fig. 2. Localization of LRP1 and GFAP in serial sections from a diabetic retina (Case #9). The H and E staining demonstrates a very thin retina (A) with limited blood vessels ($CD34^{+}$) in this area (B). LRP1 is present in photoreceptor OS, innermost retina and around the blood vessel (C) and GFAP labeling of astrocytes is in relatively the same areas of inner retina (D). (A hematoxylin and eosin; B–D APase; Bar = 20 μm).

compared to controls (Table 2a). In choroid, LRP1 staining was significantly higher in BrM, CC and choroidal stroma compared to the normal eyes ($p \leq 0.05$) (Table 2b), whereas the α_2M expression was significantly increased in RPE, choroid arteries, and choroidal stroma compared to control subjects ($p \leq 0.05$) (Table 2b).

There was a significant difference in staining for LRP1 in sickle cell eyes in the INL compared with normal eyes ($p \leq 0.05$) (Table 2a). The α_2M immunoreactivity was significantly different in INL and photoreceptors ($p \leq 0.05$) compared to normal eyes (Table 2a).

4. Discussion

The present study provides the first analysis of LRP1 and α_2M expression in normal human retina and choroid as well as in human ischemic retinopathies. In normal eyes, a weak and diffuse LRP1 localization was observed at the ILM, astrocytes, RPE, BrM-CC and choroidal stroma, whereas the most prominent immunoreactivity for the LRP1 ligand α_2M was associated with retinal and choroidal blood vessels, choroidal stroma and BrM-CC. A differential staining pattern and intensity between LRP1 and α_2M was observed in diabetic and sickle cell retinas compared with normal eyes. The most prominent sites of LRP1 immunoreactivity in the diabetic eyes were the ILM, astrocytes, photoreceptors, RPE, CC and choroidal stroma, whereas α_2M staining was most prominent in retinal blood vessel walls and in RPE and throughout choroid. In the sickle cell eyes, LRP1 was present in both avascular and vascular areas of retina, but the distribution and intensity was different. In avascular areas, the LRP1 staining was most pronounced in ILM and INL, which corresponds with Müller cell processes and bodies, while it was confined to the ILM area in vascular retina. In the avascular area, α_2M was decreased in both neural retina and choroid, being limited only to choroidal stroma, whereas in vascular areas it was in RPE-BrM and choroidal stroma (Fig. 4). In areas with CNV, the LRP1 staining was significantly decreased in RPE-BrM and choroidal stroma while α_2M was elevated in these areas (Fig. 6).

It has been reported that LRP1 is expressed in Müller cells isolated from normal rabbit and human retina (Birkenmeier and Kunath, 1996). In our previous work, we have demonstrated that the LRP1 was expressed in Müller cells in an *in vivo* rat model of oxygen-induced retinal NV (Sanchez et al., 2006). In the present report, we show that enhanced LRP1 expression in human eyes with proliferative retinopathy was prominent in regions of inner neural retina where Müller cell end feet and astrocytes are present. In sickle cell avascular periphery, however, LRP1 appeared to be Müller soma in the inner nuclear layer. LRP1 in brain has been localized to astrocytic foot processes (Tooyama et al., 1995). The most prominent localizations in normal retina and in ischemic retinopathies was astrocytes.

As mentioned above, using the oxygen-induced retinopathy rat model we have previously demonstrated that LRP1 is involved in retinal NV, showing hypoxia dependent-LRP1 expression mainly in Müller cells (Sanchez et al., 2006). In the present work, two different human diseases that have hypoxic areas of retina demonstrated different localizations in the LRP1 expression; diabetic retina had LRP1 predominantly in innermost retina while sickle cell retina in non-perfused periphery had innermost retina and a Müller cell localization. Labeling of what appeared to be Müller cells was most dramatic in the non-perfused and border regions of sickle cell retina, whereas the labeling in the perfused area of sickle cell retina resembled the diabetic retina. Both ischemic retinopathies have hypoxic areas of retina but in sickle cell retinas it is confined mostly to periphery (area with Müller cell labeling), whereas in diabetic retina hypoxic areas may be diffuse. This difference between diabetic and sickle cell retina may be due to the nature of the two

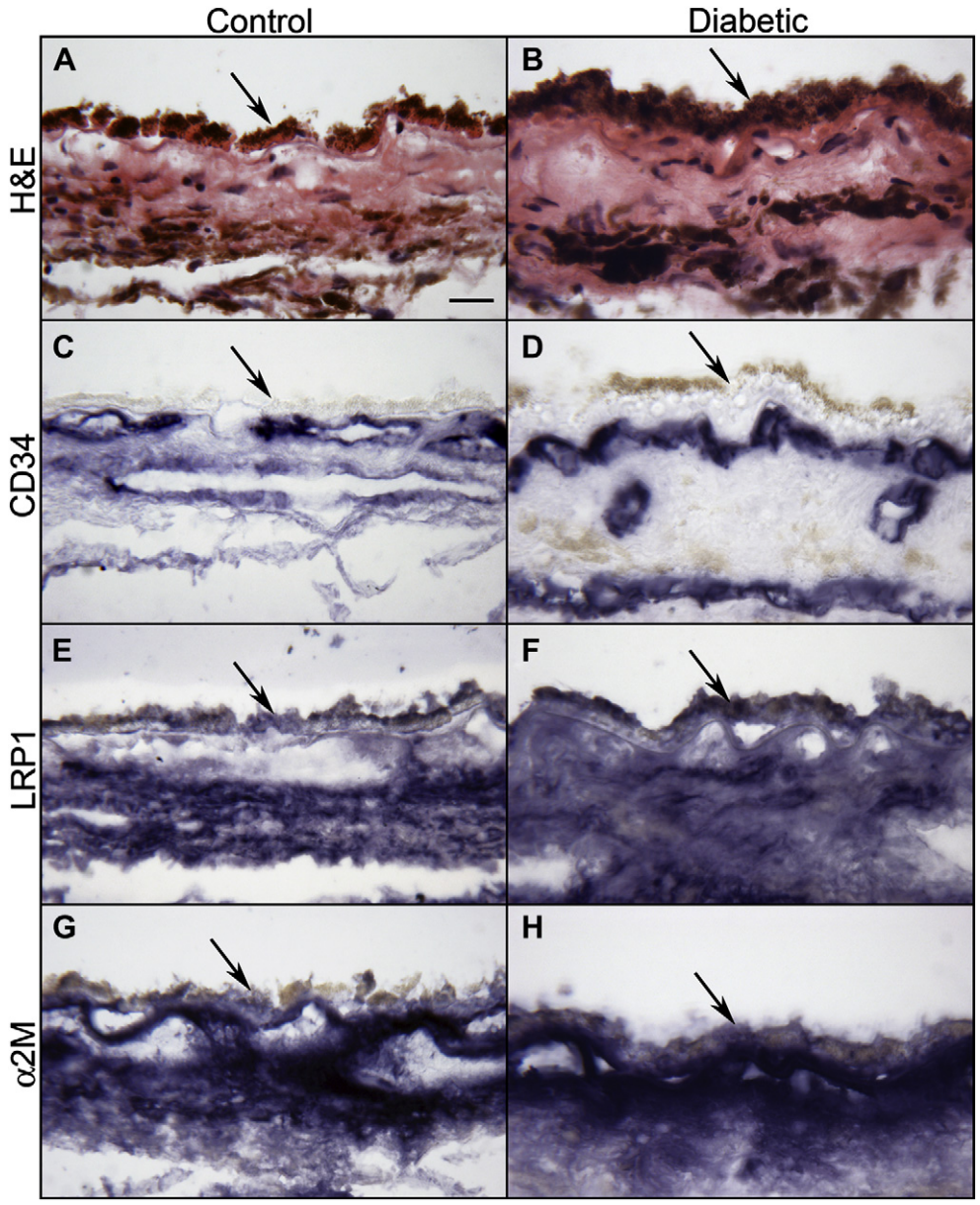

Fig. 3. Comparison of choroid in a control subject (Case #7)(A, C, E, G) and a diabetic subject (Case #15). (B, D, F, H). The H and E stained sections demonstrate that the control choroid (A) is normal and appears similar to the diabetic choroid (B). CD34 is present in the choriocapillaris and a large choroidal vein in the control subject (C) while it is more intense in constricted choriocapillaris and intermediate choroidal blood vessels in the diabetic subject (D). LRP1 immunoreactivity is present at low levels in RPE and CC, and intensely in stroma in the control choroid (E), while it is more prominent in RPE and throughout the entire choroid (E) in the diabetic subject. α_2M is very intense in inner choroid in the control (G) and diabetic subject (H). (A–B hematoxylin and eosin; C–J H APase; Bar = 20 μm).

diseases: diabetes is a metabolic disease that may have occlusions throughout retina; sickle cell occlusions are caused by sickle RBCs adhering and occluding blood vessels mechanically, predominantly in peripheral retina.

Our data provides evidence for a relationship between LRP1 expression and presumed retinal hypoxia in human eye pathologies, making these findings relevant from the clinical point of view. Although the molecular and cellular mechanisms as well as the clinical significance of this hypoxia-increased LRP1 expression in the human retina is for the moment unknown, previous works have demonstrated that LRP1 is inducible by hypoxia in other types of cells such as cervical and squamous carcinoma cells (Koong et al., 2000) as well as in non-renal derived cell lines (Wykoff et al., 2000). Nevertheless, further studies are required in order to establish the relationship between hypoxia and LRP1 expression in Müller cells, which could prove to be an early event in the development of NV during ischemic retinopathies.

Increase in vascular permeability is widespread in diabetic retinopathy and occurs at sites of NV in both retinopathies included in this study. α_2M is a prominent serum protein. Localization observed was different between this ligand and its receptor. Serum proteins like albumin are prominent in the inner photoreceptor matrix and around blood vessels in diabetic retinopathy (Kunz Mathews et al., 1997). Both LRP1 (Fig. 1F) and α_2M (Fig. 1H) were prominently localized to photoreceptors and around blood vessels, respectively. LRP1 was not most prominent around blood vessels while α_2M was in blood vessel wall. LRP1 in blood vessel wall was abluminal suggesting that it was in pericytes or smooth muscle cells not endothelial cells. This is in agreement with the observations of Lillis et al. in brain (Lillis et al., 2005). Unlike leakage of

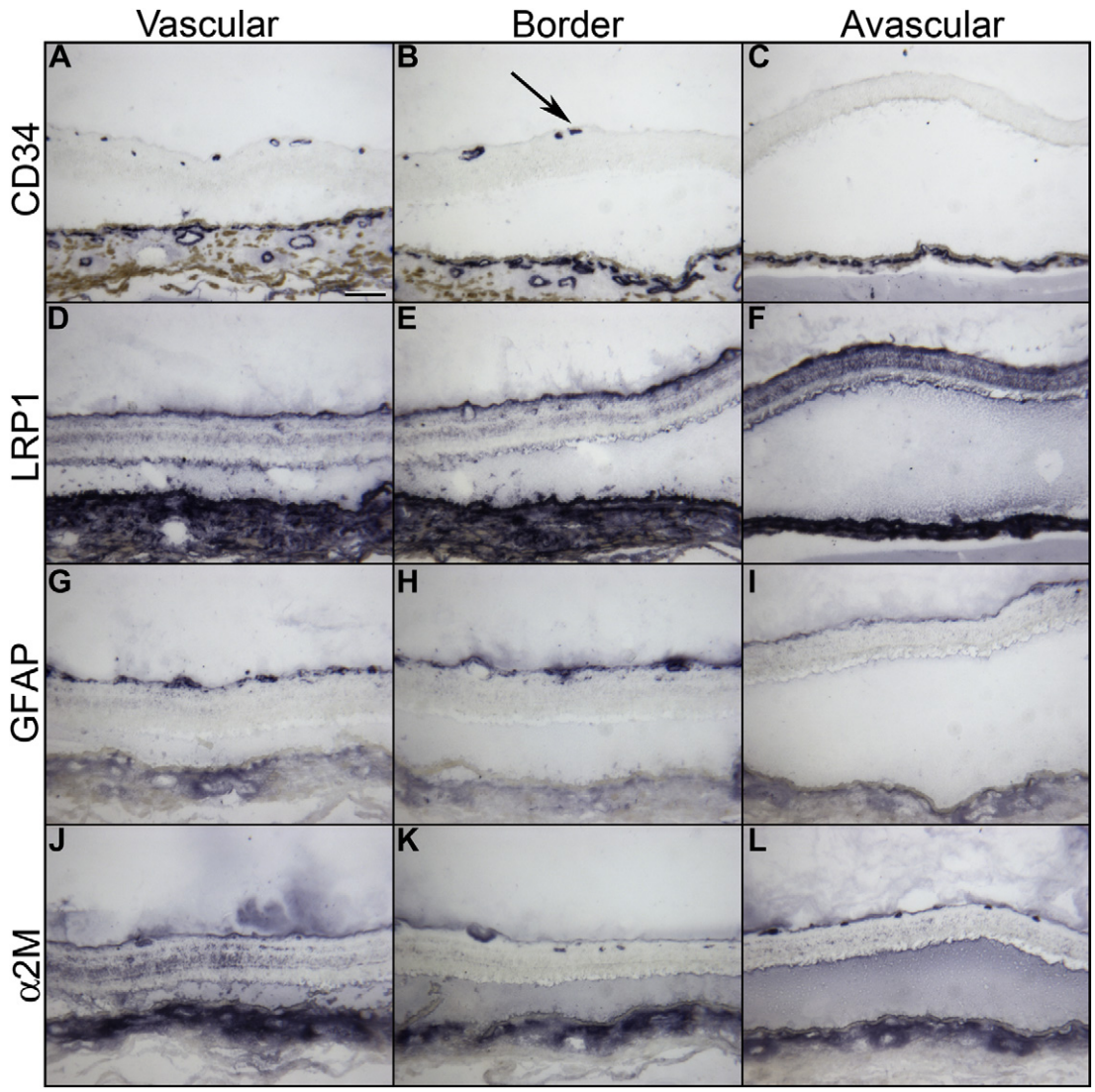

Fig. 4. Three areas in a sickle cell eye (Subject #17): perfused (A, D, G, J), border of perfused and non-perfused (B, E, H, K), and non-perfused (C, F, I, L). CD34 labeling demonstrates retinal blood vessels in the perfused (A), and border areas (B), but no viable blood vessels are present in the non-perfused area (C). LRP1 immunoreactivity is confined to some retinal blood vessels and innermost neural retina in the perfused area (D) and border region (E), whereas neuronal layers are also positive in the non-perfused region (F). α_2M is only prominent in the perfused area of the retina (J). (All APase; Bar = 100 μm).

albumen in diabetic retina, neither protein had a halo of immunoreactivity around veins (Kunz Mathews et al., 1997). In addition, other obvious sites of vascular and barrier leakage like CNV had reduced immunoreactivity for LRP1 while α_2M was elevated near CNV as are other serum proteins (Fig. 6).

α_2M analysis revealed that its localization was diffuse, sometimes coincident with the localization of LRP1 in neural retina in both diabetic and sickle cell subjects but most prominent around blood vessels. Comparison of the two antigens suggests that the receptor LRP1 had a cellular localization whereas α_2M was diffusely in the milieu at sites of increased permeability. The difference in localization between the ligand and receptor may be due to the many ligands that LRP1 binds. LRP1 is known to bind proteins involved in lipoprotein metabolism (chylomicrons, VLDL remnants), proteases and protease inhibitors (α2-Macroglobulin, MMPs, plasminogen activators and inhibitors), extracellular (thrombospondin1 and 2, fibronectin) and intracellular proteins (calreticulin), and growth factors (PDGF, connective tissue growth factor) (Lillis et al., 2005). Both α_2M and LRP1 immunoreactivites were associated with photoreceptors in some subjects but only α_2M was significantly elevated in diabetic and sickle cell retinas (Table 2a). It is known that α_2M is a soluble protein constitutively expressed principally by the liver (Chu and Pizzo, 1994). It has been also demonstrated that serum proteins enter the retinal tissue after the breakdown of the blood-retinal barrier in ischemic diseases like diabetic retinopathy (Engerman, 1989), however, the appearance of α_2M around blood vessels was unlike albumen in diabetic retina (Kunz Mathews et al., 1997). Previously, we have demonstrated that α_2M concentration is increased in vitreous samples from proliferative diabetic retinopathy and in retinas of rats with oxygen-induced retinal NV (Sanchez et al., 2006, 2007). However, it has been reported that α_2M mRNA is highly expressed during experimental glaucoma in rat (Ahmed et al., 2004), which suggests that α_2M could also be synthesize by retinal cells. In addition, a recent *in situ* mRNA hybridization study showed that α_2M is preferentially expressed in the inner and outer nuclear layers in glaucoma, whereas α_2M protein was detected in ganglion cells, Müller cell end feet and astrocytes. The mechanism suggested by the authors is that α_2M is

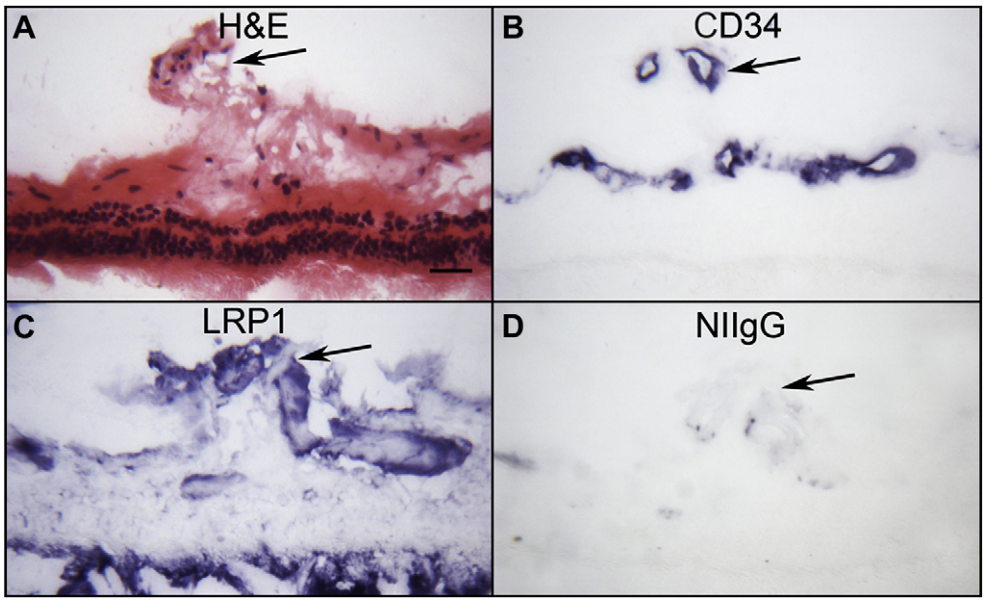

Fig. 5. A sea fan neovascular formation (arrow) is apparent in this sickle cell subject (Subject #18) with H and E staining (A) and CD34 immunolabeling (B). LRP1 is prominent in the sea fan and also in the photoreceptor outer segments (C). There is very little nonspecific IgG binding with a non-immune IgG used instead of primary antibody (D). (A hematoxylin and eosin; C–D APase; Bar = 30 μm).

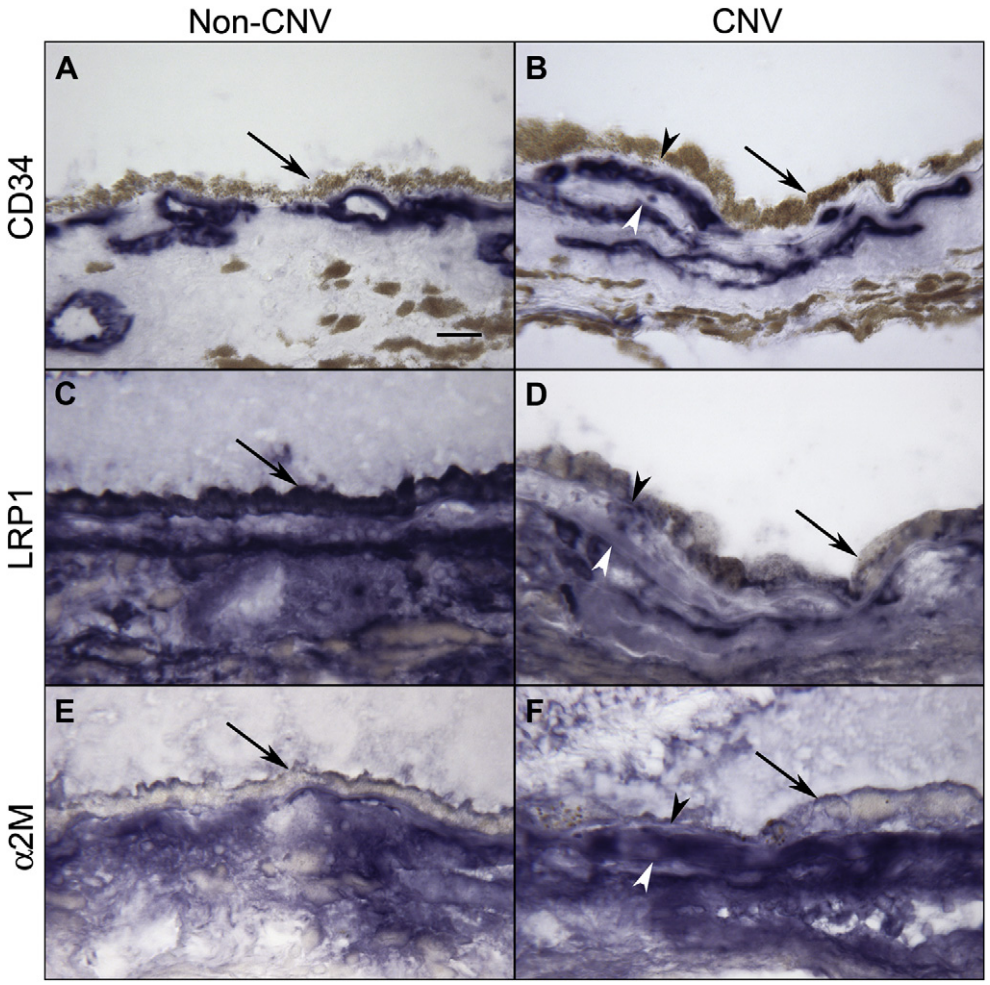

Fig. 6. Comparison of two areas of choroid in a sickle cell subject (Subject #17) with (B, D, F) and without choroidal neovascularization (CNV) (A, C, E). CD34 labeling demonstrates a normal choriocapillaris and intermediate blood vessels in the normal area (A) and a constricted CNV lumen (between arrowheads) and choriocapillaris in the pathologic area (B). In the area without CNV, LRP1 immunoreactivity is intense in RPE and stroma beneath the choriocapillaris (C), while levels are low in the CNV and greatly reduced in RPE (arrow) and choroid in this area (D). α_2M immunoreactivity is moderate in the normal area (E) and greatly elevated in the area with CNV (F). (All APase; Bar = 20 μm).

processed and secreted by glia and then, this protein is bio-available to ganglion cells which constitutively express LRP1 (Shi et al., 2008). Our data clearly demonstrate that in both diabetic and sickle cell retinopathy the LRP1 expression was in astrocytes, ILM, Müller cells and photoreceptors. Further studies are needed in order to elucidate the putative site of synthesis as well as the potential role and mechanism of α_2M in ischemic diseases.

Finally, our data suggest that enhanced LRP1 and α_2M expression in retinal regions in ischemic retinal diseases, as in diabetes and sickle cell retinopathy, are coincident with the localization of astrocytes and Müller cell processes. We realize that the results in this manuscript rest on a small number of cadaver eyes of varying postmortem time from patients with diverse medical histories, both of which could have affected the localization reported. However, there was significantly more LRP1 in some structures in sickle cell retina and diabetic retina groups compared to control subject group, suggesting that upregulation of LRP1 occurs in ischemic retinopathies. Further studies are required to determine the role of α_2M/LRP1 system in these processes and how that is related to the pathogenesis of ischemic retinal vascular disease. Furthermore, there was a significant increase in both LRP1 and α_2M in diabetic choroid, which may be related to diabetic choriodopathy in which areas of choriocapillaris are lost (Cao et al., 1998). The increased presence of LRP1 may be related to hypoxia in the diabetic eye. However, it is inexplicable that LRP1 was reduced in choroid in areas with CNV unless the new blood vessels actually provide enough blood flow to alleviate hypoxia.

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