EXTENDED REPORT

Localisation of SDF-1 and its receptor CXCR4 in retina and choroid of aged human eyes and in eyes with age related macular degeneration

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Aim: To examine the immunolocalisation of stromal cell derived factor 1 (SDF-1) and its receptor CXCR4 in aged control human donor eyes and eyes with age related macular degeneration (AMD).

Methods: Postmortem eyes from eight aged control donors (mean age 79.8 years) and from 12 donors with AMD (mean age 83.9 years) were cryopreserved and sectioned through the macular region. SDF-1 and CXCR4 were localised using streptavidin alkaline phosphatase immunohistochemistry and then sections were bleached. Three independent masked observers scored the immunohistochemical reaction product.

Results: In aged control retinas, SDF-1 immunoreactivity was most intense in inner photoreceptor matrix (IPM). CXCR4 showed a similar pattern of immunostaining, but was more prominent in inner segments of photoreceptors. In aged control and AMD choroid, SDF-1 and CXCR4 localisations were most prominent in retinal pigment epithelial (RPE) cells and choroidal stroma. However, the intensity for SDF-1 was significantly reduced in RPE ($p < 0.0001$) and choroidal stroma ($p < 0.05$) in late AMD eyes. SDF-1 and CXCR4 immunoreactivities were weak or nearly absent in disciform scars with choroidal neovascularisation (CNV). Circulating cells, presumably leucocytes, were most intensely positive for CXCR4.

Conclusions: These results show that changes in distribution and relative levels of SDF-1/CXCR4 were not evident in early AMD. This suggests that SDF-1/CXCR4 may not contribute to the formation of CNV in AMD, in that CXCR4+ cells were not incorporated into neovascularisation. However, the examples of CNV studied were within disciform scars, so the authors cannot comment on the role of SDF-1/CXCR4 in the early stages of CNV formation.

Angiogenesis in the form of choroidal neovascularisation (CNV) is an important pathobiological event encountered in a variety of chorioretinal diseases.¹ CNV is a major cause of vision loss in patients with age related macular degeneration (AMD). The pathogenesis of CNV is clearly multifactorial, but generally considered to be driven by growth factors promoting angiogenesis. The historical paradigm was that the cellular components of the new vessel complex (endothelial cells, smooth muscle cells, and pericytes) were derived from cells resident within adjacent pre-existing blood vessels—that is, by angiogenesis. However, recent studies in animal models suggest that circulating endothelial precursor cells (EPCs) and haematopoietic stem cells (HSCs) participate in this process and other pathophysiological angiogenesis, including retinal neovascularisation.^{2–4}

Stromal cell derived factor 1 (SDF-1) is a member of the CXC chemokine subfamily that was originally isolated from murine bone marrow stromal cells and functions as a chemoattractant for leucocytes.⁵ SDF-1 is involved in HSC and EPC cell homing^{6–7} and recruitment of HSCs from the bone marrow to target tissue.^{8–9} Recently, it has been suggested that members of the CXC chemokine family can either promote or inhibit vasculogenesis/angiogenesis depending on the presence or absence of a structural functional domain ELF.¹⁰ SDF-1 lacks an ELF domain and therefore should be antiangiogenic. However, SDF-1 has been reported to be angiogenic in dermis¹¹ yet have no effect in the corneal micropocket assay.¹⁰ SDF-1 also acts as an angiogenic agent in several in vivo and in vitro model systems^{7–12} and may have a role in proliferative retinopathy by providing the directional cues necessary for EPCs to reach sites of ischaemia.¹³

CXCR4, a seven transmembrane spanning G protein coupled receptor, is the only known receptor for SDF-1 and is expressed on various endothelial cells, HSCs, and EPCs.^{8–14–15} CXCR4 is also expressed on lymphocytes, monocytes, neutrophils, microglia, and ganglion cell precursors in retina.^{16–17} Its expression is increased after treatment with VEGF or bFGF.⁷ In brain tumours, CXCR4 is immunohistochemically expressed in regions of angiogenesis, degeneration, and necrotic changes.¹⁸ Mice deficient in either SDF-1 or CXCR4 had defective formation of large vessels supplying the gastrointestinal tract, severely reduced B lymphopoiesis, reduced myelopoiesis in fetal liver, and a virtual absence of myelopoiesis in bone marrow.^{19–20}

Considering the importance of the chemokine SDF-1 and its receptor, CXCR4, in regulation of leucocyte trafficking, modulation of haematopoietic cell proliferation, adhesion to extracellular matrix molecules, and regulation of angiogenesis like CNV, we investigated SDF-1 and CXCR4 expression in the retina and choroid of aged human control donor eyes and in eyes with AMD. To our knowledge, this is the first study describing the distribution of SDF-1 and CXCR4 in the adult human eye.

Abbreviations: ABC, avidin-biotin complex; AMD, age related macular degeneration; BLD, basal laminar deposits; BM, Bruch's membrane; BSA, bovine serum albumin; CC, choriocapillaris; CNV, choroidal neovascularisation; COPD, chronic obstructive pulmonary disease; DET, death to enucleation time; DM, diabetes mellitus; EPCs, endothelial precursor cells; GA, geographic atrophy; HSCs, haematopoietic stem cells; HTN, hypertension; ILM, internal limiting membrane; IPM, inner photoreceptor matrix; NBT, nitroblue tetrazolium; PMT, postmortem time; RPE, retinal pigment epithelium; SDF-1, stromal cell derived factor 1; TBS, TRIS buffered saline; VEGF, vascular endothelial growth factor

Table 1 Characteristics of human donor cryopreserved eyes

	Time (hours)					
Case	DET	PMT	Age/race/sex	Primary cause of death	Medical history	Ocular diagnosis
Aged						
1	2.5	33	75/W/F	Heart disease		Normal
2	7	27	76/W/F	Lung cancer	HTN, CNF	Normal
3	1	26	77/W/M	COPD	HTN	Normal
4	2.5	28	80/W/M	COPD		Normal
5	7.15	28	80/W/M	Intracranial haemorrhage	HTN, angioplasty	Normal
6	3	15	82/W/M	Metastasis brain cancer		Normal
7	3	16	83/W/M	Cardiac respiratory arrest		Normal
8	5	26	86/W/F	Respiratory failure		Normal
AMD						
9	3.5	34	61/W/M	Metastasis oesophageal cancer		AMD, early
10	3.5	42	69/W/F	Subarachnoid haemorrhage	Pulmonary fibrosis	AMD (GA), late
11	4	33	74/W/M	Prostate cancer		AMD, early
12	7	30	75/W/M	Aspiration pneumonia		AMD (GA), late
13	3	33	79/W/M	Pneumonia	HTN, asthma	AMD, early
14	5	29	81/W/F	Myocardial infarction	HTN	AMD, early
15	3	12	83/W/M	Prostate cancer	DM, HTN	AMD, early
16	4	20	93/W/F	Multisystem failure	DM, HTN	AMD (disciform scar), late
17	3	36	94/W/M	Cardiac failure		AMD (disciform scar), late
18	3.5	?	95/W/M	Cardiomyopathy		AMD (disciform scar), late
19	2	33	98/W/F	Old age		AMD, early
20	4.5–5	11	105/W/M	COPD		AMD (disciform scar, GA), late

DET, death to enucleation time; PMT, postmortem time (death to fixation); W, white; AMD, age related macular degeneration; DM, diabetes mellitus; HTN, hypertension; COPD, chronic obstructive pulmonary disease; GA, geographic atrophy.

MATERIALS AND METHODS

Donor eyes

Human donor eyes were obtained with the assistance of Janet Sunness, MD (Great Baltimore Medical Center, Baltimore, MD, USA) and Carol Applegate and the National Disease Research Interchange (NDRI; Philadelphia, PA, USA) within 11–36 hours after death. Eyes of the following donors were used in the study: 12 subjects with AMD (age range 61–105 years; mean age 83.9 years); eight aged control donors (age range 75–86 years; mean age 79.8 years) with no history of chorioretinal disease. All donors were white. Table 1 has the characteristics of each subject. The protocol of the study adhered to the tenets of the Declaration for Helsinki regarding research involving human tissue and was approved by the Johns Hopkins JCCI. The diagnosis of AMD was made by reviewing ocular medical history on the eye bank transmittal sheet and the postmortem gross examination of posterior eyecup, using transmitted and reflected illumination with a dissecting microscope (Stemi 2000; Carl Zeiss Meditec, Inc, Thornwood, NY, USA).

Tissue preparation

After the anterior segment of the eye was removed, the posterior eye cup was fixed in 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) with 5% sucrose at room temperature for 1 hour. The tissue was cut into coulottes of vitreous/retina/choroid complex and cryopreserved as previously described.²¹ Serial 8 µm sections were cut from the inferior macula.

Immunohistochemistry

Streptavidin alkaline phosphatase (APase) immunohistochemistry was performed on cryosections using a nitroblue tetrazolium (NBT) development system as previously described.²² After permeabilising with absolute methanol, sections were blocked with 2% normal goat or rabbit serum in TRIS buffered saline (TBS; pH 7.4 with 1% bovine serum albumin, BSA) and with an avidin-biotin complex (ABC) blocking kit (Vector Laboratories, Inc, Burlingame, CA, USA). Sections were then incubated at 4°C overnight with 1:100 goat anti-human SDF-1 antibody (sc-6193, Santa Cruz, CA,

Figure 1 SDF-1 and CXCR4 immunoreactivity in retina (top) and choroid (bottom) from an aged control eye (case 3). Intense SDF-1 immunoreactivity is present in the inner photoreceptor matrix with significantly less present in some retinal vessels. CXCR4 has a similar pattern of immunostaining, but is more prominent in the inner segment area of photoreceptors and associated with the endothelial cells of blood vessels. In choroid, immunostaining of SDF-1 is most prominent in retinal RPE cells and less intensively positive in choroidal blood vessels and choroidal stroma, where it appeared diffuse. CXCR4 is less prominent in RPE than SDF-1 but comparable in choroid. Note the leucocytes within the choroidal blood vessel lumen are intensively labelled with CXCR4 (arrows). Pigment in the choroidal sections was bleached from RPE and choroidal melanocytes. Bar = 20 µm.

USA), 1:5000 rabbit anti-human CXCR4 antibody (NLS1380, Novus Biologicals, Littleton, CO, USA), or mouse anti-human CD-34 (1:800; Signet Laboratory, Dedham, MA, USA) antibody in adjacent tissue section to label blood vessels. To demonstrate the validity of antibody binding, anti-SDF-1 antibody was pre-incubated with a 200-fold excess of peptide or human serum albumin overnight at 4°C before use. As a negative control, the primary antibodies were omitted and no staining was observed. The pigment was bleached from retinal pigment epithelium (RPE) and choroidal melanocytes as described previously.²² Finally, cover slips were mounted with Kaiser's glycerogel.

Three independent masked observers blindly graded the relative intensity of the immunoreactivity for both antibodies in retinal and choroidal structures, using a previously described eight point grading system.²³

Statistical analysis

Mean score (SD) from the graders was calculated for each retinal and choroidal structure. The Kolmogorov and Smirnov method was used to determine the Gaussian distribution of these scores. The *p* values were determined by comparing mean scores from the aged control eyes with scores from eyes with AMD using the Student's *t* test and assuming unequal variance and two tails. The *p* value <0.05 was considered significant. Statistical analysis was done with InStat software (version 2.0, GraphPad Software, San Diego, CA, USA).

RESULTS

Immunolocalisation of SDF-1 and CXCR4 in aged control human retina/choroid

In retina, the immunostaining of SDF-1 was most intense in inner photoreceptor matrix (IPM) and present to a lesser extent in internal limiting membrane (ILM) and large retinal blood vessels. CXCR4 showed a similar pattern of immunostaining, but was more prominent in inner segments of photoreceptors and associated with the endothelial cells of some retinal blood vessels (fig 1). There was no apparent difference in immunoreactivity level of either SDF-1 and CXCR4 antibodies in ILM and large retinal blood vessels. Immunostaining of both antibodies was very weak or absent in the inner neural retina.

In choroid/RPE complex, immunostaining of SDF-1 was most prominent in both apical and basal portions of RPE cells (fig 1). The immunostaining for both SDF-1 and CXCR4 in large choroidal vessels, the choriocapillaris, and intercapillary septa was less intense and distinct and diffuse throughout choroidal stroma (fig 1). Immunoreactivity for SDF-1 and CXCR4 was not uniform but rather heterogeneous. So the scores of immunoreactivity represent the graders' overall impression of the immunoreactivity in a particular structure throughout the whole tissue section. In some choroidal tissue sections, circulating cells, presumably leucocytes, within choroidal vascular lumens as well as in choroidal stroma were also intensely immunostained for CXCR4 (figs 1 and 2). SDF-1 immunoreactivity was blocked by preincubation of the antibody with 200-fold excess peptide used as antigen (data not shown).

Figure 2 Immunolocalisation of SDF-1 and CXCR4 in retina (top) and choroid (bottom) in an early AMD eye (case 15). The pattern and intensity of immunostaining of both antibodies appeared identical to the aged control eye in figure 1, the CXCR4 immunoreactivity is more weakly labelled in RPE than the SDF-1 immunoreactivity. Bar = 20 μ m.

Figure 3 Mean immunoreactivity scores (SD) for retinal and choroidal structures of aged control (shaded), early AMD (solid), and late AMD (open) eyes. The immunoreactivity scores for SDF-1 (A) were significantly decreased in RPE ($p < 0.0001$; $p < 0.0075$) and choroidal stroma (both $p < 0.05$) in late AMD eyes compared with the aged control and early AMD eyes, respectively. There was no significant difference in the immunoreactivity scores for the CXCR4 in all the structures graded between AMD and aged control eyes (B). Ch str, choroidal stroma; Cir cells, circulating cells; ILM, internal limiting membrane; IPM, inner photoreceptor matrix; RPE, retinal pigment epithelium; * $p < 0.05$.

Immunolocalisation of SDF-1 and CXCR4 in AMD retina/choroid

Retina and choroid of early AMD eyes had a pattern and intensity of SDF-1 and CXCR4 staining similar to aged control eyes (fig 2). CXCR4 immunostaining appeared lower in RPE cells of early AMD eyes (fig 2) compared to aged control eyes. However, there was no significant difference in immunoreactivity scores for SDF-1 and CXCR4 in retinal and choroidal structures between aged control and early AMD eyes (fig 3).

Haematoxylin and eosin staining was used for the morphology of the choroid in aged control and AMD eyes. Basal laminar deposits (BLD) or drusen were negative for SDF-1 and CXCR4 immunoreactivity (data not shown). The identity of BLDs was confirmed with periodic acid Schiff staining on serial sections.

In late AMD eyes, expression of SDF-1 was variable in retina. As shown in figure 4 (case 18), intense immunoreactivity for SDF-1 was associated with ILM, nerve fibre layer, and retinal blood vessels in some eyes, whereas CXCR4 immunoreactivity was weak in nerve fibre layer and retinal blood vessels. This was not a constant finding in late AMD eyes; only two late AMD eyes had intense SDF-1 immunoreactivity in the nerve fibre layer. However, the immunoreactivity score for SDF-1 was significantly lower in RPE cells ($p < 0.0001$) and choroidal stroma ($p < 0.05$) in late AMD eyes than in aged control eyes (fig 3). There was significantly lower SDF-1 in RPE in late AMD eyes than in early AMD eyes ($p = 0.0075$). There was no significant difference in immunoreactivity scores for SDF-1 in choriocapillaris, intercapillary

Figure 4 Immunolocalisation of SDF-1 and CXCR4 in retina and choroid from a late AMD eye (case 18) shows intense SDF-1 immunoreactivity associated with ILM, nerve fibre layer, and retinal blood vessels, whereas CXCR4 immunoreactivity is weakly labelled in nerve fibre layer and retinal blood vessels. Retinal endothelial cells and presumably an adherent leucocyte (arrowhead) are positive for CXCR4. In choroid, immunostaining of SDF-1 and CXCR4 is very weak or negative in RPE, but the reaction product appeared more diffuse in choroidal stroma. Leucocytes are positive for CXCR4 (arrows). R, retina; C, choroid. Bar = 50 µm.

septa, and large choroidal vessels in AMD eyes compared with aged control eyes. All mean immunoreactivity scores for the retinal and choroidal structures of the aged control versus AMD eyes are shown in figure 3.

In late AMD eyes (cases 16–18 and 20), disciform scars were present and had small CNV formations. The specimen from a late AMD eye (case 16) having a disciform scar with small CNV formation within the scar showed no SDF-1 and CXCR4 immunoreactivity in CNV, whereas both antibodies weakly labelled the disciform scar (fig 5). The immunostaining for both antibodies in choroidal vessels, including the choriocapillaris and choroidal stroma, was diffuse. Some pigmented cells in outer retina, perhaps “trans-differentiated RPE cells,”²⁴ were intensively labelled with SDF-1, whereas no CXCR4 was associated with migrating cells (fig 5).

DISCUSSION

This study demonstrates for the first time the immunolocalisation of SDF-1 and its receptor CXCR4 in normal aged human retina and choroid. The most prominent localisation of SDF-1 was in RPE cells and IPM, while the most prominent localisation of CXCR4 was in cells within the lumen of blood vessels. The localisation of SDF-1 and CXCR4 was similar in early AMD and aged control eyes, suggesting that SDF-1/CXCR4 may not have a prominent role in the early stages of CNV formation in AMD.

The finding of SDF-1 prominently localised in RPE was not surprising in that Crane *et al* found that RPE cells in culture can constitutively produce SDF-1.²⁵ Furthermore, they found by flow cytometry that RPE express CXCR4, which we also observed in RPE in tissue sections. The high levels of SDF-1 in photoreceptor outer segments or IPM and CXCR4 in

Figure 5 Sections of retina and choroid from late AMD eye (case 16) with a disciform scar and small CNV formation (CD34+, arrows) within the scar showing negative SDF-1 and CXCR4 immunoreactivity associated with CNV, whereas both antibodies weakly label the disciform scar itself (asterisk). Note the apparent trans-differentiated RPE cells in retina that are intensively labelled with SDF-1 (arrowheads). The immunoreaction product from both antibodies appeared more diffuse in choroid. Haematoxylin and eosin (H&E) staining demonstrate the morphological changes in retina and choroid. R, retina; C, choroid. Bar = 100 µm.

photoreceptor inner segments were unexpected. Photoreceptors are not considered to signal leucocyte or EPC homing and inner segments are not mobile so the rationale for CXCR4 in these structures is unclear. Perhaps RPE produced SDF-1 diffuses and binds to matrix components of the IPM and choroidal stroma, another site where SDF-1 and CXCR4 were prominent in choroid. Why exactly CXCR4 is associated with inner segments is unknown at this time unless there are soluble forms of CXCR4 that are shed and diffuse to bind to other proteins or matrix components.

Some reports have shown that resident RPE cells directly produced various angiogenic factors and thus contribute to the pathogenesis of AMD.^{26–27} Since SDF-1 appears to be constitutively produced by RPE and is present in choroidal stroma of all subjects, perhaps an increase in circulating CXCR4+ cells during inflammatory processes results in accumulation of these cells in RPE/Bruch’s membrane (BM)/choriocapillaris (CC) complex and further activation of them occurs because RPE produce cytokines. These ocular infiltrated inflammatory cells may then produce angiogenic factors that have a role in formation of CNV. Our recent immunohistochemical study of VEGF demonstrated that leucocytes had the greatest levels of vascular endothelial growth factor (VEGF) in RPE/BM/CC complex.²⁸ Several laboratories have suggested the importance of inflammation and recruitment of inflammatory cells in the initial phase of CNV.^{29–30}

Recruitment of endothelial precursor cells to sites of injury is a well established phenomenon in several different animal models of angiogenesis and vascular repair.^{31–32} This has been clearly demonstrated in experimental retinal and choroidal neovascularisation.^{2–4–33} The study by Sengupta *et al* suggests that SDF-1 is the attractant for these precursors.³⁴

In human AMD, the cellular composition of CNV has not been well defined. Clearly, endothelial cells are essential in the formation of the lumens of perfused new capillaries. However, CNV are fibrovascular lesions that also consist of vascular smooth muscle cells, poorly differentiated myofibroblastoid cells, proliferating RPE cells, and inflammatory cells.^{35–37} These cells are typical of a wound healing response. Although the laser induced CNV is not identical to CNV in

AMD, this model shares enough biological similarity that preclinical studies of pathogenesis and drug efficacy are said to predict human response. Like human AMD, it is a wound healing event in some ways. Monocytes³⁸ and neutrophils³⁹ are recruited to the site of laser injury and preventing this infiltration lessens the angiogenic response. Like EPCs, both of these cell types can express CXCR4,^{40–41} so recruitment to laser induced CNV may involve SDF-1. Blocking SDF-1 prevents EPCs from homing to laser induced CNV.³⁴ Therefore, it is surprising that we did not observe a significant increase in SDF-1 in early or advanced AMD or human CNV. However, a shortcoming of our study is the lack of early CNV in our specimens. All of our CNV formations were within disciform scars and, therefore, were end stage.

This study suggests that the chemokine SDF-1 and its receptor, CXCR4, are not related to early AMD and, therefore, may not contribute to the formation of CNV in AMD. However, the examples of CNV studied were within disciform scars, so we cannot comment on SDF-1/CXCR4 in developing CNV. Furthermore, the data suggest that SDF-1 levels may be less in advanced AMD than in normal aged choroidal stroma and RPE. This suggestion is probably the result of the decrease in RPE cell number and presence in advanced AMD and the significant thinning of choroidal stroma with age. This study presents the interesting scenario of constitutive production of a potent chemokine for homing of leucocytes and EPCs. Why this does not result in accumulation of CXCR4+ cells in RPE/BM/CC normally remains to be elaborated.

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