

CHAPTER 5

ROLE OF FAS-LIGAND IN AGE-RELATED MACULOPATHY NOT ESTABLISHED

ABSTRACT

Purpose: Fas-ligand (FasL) expression on retinal pigment epithelium (RPE) is hypothesized to have an inhibitory effect on human ocular neovascularization.

Methods: We studied FasL expression in the aging RPE and in early and late stages of age-related maculopathy (ARM). Immunohistochemistry with antibodies against FasL was performed on paraffin-embedded sections of 23 human eye bank eyes (aged 45 to 96 years) and 12 eyes with neovascular AMD.

Results: FasL expression in RPE was not related to age or to the presence of early ARM. Furthermore, FasL expression in RPE was similar in subretinal and sub-RPE choroidal neovascular membranes (CNVM).

Conclusions: It appears to be unlikely that FasL expressed on RPE controls the extension of CNVM from sub-RPE to subretinal.

INTRODUCTION

Age-related maculopathy (ARM) is the major cause of blindness in people over 65 years in the Western world.³ Late stages of ARM, also called age-related macular degeneration (AMD), include geographic atrophy and neovascular macular degeneration. The neovascular form is characterized by choroidal neovascular membranes (CNVM). In CNVM new vessels grow beneath the retinal pigment epithelium and the retina from the underlying choroid. Many growth factors have been identified that might influence angiogenesis in CNVM, such as VEGF, bFGF and somatostatin.^{145,159,160,189,190,246-248}

Recently, the role of Fas and its natural ligand, Fas-ligand (FasL) has been acknowledged in the process of angiogenesis.²⁴⁹ Fas and FasL are important for apoptosis in T-lymphocytes but are also expressed on non-lymphoidal tissue. In the eye Fas-FasL interactions appear to be an important mechanism for the maintenance of immune privilege by inducing apoptosis of invading lymphocytes.⁶³ Kaplan and coworkers⁶⁴ studied the role of FasL in surgically excised CNVMs of patients with AMD. They demonstrated FasL-positive RPE cells in close proximity to and surrounding Fas-positive vascular endothelial cells in new vessels. They also found an increased incidence of neovascularization in Fas-deficient and FasL-defective mice compared with normal mice. Fas-FasL interaction on RPE induced apoptosis of cultured choroidal endothelial cells. They concluded that FasL expressed on RPE may control the growth and development of subretinal neovascularization. They hypothesized that with RPE senescence, subretinal neovascularization in AMD may result from a decreased inhibitory effect of FasL-positive RPE cells on angiogenesis.

The purpose of our study was to investigate FasL expression in the aging RPE and in early stages of ARM, and to study FasL expression on RPE in subretinal (clinically defined as classic) CNVMs, as well as subretinal RPE (clinically defined as occult) CNVMs.

MATERIALS AND METHODS

The study was performed according to the tenets of the Declaration of Helsinki. Enucleation or surgical excision of subfoveal CNVs was performed after obtaining informed consent of the patient.

Patient materials

All eyes were retrieved from the files from the Ophthalmic Pathology Department of the University Hospital of Rotterdam. For determination of FasL expression on RPE related to age and early ARM, 22 human eye bank eyes were used. The donors were 45 to 96 years of age (mean 75 years) with postmortem time from 2 to 11 hours. The donors had no history of eye disease, and the samples were macroscopically and microscopically checked for retinal diseases that might stimulate angiogenesis. The macular area (about 1 cm²) was dissected from the ocular tissue, fixed in phosphate buffered formaldehyde and embedded in paraffin. Sections of 5 µm were made and classified for the presence of ARM as described before¹⁴⁴ (Table 5.1).

Furthermore, FasL expression on RPE in CNVM was determined on 12 eyes (6 enucleated eyes, 4 donor eyes and 2 surgically removed subretinal neovascularizations) of 11 patients with neovascular AMD, described before²⁴⁸ (Table 5.2). All eyes were processed for routine diagnostic procedures by fixation in formaldehyde and embedded in paraffin. Five µm sections were prepared for immunohistochemistry.

TABLE 5.1. CLASSIFICATION OF HUMAN MACULAE AND FASL EXPRESSION IN RPE

Case no.	Age	PM	Classification of macula	FasL expr RPE
1	38	9.5	no ARM	1
2	45	8.5	no ARM	2
3	55	7	no ARM	2
4	64	10	ARM	2
5	65	8	ARM	0
6	67	11	ARM	3
7	74	8	ARM	1
8	74	8	ARM	2
9	76	7	no ARM	3
10	77	9	no ARM	0
11	77	9	no ARM	2
12	80	9.5	no ARM	2
13	81	5.5	no ARM	n.c.
14	81	7	ARM	0
15	81	7	ARM	2
16	85	4.5	ARM	3
17	86	5	ARM	2
18	86	10	ARM	3
19	87	5	ARM	0
20	88	8	ARM	0
21	91	4.5	no ARM	3
22	96	2	ARM	2

Categories of FasL expression: 0 (0 – 25% positive cells), 1 (26 – 50% positive cells), 2 (51 – 75% positive cells) and 3 (76 – 100%). PM = postmortem time in hours; ARM = age-related maculopathy; n.c. = not classifiable.

Immunohistochemistry

Polyclonal rabbit antibodies against Fas (C20) and FasL (N20) for immunohistochemistry were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The sections were deparaffinated and rehydrated. After blocking with normal goat serum (Dako, 1:10) for 15 minutes, the slides were incubated with the antibodies (Fas, 1:500; FasL 1:100) for 1 hour. The sections were further incubated with biotinylated multilink antibodies for 30 minutes, followed by alkaline phosphatase-labeled antibiotin (both Biogenex, San Ramon, USA) for 30 minutes. The complex was visualized by incubating the sections with new fuchsin for 30 minutes in the dark. The slides were counterstained with Mayer's hematoxylin, mounted and examined by light microscopy. We graded the expression in 4 categories of positive cells: 0 (0 – 25%), 1 (26 – 50%), 2 (51 – 75%) and 3 (76 – 100%). Negative controls for immunohistochemistry included 1) omission of the primary antibody, 2) incubation with an irrelevant polyclonal rabbit antibody and 3) preabsorption of the antibodies with a tenfold of the immunizing receptor peptide for 4 hours. The manufacturer has described the specificity of the antibodies.

RESULTS*FasL expression in aging human macula and early ARM*

FasL protein was found mostly in a membranous pattern at the basal side of the RPE (Figure 5.1A). Incidental cells stained in a more diffuse pattern. In early ARM, FasL staining was similar to non-ARM maculae (Figure 5.1B).

FasL expression in RPE cells was not related to age (the Spearman coefficient, $r = -0.14$, $P = 0.95$), nor to presence of early ARM (logistic regression adjusted for age, FasL > 25% vs. FasL < 25%; odds ratio = 2.3; 95% CI: [0.2 to 28.2]) (Table 5.1). In negative controls, no staining was detected.

FasL expression on RPE in CNVM

In CNVMs, strong FasL and less intense Fas staining were found in RPE monolayers (Figure 5.1C-D, Table 5.2). Endothelial cells of newly formed vessels had both FasL and Fas expression in most cases. FasL staining in RPE was similar in sub-RPE (Figure 5.1C) and subretinal CNVMs (Figure 5.1D), as well as in fibrovascular and fibrocellular CNVMs. In negative controls with CNVMs, no staining was detected.

TABLE 5.2. PATIENT DATA AND EXPRESSION OF FASL AND FAS IN EYES WITH CNV

No.	Age/ sex	OD/ OS	Clinical description	Histological classification CNV			Fas/FasL expression					
				subretinal/ sub-RPE	FV/FC	other	Fas			FasL		
							EC	RPE	EC	RPE	EC	RPE
1. CNV1	79/M	U	Surgically excised CNV	mixed	FV and FC	hemorrhage	1	1	2	2	0	
2. CNV2	79/F	U	Surgically excised CNV	subretinal	FV and FC	hemorrhage	0	1	2	2	2	
3. CNV6	72/M	OS	disciform MD	mixed	FV and FC	BLD, hemorrhage	2	1	1	1	2	
4. CNV7	86/M	OS	disciform MD, acute glaucoma	sub-RPE	FV and FC	BLD, hemorrhage, retinal detachment; posterior uveitis	1	0	1	1	0	
5. CNV8	91/M	OS	donor eye	mixed	FC	BLD	NC	NC	NC	NC	NC	
6. CNV9	87/M	OS	donor eye	mixed	FV and FC	BLD	2	0	0	0	0	
7. CNV10	83/M	OD	painful eye, suspected uveal melanoma	mixed	FV and FC	BLD, hemorrhage, ischemic retinal disease	1	1	2	2	1	
8. CNV11	73/M	OS	disciform MD	subretinal	FC and FV		2	0	2	2	1	
9. CNV12	73/M	OD	disciform MD, post irradiation	subretinal	FV		1	1	2	2	0	
10. CNV13	82/M	OD	disciform MD	mixed	FC	confluent soft drusen	1	1	1	0	2	
11. CNV14	85/F	OS	post surgical endophthalmitis	sub-RPE	FV	endophthalmitis, uveitis	0	1	1	1	2	
12. CNV15	84/F	OD	expulsive hemorrhage, cataract	sub-RPE	FV	hemorrhage	1	1	1	1	2	

Categories of Fas and FasL expression: 0 (0 – 25% positive cells), 1 (26 – 50% positive cells), 2 (51 – 75% positive cells) and 3 (76 – 100%). U = unknown; CNV = choroidal neovascular membrane; FV = fibrovascular; FC = fibrocellular; ARM = age-related maculopathy; NC = not classifiable.

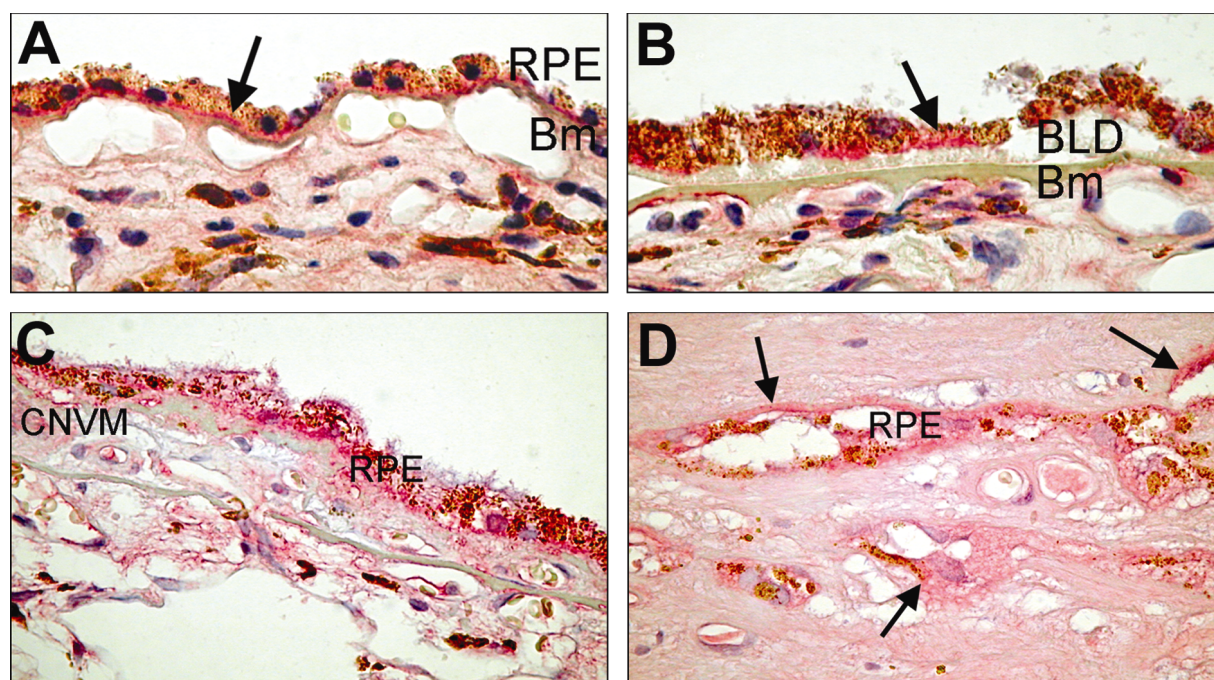


Figure 5.1 *FasL expression in RPE* (A) FasL staining of human macular RPE in 80-year old donor eye. The FasL protein is found mostly in a membranous pattern at the basal side (arrow). (B) FasL staining of 86-year old donor eye with ARM. (C) FasL staining of subRPE choroidal neovascular membrane (CNVM) secondary to ARM. (D) FasL staining on RPE (arrows) of mixed subRPE and subretinal CNVM secondary to ARM. RPE = retinal pigment epithelium, Bm = Bruch's membrane, BLD = basal laminar deposits, CNVM = choroidal neovascular membrane (original magnification $\times 400$, counterstaining Mayer's hematoxylin).

DISCUSSION

Our results show similar expression of FasL in the RPE in maculae of different age and ARM status. This might indicate that FasL expression in the RPE is not age-related or related to the presence of early ARM. This is not in line with Kaplan's hypothesis of FasL reduction with RPE senescence.⁶⁴

In earlier studies, immunohistochemical Fas and FasL expression was found constitutively in the normal human retina and the choroid.²²⁴ Both were also detected in cultured human RPE.²⁵⁰

Furthermore, we found similar FasL RPE expression in subretinal and in sub-RPE CNVM secondary to ARM. In subretinal CNV, the fibrovascular tissue grows through the RPE beneath the retina, while in sub-RPE CNV the neovascularization is restricted to the sub-RPE level. From experimental studies, and a study on excised human subretinal CNVMs it was postulated that decreasing FasL expression in sub-RPE CNVMs fails to inhibit subretinal extension of CNVM.⁶⁴ We did not find decreased expression in sub-RPE or mixed sub-RPE/subretinal CNVMs compared with subretinal CNVMs. Kaplan and coworkers used surgically excised CNV, which are mainly localized subretinal, while CNVMs in our study were localized mainly sub-RPE and mixed sub-RPE/subretinal.

This might partly explain the different expression pattern of Fas and FasL in our study compared to the results of Kaplan and coworkers. However, subretinal CNVMs are less common in ARM.¹²⁰ Therefore, it appears to be unlikely that FasL expression in RPE controls the extension of CNVM from sub-RPE to subretinal in human ARM. It may be possible that the Fas-FasL system is still important in this process, but through soluble- rather than membrane bound factors. Further investigation about the role of FasL in ARM is necessary.

