6

Fibrovascular Membranes Associated with PDR: Development of Molecular Targets by Global Gene Expression Profiling

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1. Introduction

Diabetic retinopathy (DR) is one of the leading causes of decreased vision and blindness in industrialized countries (Bhavsar, 2002). Much of the retinal damage that characterizes advanced proliferative diabetic retinopathy (PDR) results from retinal neovascularization (Simo, et al., 2006). When the newly formed vessels are associated with fibrous proliferations that form fibrovascular membranes (FVMs) on the surface of the neuroretina, traction retinal detachments can develop, resulting in potentially severe loss of vision (Hiscott, et al., 2000).

FVMs are characterized by the migration and proliferation of various types of cells, e.g., retinal glial cells, fibroblasts, macrophages/monocytes, hyalocytes, laminocytes, and vascular endothelial cells. It has been postulated that the formation of FVMs represents a wound healing process (Hiscott, et al., 2000). To date, the factors regulating the development and progression of FVMs have not been fully determined. Moreover, despite improvements in vitreal surgical techniques, panretinal photocoagulavtion, and the use of intravitreal anti-VEGF drugs such as Ranibizumab, the prognosis for DR is still poor especially in advanced cases of PDR. It is therefore required to develop better treatments that are based on the pathogenesis of FVMs.

2. Conventional studies investigating the mechanisms of FVM formation

Earlier conventional studies investigating the molecular effects of FVM formation have focused mainly on one or a few molecules or pathways. Several molecules, e.g., vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF- α), basic fibroblast growth factor (bFGF), intereleukin-8 (IL-8), apelin, tumor endothelial marker 7 (TEM7), monocyte chemoattractant protein-1 (MCP-1;CCL2), erythropoietin, angiopoietin-2, advanced glycation end product, nuclear factor kappa-B, and activator protein-1 have been detected in FVMs and/or vitreous fluid collected from patients with PDR (Simo, et al., 2006; Yoshida, et al., 1998; Yoshida, et al., 1999; Yoshida, et al., 2010; Watanabe, et al., 2005).

Retinal hypoxia is assumed to be the common mechanism which initiates a series of events leading to retinal neovascularization (Ishikawa, et al., 2010). Cellular inflammation is initiated at the blood-microvascular endothelial-cell interface, and leukocytic infiltration has been observed after retinal hypoxia. Several mediators of retinal neovascularization have been determined in well-established murine models of oxygen-induced retinopathy (OIR) (Smith, et al., 1994).

Chemokines, a family of structurally related cytokines involved in the activation and directed migration of leukocytes, may be pathophysiologically key mediators of inflammation (Singh, et al.). Two well-studied CC chemokines are MCP-1 and macrophage inflammatory protein-1α (MIP-1α;CCL3). Both MCP-1 and MIP-1α have been shown to mediate the recruitment of leukocytes and induction of neovascularization in several inflammatory diseases.

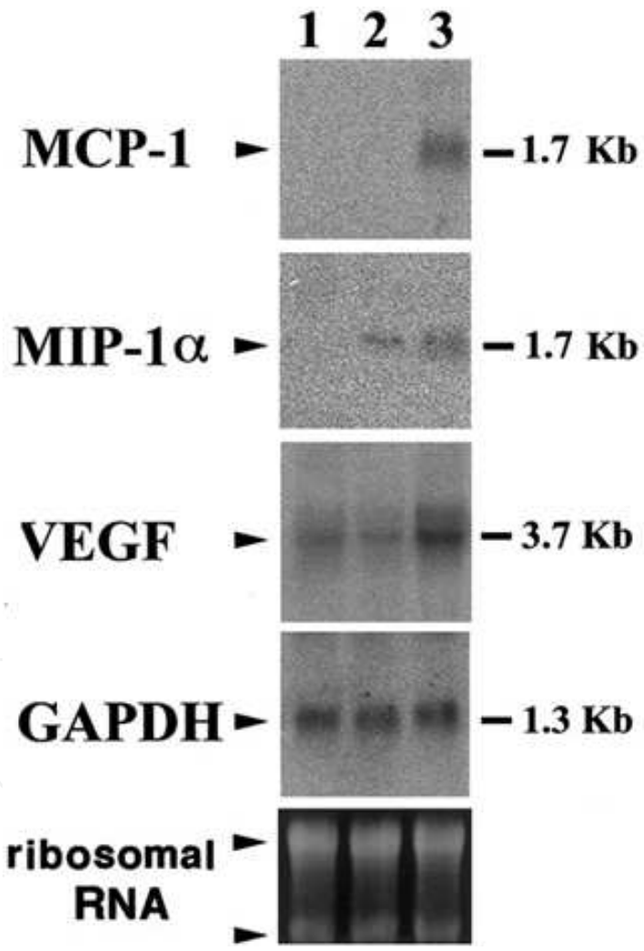

Fig. 1. Northern blot determination of mRNA expression of MCP-1, MIP-1α, and VEGF in murine OIR. Representative blots of three independent experiments are shown. Lane 1, Control retina (P12); lane 2, retina after 5 days exposure to hyperoxia (P12); lane 3, retina 12 h after hypoxia (P12.5). For control, the same blot was stripped and reprobed with GAPDH, and 18 S and 28 S ribosomal RNA were used for equal loading of RNA. Each lane contains 10 μg total RNA. Reproduced with permission from Yoshida et al. [17]

In order to determine the role played by MCP-1 and MIP-1α, we have investigated whether these chemokines can be induced in murine OIR (Singh, et al.). The expression of the mRNAs of MCP-1 and MIP-1α was very low or undetectable in the retinas of the normal controls and in P12 mice killed just 5 days after hyperoxia (Fig. 1). A dramatic increase in MCP-1 mRNA expression was observed 12 h after the onset of hypoxia. The profile of MIP-1α mRNA expression was similar to that of MCP-1 mRNA, except that a slight increase of MIP-1α mRNA was detected in the retinas of mice 5 days (P12) after hyperoxia.

In contrast to MCP-1 and MIP-1α, a steady level of VEGF mRNA expression was found in the retinas of control, normal mice (Fig. 1). Hyperoxia resulted in a significant down-regulation in the level of VEGF mRNA, and the subsequent hypoxia led to a significant up-regulation of VEGF expression compared with that in the control retinas.

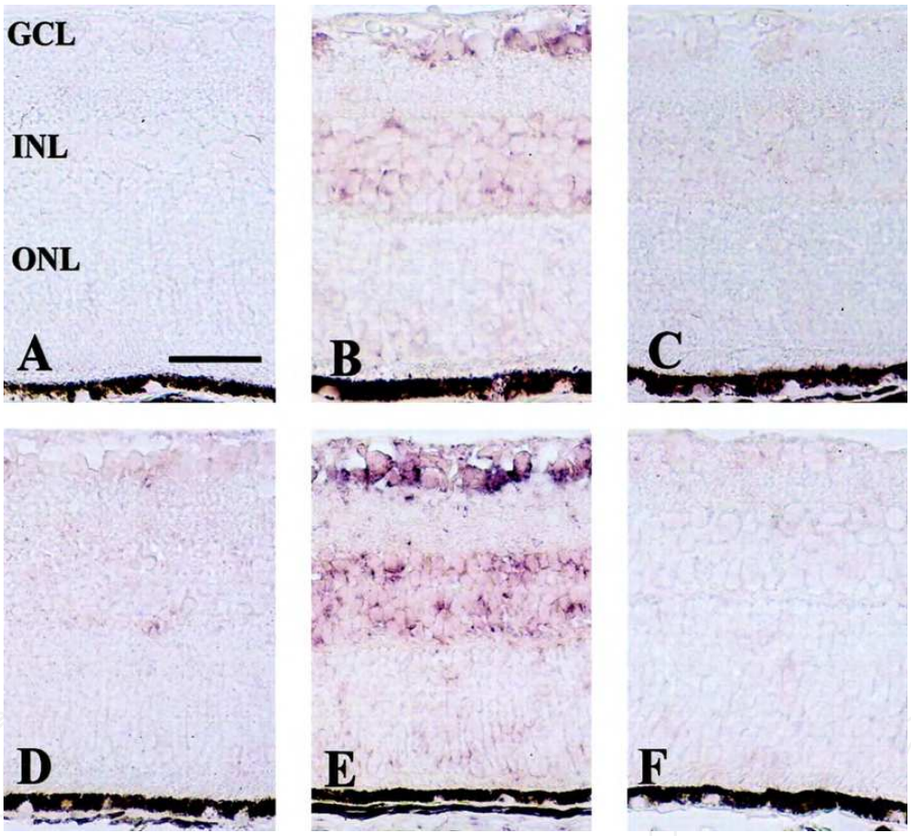

Fig. 2. In situ hybridization for MCP-1 and MIP-1α in the retinas of murine OIR. Hybridization was performed with antisense (A, B, D, E) or sense (C, F) probes specific for MCP-1 (A–C) and MIP-1α (D–F). A, D, Control normal retina (P12); B, C, E, and F, retina 12 h after hypoxia (P12.5). GCL, Ganglion cell layer; INL, inner-nuclear layer; ONL, outer-nuclear layer. Original bar = 50 μm. Reproduced with permission from Yoshida et al. [17]

In retinal hypoxia, the inner retina, which is supplied by retinal vessels, is hypoxic, whereas the outer retina, supplied by the choroidal vessels, is not. In situ hybridization for MCP-1 and MIP-1α showed a prominent increase of positive cells located in the inner retina 12 h after the hypxia (P12.5) in comparison to the lower level of staining in the nonhypoxic

retinas of control mice (Fig. 2). The inner retinal layer where ganglion cells and astrocytes are located was the most prominent cellular site of MCP-1 and MIP-1α gene expression in the hypoxic inner retina. This suggests that MCP-1 and MIP-1α expressed in this region attract resident microglia, hyalocytes, and/or bone marrow-derived monocyte lineage cells (BM-MLCs) through the blood-retinal barrier toward the superficial layer of the retina where neovascularization occurs.

Among the environmental stimuli, gene expression by macrophages/BM-MLCs following hypoxia is becoming increasingly well-characterized to have angiogenic potential(Lewis, et al., 2007). In murine OIR, macrophages/BM-MLCs in the ischemic retina exhibited thicker and more distended processes compared with those in normal, control retinas (Ishikawa, et al., 2011). We assume that such "hypoxia-activated" BM-MLCs have the potential to produce an array of angiogenic cytokines and growth factors including TNF-α and VEGF, which can contribute to the progression of retinal neovascularization/revascularization. TNF-α is an angiogenic molecule produced by hypoxic monocytes/macrophages (Yun, et al., 1997) and is a likely mediator of retinal neovascularization/revascularization. It has been reported that TNF-α level is higher in patients with PDR(Limb, et al., 2001), and we have detected this molecule in the macrophages/BM-MLCs in murine OIR (Yoshida, et al., 2004). Moreover, we have demonstrated that TNF-α up-reguletes the production of IL-8, VEGF, bFGF, or MCP-1 in retinal vascular cells and/or glial cells adjacent to microvessels triggering neovascularization/revascularization in an autocrine or paracrine manner (Yoshida, et al., 2004; Yoshida, et al., 1997). These processes are likely to be important in promoting macrophages/ BM-MLCs-related retinal neovascularization/revascularization in hypoxic retinas.

3. Global gene expression profiling of FVMs

Despite earlier studies investigating the molecular effects of retinal hypoxia, the molecular events taking place in hypoxic retinas that may lead to retinal neovascularization remain undetermined. The recent technological advancements in genomics, such as advent of microarray technology, have opened up new avenues to identify all the genes and their products that are expressed in a particular tissue.

To determine the factors that are activated during retinal hypoxia, we performed a gene expression profiling of hypoxic retinas obtained from a murine OIR using gene microarray thechnology(Ishikawa, et al., 2010). Our analyses showed that retinal hypoxia were associated with specific changes in the patterns of gene expression. These alterations may reflect the postischemic inflammation, and subsequent neural and vascular remodeling, and pathologic neovascularization in retinas of murine OIR (Fig. 3).

Several genes reported to be involved in retinal hypoxia, such as *Vegfa*(Yoshida, et al., 2003), *Hif1a*(Ozaki, et al., 1999), and *Mip1a*(*Ccl3*)(Yoshida, et al., 2003), were also detected to be differentially expressed in the hypoxic retina in our study, confirming that our microarray analyses were trustworthy. Interestingly, the most up-regulated gene among the differentially-expressed genes in hypoxic retinas was the *Mip1β(Ccl4)* and not *Vegfa* and other chemokines.

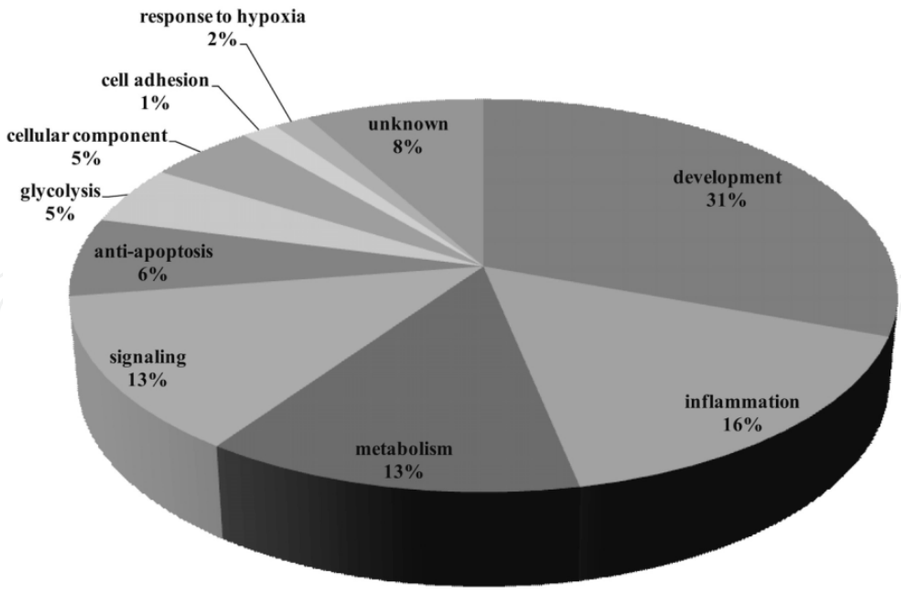

Fig. 3. Distribution of Gene Ontology terms in genes regulated by hypoxic retinas. Ontology of genes upregulated by hypoxia. Reproduced with permission from Ishikawa et al. [8]

MIP-1β (CCL4) is a member of the CC chemokine family that are characterized by their ability to direct migration of leukocytes into inflamed tissue. MIP-1β was first isolated from the culture medium of LPS-activated macrophages, and it recruited macrophages/microglia to sites of injury in patients with sepsis, arthritis, and systemic sclerosis. Because MIP-1β is not known to be a hypoxia-responsive gene in the retina, it may play more roles in hypoxic retinas than previously envisaged. Therefore, further studies are required to determine the role played by MIP-1β in hypoxia-induced retinal neovascularization/revascularization (Ishikawa, et al., 2011).

Expressed sequence tag (EST) analysis, another method of global gene expression profiling, permit the identification of genes expressed in particular tissues in a completely unambiguous manner (Wistow, 2006). ESTs may also reveal comprehensive data on transcript and gene variants. These represent important sources for the search for as yet incompletely characterised genes and pathways as exemplified by the NEIBank project in the eye research field (Wistow, 2006). We hypothesized that a comprehensive analysis of gene expression in FVMs may open up new avenues in enhancing our understanding of the formation of FVMs, and such an effort should lead to further advances in the surgical and medical treatment of FVMs (Yoshida, et al., 2010).

To overcome the limitation of the starting amount of RNA from the FVMs obtained from patients with PDR, We chose to employ Switching Mechanism at the 5′ end of RNA Transcript (SMART) technology, an exponential PCR-based technology. With this technology, we have successfully constructed a complementary DNA (cDNA) library from the FVMs and sequenced more than 2800 cDNAs (Yoshida, et al., 2010). We next performed sequence similarity searches to compare every EST to those in public databases. For ESTs with known gene matches in public databases, functional annotation was retrieved from the human cDNA database (Ensembl) and analysed by FatiGO.

Among the 625 non-redundant clusters, 515 (82%) matched Ensembl. The remaining 110 (17%) corresponded to potentially novel ESTs or untranslated sequences. Among those database-matched, 515 clusters were subdivided by functional subsets of genes related to ribosomal activity, oxidative phosphorylation, focal adhesion, cell adhesion, and other functions by FatiGO analysis (Fig. 4). This suggests that many subsets of functional genes are expressed in FVMs. Thus, there are many complex interactions among the molecules that are encoded by those genes. Among these, ferritin, light polypeptide (*FTL*) and metastasis associated lung adenocarcinoma transcript 1 (*MALAT1*) appeared to be the most abundant transcripts in the FVMs (Yoshida, et al., 2010).

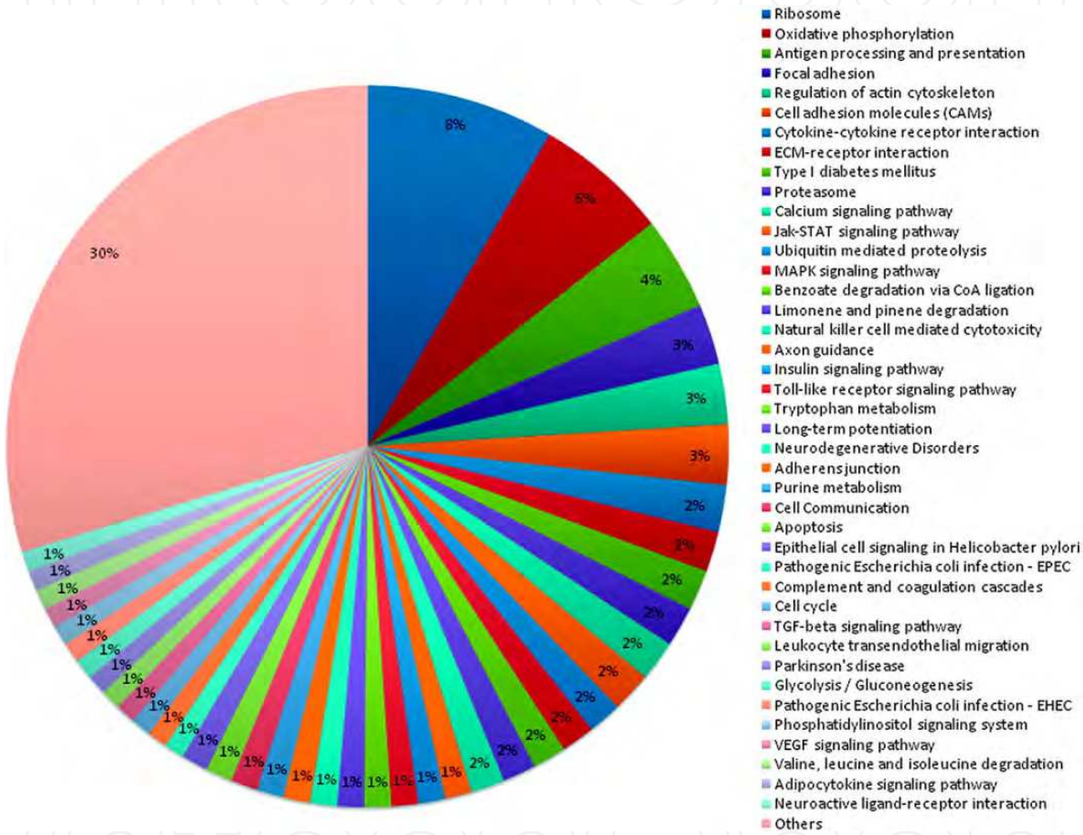

Fig. 4. The known human genes identified in the human fibrovascular membranes (FVMs) are grouped according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) functional categories. Reproduced with permission from Yoshida et al. [20]

The importance of the production of extracellular matrix by FVMs is well recognized (Hiscott, et al., 1999). In agreement with this notion, functional annotation of ESTs determined that those genes related to cell adhesions and focal adhesions are highly expressed in FVMs. Until now, among the components in extracellular matrix, type II collagen, SPARC and FN, major components of the vitreous, are well-known molecules. However, our EST analysis demonstrated the expression of *COL1A2, COL3A1, MXRA8,* and others, in addition to *FN, SPARC* as cellular adhesion components. This indicates that cells that comprise the FVM actively produce a variety of adhesion molecules and are actively involved in cell migration and proliferation (Yoshida, et al., 2010).

4. Development of molecular targets by global gene expression profiling of FVMs

Recently, several trials of anti-VEGF therapy such as Ranibizmab have been applied on patients with intraocular neovascular diseases. However, undesirable side effects including brain and retinal vein occlusion have been reported (von Hanno, et al., 2010). Therefore, identifying the other molecular targets that can be treated less invasively is definitely still a goal. Because we were able to determine the existence of several potential factors other than VEGF by global gene expression profiling of FVMs (Yoshida, et al., 2010), we postulate that some of the extracted genes may be additional novel candidates for molecular targeting therapy. Among newly identified genes from FVMs, we found that tumor endothelial marker 7 (TEM7) and periostin were expressed more strongly in FVMs than in idiopatic epiretinal membranes (ERMs) (Yoshida, et al., 2011; Yamaji, et al., 2008). This led us to hypothesize that these two molecules play key roles in the maintenance and/or development of FVMs.

a. Tumor endothelial marker 7 (TEM7)

As described earlier, recent technological improvements in cellular fractionation and genomics have led to the identification of several markers preferentially expressed on vascular endothelial cells of human tumors (Nanda and St Croix, 2004). Among these markers, the tumor endothelial markers (TEMs) are a group of cell surface proteins preferentially expressed on the endothelial cells of various cancer cells.

Of these cell surface markers, TEM7, also known as plexin domain-containing 1 (PLXDC1), is especially attractive because it is the most abundant isoform among the TEMs. TEM7 protein is overexpressed in the neovascular vessels of human solid tumors such as lung, colon, and esophageal cancers (Nanda and St Croix, 2004). The full-length form of TEM7 has sequence characteristics of cell surface proteins, including signaling peptides, plexin-semaphorin-integrin (PSI) domain, and transmembrane domain(s). In addition, TEM7 is expressed as a complex pattern of transcripts derived by alternative splicing with potentially different activities and biological functions. These transcripts are predicted to be intracellular (TEM7-I), secreted (TEM7-S), or on the cell surface membrane (TEM7-M) of the endothelial cells of tumors.

We asked whether the mRNA of TEM7 is expressed in the neovascular endothelial cells of FVMs surgically removed from patients with PDR (Fig. 5) (Yamaji, et al., 2008). The mRNA of TEM7 was enhanced in 10 of 10 FVMs obtained from PDR patients but was barely detected in the five idiopathic ERMs (control). In addition, RT-PCR with specific primer pairs yielded multiple bands suggesting the presence of splice variants of TEM7 in the FVMs. The mRNAs of IL-8 were detected in 10 of 10 FVM specimens, VEGF in 8 of 10 FVM specimens, and VEGFR2 in 9 of 10 FVM specimens. In contrast to TEM7, these 3 angiogenic molecules were also upregulated in some of the control idiopathic ERMs (3 of 5, 3 of 5, and 2 of 5, respectively).

To determine the location of the protein of TEM7 in FVMs, we next double-stained the FVM sections with an anti-TEM7 monoclonal antibody (IM193), which specifically detects TEM7-M, and an antibody to CD34, an endothelial cell marker (Fig. 6). Consistent with previous

results on the staining patterns of tumors with neovascularization (Nanda and St Croix, 2004), the antibody specifically labeled the neovascular endothelial cells in the FVMs (Fig. 6).

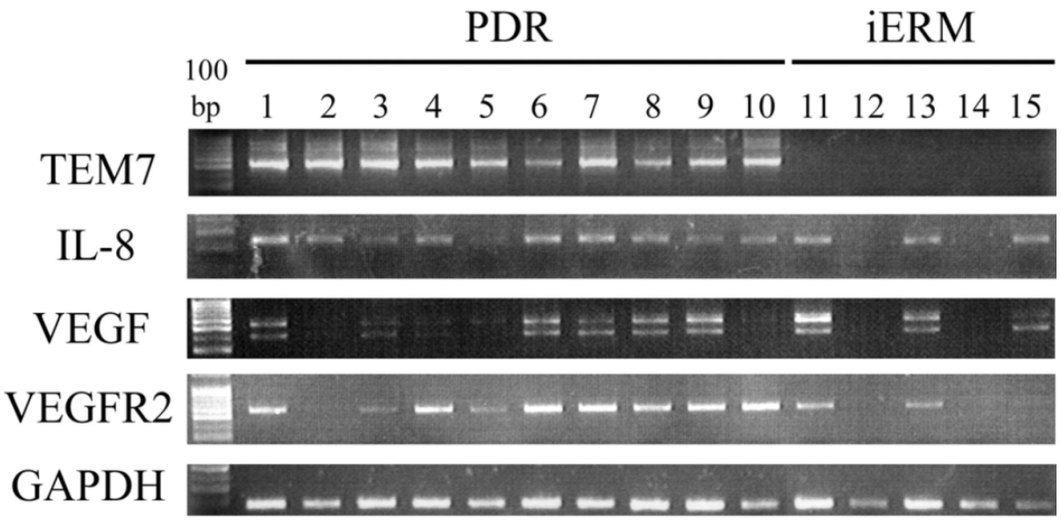

Fig. 5. RT-PCR analysis of TEM7, IL-8, VEGF, VEGFR2, and GAPDH in fibrovascular membranes derived from patients with proliferative diabetic retinopathy (PDR; patients 1–10) and in epiretinal membranes from eyes with idiopathic epiretinal membranes (iERMs; patients 11–15). After 35 cycles, 8 µL each sample was electrophoresed through a 2% Tris-acetate-EDTA agarose gel, and the fractionated products were stained with ethidium bromide. Note the distinct high expression of the mRNA of TEM7 in the fibrovascular membranes derived from patients with PDR compared with control iERMs. Reproduced with permission from Yamaji et al. [24]

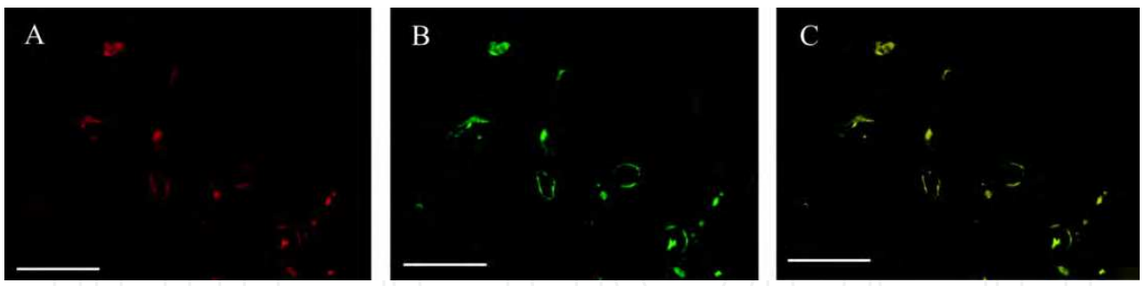

Fig. 6. Double staining for TEM7 and CD34 in the FVM. (A) Neovascular endothelial cells are visible after specific staining with CD34 in the FVM. (B) Specific staining for TEM7 in the same section shows an identical staining pattern. (C) Double staining for TEM7 and vascular endothelial cells in the same sample shows positive cells for both antibodies. The *yellow* staining is caused by the overlapping of the *red* and the *green* colors, showing colocalization of TEM7-M with the pan-endothelial marker CD34. Sale bars, 50 µm. Reproduced with permission from Yamaji et al. [24]

To determine a more exact location of the TEM7 protein within the neovascular endothelial cells, we performed immunoelectron microscopy using monoclonal anti-TEM7 antibody (IM193). Electron microscopy revealed that TEM7-M was expressed at the tight junctions and at the luminal surfaces of the vascular endothelial cells (Fig. 7)

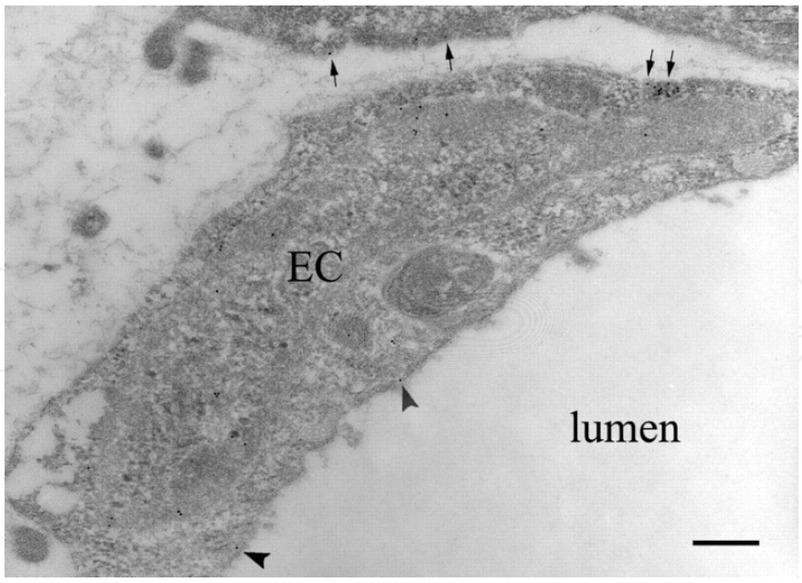

Fig. 7. TEM7 staining for transmission electron microscopy with IM193 antibody to detect TEM7-M. Staining is observed on the tight junctions (*arrows*) and on the luminal surfaces of endothelial cells (ECs; *arrowheads*). Scale bar, 200 nm. Reproduced with permission from Yamaji et al. [24]

Considerable effort has been invested recently to develop agents that block the formation of new blood vessels. For example, Bebacizumab, a selective VEGF inhibitor, was recently found to be effective in the regression of retinal and iris neovascularization secondary to PDR, but because of its cytostatic property, its effect may be limited to established vasculature. Therefore, it has become apparent that targeted destruction of the established vasculature is another option for therapeutic opportunities. In this regard, the presence of membrane-bound TEM7 on the luminal surfaces of neovascular endothelial cell is of interest (Fig. 7). Recently, cortactin, a monomeric protein that can be activated by external stimuli to promote polymerization and rearrangement of the actin cytoskeleton, and nidogen, a component of the basement membrane, were identified as proteins capable of binding to the extracellular region of TEM7 (Lee, et al., 2006; Nanda, et al., 2004). Because the retinal vessels are the only vessels that can be observed in situ, it may be possible to use a more selective ligand-based neovascular endothelial cell targeting strategy by delivering the bioactive molecules to the blood-retinal neovascular endothelial interface. For example, photodynamic therapy using such TEM7-binding partners labeled with photosensitive biomolecules may open new possibilities for a lower invasive therapy of retinal neovascularization.

b. Periostin

Periostin is a matricellular protein and is a member of the fasciclin family (Takeshita, et al., 1993). It contains an N-terminal secretory signal peptide, followed by a cysteine-rich domain, four internal homologous repeats, and a C-terminal hydrophilic domain (Horiuchi, et al., 1999). The high degree of structural and sequence homology of periostin with fasciclin 1 and transforming growth factor β–induced (TGFBI) suggests that periostin plays a role in cell adhesion and migration (Horiuchi, et al., 1999). In addition, periostin is expressed as a

complex pattern of transcripts derived by alternative splicing with potentially different activities and biological functions (Yoshida, et al., 2011).

Periostin expression is altered in different diseases, including neoplasias, cardiovascular disease, and wound repair (Kanno, et al., 2008). Periostin is overexpressed in various human cancers such as pancreas, colon, ovary, oral squamous cell carcinoma, and lung, and its overexpression is correlated with the aggressiveness of the tumor and with poorer survival. Moreover, tumor cell lines engineered to overexpress periostin have accelerated the growth and higher angiogenic and metastatic potential in immunocompromised animals. In the heart, periostin plays a key role in the progression of cardiac valve complex degeneration by inducing angiogenesis and MMP production (Hakuno, et al., 2010). Periostin is also an element of bone marrow fibrosis and subepithelial fibrosis of bronchial asthma (Takayama, et al., 2006).

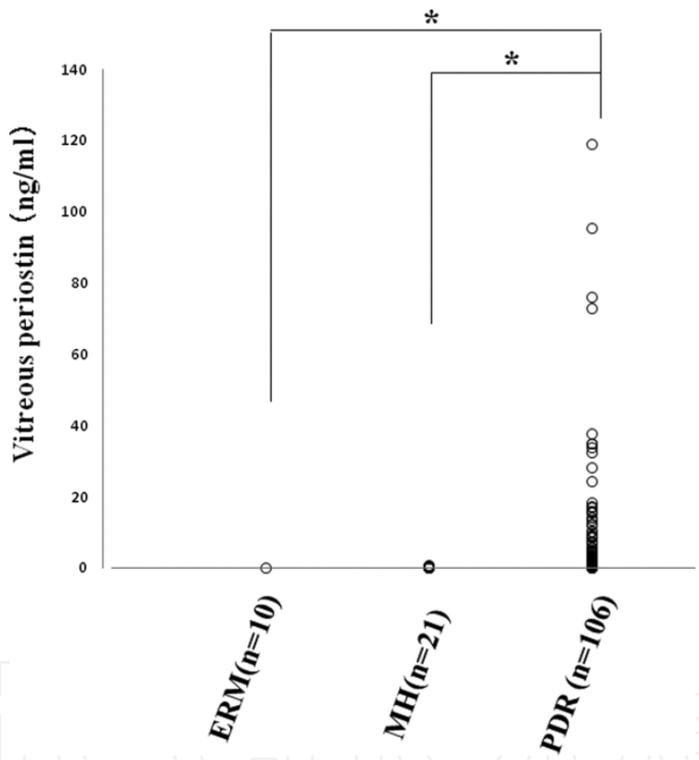

Fig. 8. Periostin levels in vitreous samples from eyes with nondiabetic (epiretinal membrane and macular hole) ocular diseases and eyes with proliferative diabetic retinopathy. ERM, epiretinal membrane. *$P < 0.001$. Reproduced with permission from Yoshida et al. [23]

We examined the amount of periostin in the 106 vitreous samples of patients with PDR collected during vitrectomy, and in the 31 vitreous samples obtained from patients during macular hole or ERM surgery (Yoshida, et al., 2011). We found that the concentration of periostin in the vitreous of patients with PDR was significantly higher than that in the vitreous of patients without PDR (Fig. 8). The concentration of periostin in the vitreous of patients with PDR was significantly correlated with the presence of FVMs but that of VEGF was not correlated (Yoshida, et al., 2011). The differences in the correlations between periostin and VEGF are probably because VEGF is upregulated in the retina at an earlier

stage in response to hypoxia before the fromation of FVMs (Ishikawa, et al., 2010; Yoshida, et al., 2003).

Immunohistochemical analyses with the anti-periostin antibody revealed that periostin was expressed in the vascular pericytes that were α-SMA positive (Yoshida, et al., 2011). Periostin is reported to promote angiogenesis by an up-regulation of the VEGF receptor, Flk-1/KDR, by endothelial cells through an integrin αVβ3-focal adhesion kinase-mediated signaling pathway (Shao, et al., 2004). This suggests that periostin may play a role in promoting and/or maintaining vasculature in FVMs in a paracrine fashion. In addition, periostin was also expressed in myofibroblast-like cells in the stroma of FVMs (Yoshida, et al., 2011). It has been reported that a stable expression of a periostin in 293T cells causes the cells to undergo fibroblast-like transformation, and the cells expressing ectopic periostin increased cell migration, invasion, and adhesion (Yan and Shao, 2006). These findings suggest that periostin-expressing myofibroblast-like cells may play a role in the invasive properties of FVMs. Taken together, these results indicate that periostin may play specific roles in the formation and/or maintenance of FVMs.

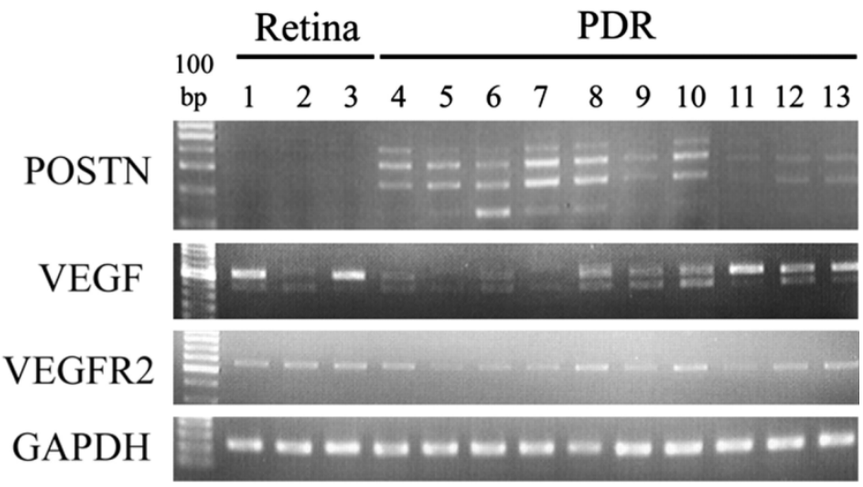

Fig. 9. RT-PCR analyses of periostin (POSTN), VEGF, VEGFR2, and GAPDH in FVMs derived from patients with proliferative diabetic retinopathy (PDR; *lanes 4–13*) and from control retinas (*lanes 1–3*). After 35 cycles, 8 μL each sample was electrophoresed through a 2% Tris-acetate-EDTA agarose gel, and the fractionated products were stained with ethidium bromide. Note the high expression of the mRNA of periostin with multiple bands in the FVMs derived from patients with PDR compared with control retinas. Reproduced with permission from Yoshida et al. [23]

Alternative splicing events occur within the C-terminal region of periostin, which is a key region that regulates cell invasiveness and metastasis (Shimazaki and Kudo, 2008). We confirmed that three spliced variants and the WT of human periostin were present in FVMs (Fig. 9) and that the periostin splice variant specifically regulated α-SMA gene expression (Yoshida, et al., 2011). It is suggested that the β strands within the C-terminal region may mediate binding interactions with other proteins such as FN or collagen. Because cell-specific isoform profiles and isoform-specific biological properties of periostin have been demonstrated (Shimazaki and Kudo, 2008), the existence of the different isoforms of periostin in FVMs may be used to vary the binding properties of periostin to other ECM

proteins. This can then result in the deregulation of crucial cellular processes such as adhesion, proliferation, differentiation, and invasion. Unraveling the role of alternative splicing of periostin in the formation of FVMs may yield the basis for the development of isoform-specific molecular targeting therapeutic strategies.

5. Conclusion

By applying global gene expression technology to FVMs and hypoxic retinas, we have successfully identified novel genes that may play key roles in the formation and/or maintenance of FVMs. We believe that the transcriptome analyses performed in our studies may provide valuable information and thus should facilitate a wide range of future studies to establish tissue-specific molecular mechanisms associated with formation of FVMs.

As described earlier, recently-introduced anti-VEGF therapy on patients with intraocular neovascular diseases can reportedly accompany undesirable side effects such as brain and retinal vein occlusion. This was partly attributed to a steady level of VEGF expression in the normal retina, suggesting a role of VEGF in keeping normal homeostasis of the retina (Fig. 1) (Yoshida, et al., 2011). The manipulation of the VEGF pathway to inhibit pathologic neovascularization could result in unexpected disturbances of the normal homeostasis in the retina and thus should be approached carefully.

Because the vitreous concentrations of periostin were not significantly correlated with those of VEGF in the patients with PDR (Yoshida, et al., 2011), it may be inferred that periostin and VEGF do not act in a directly synchronized manner in the formation of FVMs. Moreover, in contrast to VEGF, periostin and TEM7 is assumed to be nonfunctional in normal retinas, in keeping with the very low levels of periostin in the normal control retinas (Fig. 9; (Yamaji, et al., 2008)). These results raise the possibility that the two molecules might be a potential therapeutic target to regulate "disease-specific" pathways in the formation of FVMs while minimizing the unfavorable side effects to the normal retina. Therefore, modulating the expression of periostin and/or TEM7 by antibodies or antisense oligonucleotides directed against the molecule could be a novel therapeutic strategy for inhibiting the progression of FVMs associated with PDR.

6. References

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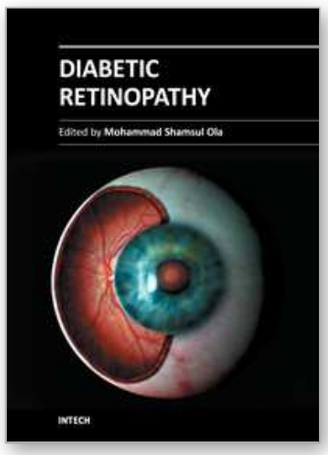

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The aim of this book is to provide a comprehensive overview of current concepts in pathogenesis, diagnosis and treatments of diabetic retinopathy. It provides a collection of topics written by excellent authors, covering discussions on advances in understanding of pathophysiology, immunological factors and emerging concepts, relating to clinical aspects and treatment strategies. The contents of the book will not only provide a resource for our knowledge but also improve diagnosis and treatment options for those patients who suffer vision loss due to diabetic retinopathy.

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