Impaired Angiogenic Response in the Corneas of Mice Lacking Osteopontin

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Purpose. To investigate the effects of loss of osteopontin (OPN) in the development of neovascularization in corneal stroma in mice. Cell culture study was also conducted to clarify the effects of OPN in transforming growth factor (TGF) β1-driven cell signaling and expression of vascular endothelial growth factor (VEGF).

Methods. Ocular fibroblasts from wild-type and OPN-null mice were used to study the role of OPN in TGFβ1 signal and VEGF expression. The effect of the absence of OPN on corneal neovascularization was evaluated in mice.

Results. In ocular fibroblast culture, loss of OPN attenuated TGFβ1 signals (Smad3 and p38) and reduced expression of VEGF. Loss of OPN attenuated neovascularization in corneal stroma in mice.

Conclusions. OPN is involved in VEGF expression in cultured fibroblasts and is required for neovascularization in corneal stroma in vivo. (*Invest Ophthalmol Vis Sci.* 2010;51:790–794) DOI:10.1167/iovs.09-3420

The cornea is an avascular tissue and must remain transparent to refract light properly. On the other hand, neovascularization in the cornea, induced in various inflammatory disorders such as trauma, infection, and extensive ocular surface damage by an alkali burn or a pathologic limbal stem cell deficiency, potentially impairs vision. Cell behavior during the process of neovascularization in an injured cornea is regulated in a complex way by various growth factors, which play critical roles in profibrogenic and proinflammatory reactions.[1–5] Major cytokines involved in injury-induced neovascularization include vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ).[6–9] However, cell behaviors are also believed to be modulated by scaffold of extracellular matrix.[10]

Osteopontin (OPN) is a secreted, noncollagenous, sialic acid-rich protein and a member of the small integrin-binding ligand N-linked glycoprotein family that plays an important role in modulating cell behaviors (Fig. 1). It is abundantly expressed in tissues during inflammation and repair. It functions as a cytokine that regulates the activities of macrophages, other immune cells, and resident tissue cells (epithelial cell types and mesenchymal cells) at sites of injury.[10–21] OPN modulates cell proliferation, migration, and matrix remodeling in vitro.[10,11,15,19] OPN function in tissue repair has been investigated.[22–24] It has been reported that loss of OPN perturbs the repair process of the cutaneous connective tissue or stromal healing in the cornea.[25,26] OPN is reportedly required for the development of neovascularization in bone formation and cancer progression.[27–31] As described, corneal neovascularization is to be suppressed for the maintenance of corneal transparency. Thus, the roles of OPN in the development of neovascularization in the cornea must be clarified for better understanding of the mechanisms of corneal opacification and neovascularization.

Materials and Methods

Experiments were approved by the DNA Recombination Experiment Committee and the Animal Care and Use Committee of Wakayama Medical University and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

TGFβ1 on VEGF Expression in OPN-Deficient Ocular Fibroblasts

To examine the effect of endogenous OPN on VEGF expression in ocular fibroblasts, we conducted real-time reverse transcription-polymerase chain reaction (RT-PCR) by using hydrolysis probes (TaqMan; Applied Biosystems, Foster City, CA).[3,4] The cells were obtained from wild-type (WT) and knockout (KO) mice, as previously reported, with a minor modification.[32] We enucleated eyeballs from postnatal day 2 mice after CO_2 asphyxia and obtained eye shells by removing intraocular tissues. The tissues were minced and explanted for the outgrowth of ocular fibroblasts. Six wells were prepared for each culture condition. The cells were grown to confluence and processed for either total RNA extraction or treatment with recombinant TGFβ1 (102-B2; R&D Systems, Minneapolis, MN) at 2 ng/mL for 24 hours, followed by total RNA extraction. RNA was extracted and processed for real-time RT-PCR for VEGF mRNA.[3,4] Data were analyzed by unpaired *t*-test.

Role of Osteopontin in Expression of Cytokines in Macrophages

Mouse macrophages were obtained from the peritoneal cavity as previously reported[32] and were allowed to adhere to 60-mm plastic dishes for 24 hours. The cells were then treated with serum-free medium for 24 hours, followed by 24-hour treatment with TGFβ1 at 2 ng/mL. RNA was extracted and processed for real-time RT-PCR for TGFβ1, MCP-1, and VEGF by using hydrolysis probes (TaqMan; Applied Biosystems).[3,4]

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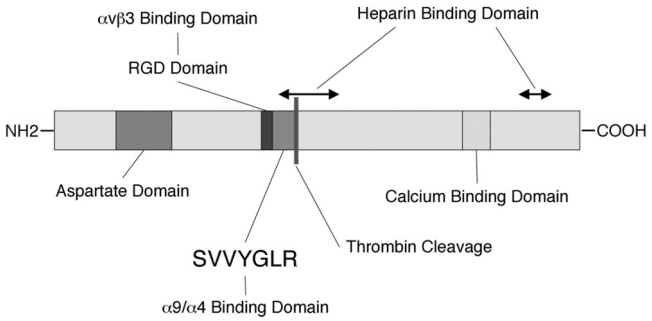

FIGURE 1. Molecular structure of OPN. OPN has a thrombin cleavage site. Peptide sequence of SVVYGLR binds to integrin $\alpha 4$ or $\alpha 9$.

Signal Transduction

To examine the effect of OPN deficiency on cytokine signaling, confluent WT or KO ocular fibroblasts in 60-mm dishes were treated with exogenous TGFβ1 (5 ng/mL) for 0.5, 1, and 3 hours and were lysed in cell lysis buffer as. Western blot analysis was performed as previously reported.[6,7] Antibodies used were rabbit polyclonal anti-p38 mitogen-activated protein kinase (MAPK) antibody (9212; 1:1000 dilution in PBS; Cell Signaling Technology, Beverly, MA) and rabbit anti-Smad3 antibody (Cabiosource 44–246G; 1:1000 dilution in PBS; Zymed, San Francisco, CA). To confirm that the protein loaded was similar in each sample, the membrane was also reacted with an antibody against glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA).

Induction of Stromal Neovascularization by Cauterization of the Central Cornea in Mice

We then conducted in vivo experiments by using a KO mouse of C57BL/6 background. Corneal neovascularization from the limbal vessels was induced by cauterization of the central cornea of an eye with the use of a disposable tool (Optemp; Alcon, Fort Worth, TX), as previously reported.[8] One eye of both WT and KO mice ($n = 120$ for each genotype; $n = 20$ on days 3, 5, 10, and 14; $n = 40$ on day 7) were treated and killed on days 3, 5, 7, 10, and 14. Eyes were then enucleated and processed for cryosectioning. Corneas of each genotype were used for histology at each time point.

Immunohistochemistry

Immunohistochemistry was performed to detect stromal neovascularization and cytokines. New vessels were detected by immunodetection of CD31 antigen, and cytokines examined were TGFβ1 and VEGF. Cryosections (7 μm) were fixed in cold acetone and processed for immunohistochemistry with rat monoclonal anti-CD31 antibody (PECAM 1:100; sc-18916; Santa Cruz Biotechnology), as previously reported.[9,33] Statistical analysis was conducted with the use of Tukey-Kramer test, and $P < 0.05$ was taken as significant.

RESULTS

Effects of Loss of OPN on Expression of Angiogenic Cytokines in Fibroblasts

In a healing cornea, expression of the major angiogenic factor VEGF might be modulated by various cytokines. In the present study, cell culture experiments were conducted to examine whether endogenous OPN affects VEGF expression. The addition of recombinant TGFβ1 at 2 ng/mL upregulated VEGF mRNA expression in cultured ocular fibroblasts approximately twofold. The upregulation of VEGF mRNA by TGFβ1 was partially counteracted by lacking OPN in cells (Fig. 2).

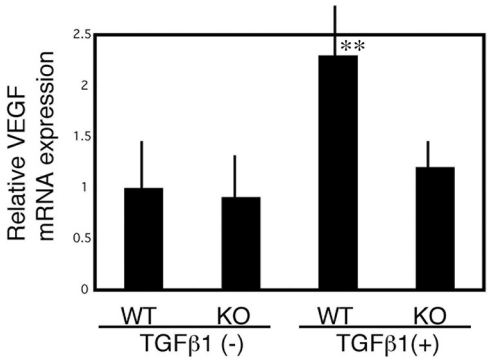

FIGURE 2. Expression of VEGF mRNA in WT and OPN-null (KO) ocular fibroblasts as examined by real-time RT-PCR. Although there is no difference in the relative expression level of VEGF mRNA between WT and KO cells in the absence of exogenous TGFβ1, VEGF mRNA expression is more marked in WT cells than in KO cells in the presence of TGFβ1 ($n = 5$ in each culture condition). $^{**}P < 0.01$.

Role of Osteopontin in Macrophage Expression of Cytokines

Loss of OPN did not affect the expression levels of TGFβ1, MCP-1, or VEGF in cultured macrophages both in the presence and absence of exogenous TGFβ1 (data not shown).

Signal Transduction

To examine the effect of OPN on fibrogenic cytoplasmic signaling, Western blot analysis was performed (Fig. 3). We first tested the level of activation of Smad3 in WT and KO fibroblasts in response to the addition of TGFβ1. Phosphorylation of Smad3 peaked at 0.5 hour after the addition of TGFβ1 and was reduced in KO cells compared with WT cells at each time point; p38MAPK is also known to be activated by TGFβ. Phosphorylation of p38MAPK peaked at 1 hour. The level of expression of phospho-p38MAPK was also reduced by the loss of OPN in fibroblasts (Fig. 3).

Neovascularization in Corneal Stroma

In WT mouse corneas, short CD31-labeled neovascularization (around 0.2 mm in length from the limbus) was detected at day 3 (Fig. 4). The length of neovascularization in the corneal stroma was observed as early as day 3, exhibited its peak at day 7, and began to decline at day 10. The length of neovascular-

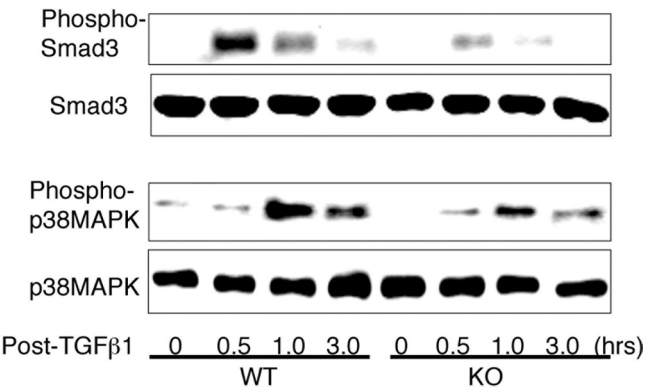

FIGURE 3. Signal transduction activated by exogenous TGFβ1 in WT and OPN-null (KO) ocular fibroblasts as examined by Western blot analysis. Both Smad3 and p38 were phosphorylated in response to exogenous TGFβ1. Loss of OPN reduces the peak phosphorylation level of Smad3 or p38 compared with that in WT cells.

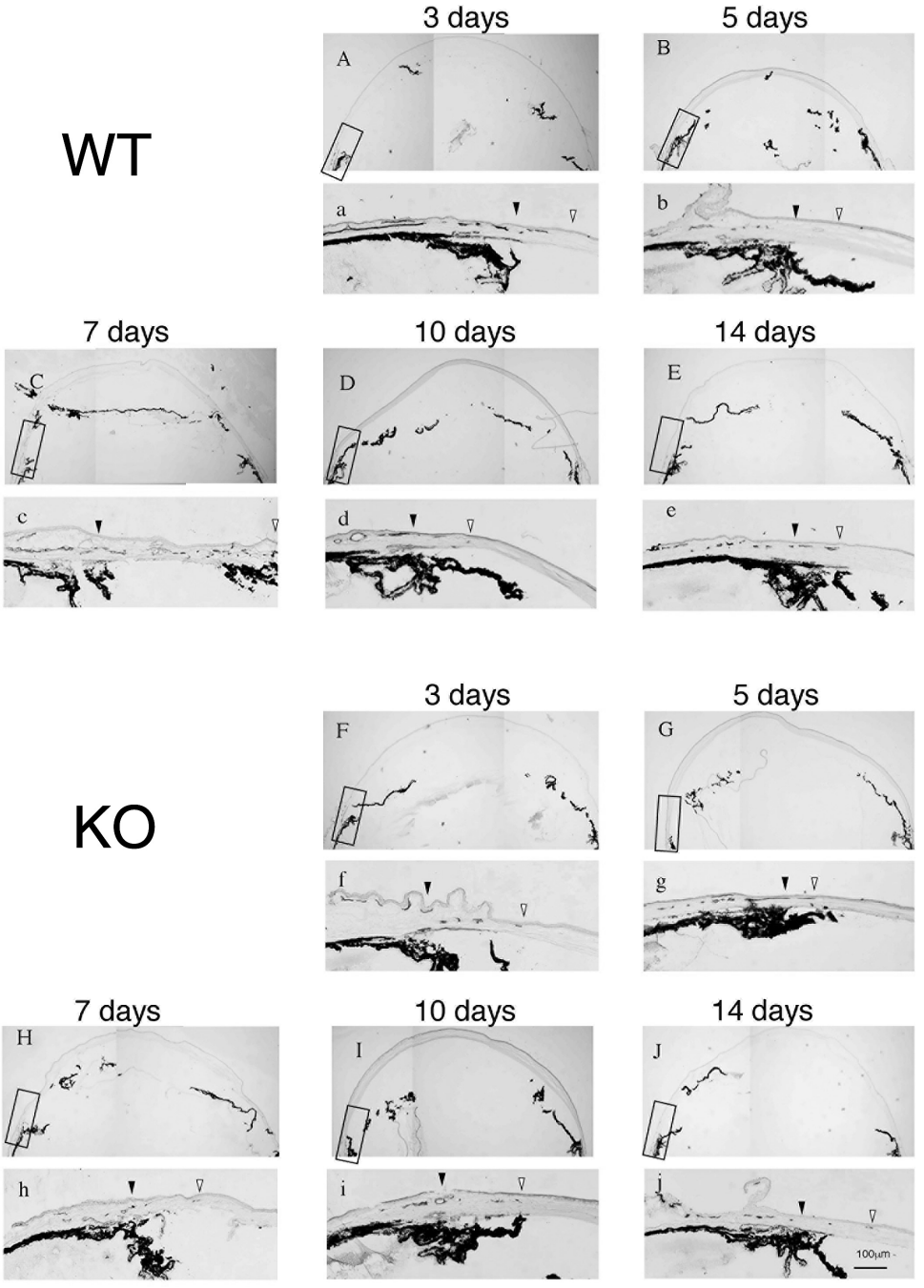

FIGURE 4. CD31-immunostaining in corneas of WT and OPN-null (KO) mice. Cauterization-induced stromal neovascularization from the limbal area is positively stained. (**A–E**; **F–J**) Low-magnification pictures of corneas of WT or KO mice at each time point. (**a–j**) Higher magnification pictures of the *boxed areas* in **A–J**. (**A**, **a**; **F**, **f**) Day 3. (**B**, **b**; **G**, **g**) Day 5. (**C**, **c**; **H**, **h**) Day 7. (**D**, **d**; **I**, **i**) Day 10. (**E**, **e**; **J**, **j**) Day 14. *Black arrows*: point of corneolimbal border. *White arrows*: leading tip of the neovascularization. Scale bar, 100 μm.

ization was shorter in KO mice than in WT mice at day 7 but not at the other four time points (Fig. 5).

DISCUSSION

The present experiments showed that loss of OPN attenuates injury-induced neovascularization in corneal stroma. Loss of OPN in ocular fibroblasts indeed suppresses the expression of angiogenic cytokines but not of cultured macrophages.

Similar angiogenic effects of OPN were reported in cultured cells and tissues. Synthetic OPN-derived peptide SVVYGLR enhances the formation of a vessel-like structure by cultured vascular endothelial cells and can induce neovascularization in artificial bone marrow scaffold biomaterials.[34] Our preliminary unpublished data also show that the SVVYGLR peptide stimulated tube-like tissue formation by human umbilical vein endothelial cells (HUVECs) cultured on a fibroblast monolayer and that the phenomenon was abrogated by adding anti-OPN antibody (Fujita N et al., unpublished data, 2009). As occurs in vitro, lack of OPN impairs the myocardial angiogenic response, leading to adverse remodeling postmyocardial infarction in animals. Moreover, OPN plays an essential role in two key aspects of tumor progression by reportedly triggering VEGF-dependent tumor progression and tumor angiogenesis.

The exact mechanisms of OPN regulation of expression of angiogenic cytokines and behaviors of vascular endothelial cells are to be investigated. However, here we showed that the loss of OPN attenuates TGFβ-related signals (Smad3 and p38) and reduces the expression of TGFβ1 and VEGF in cultured ocular fibroblasts, though the detailed signaling system in-

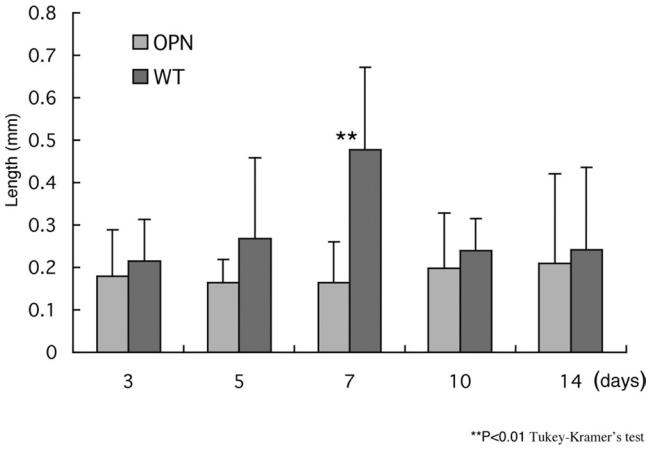

FIGURE 5. Length of the stromal neovascularization as indicated by the distance between the limbus (*black arrows* in Fig. 4) and the leading tip of the neovascularization (*white arrows* in Fig. 4) at each time point (day 3, $n = 20$; day 5, $n = 20$; day 7, $n = 40$; day 10, $n = 20$; day 14, $n = 20$). The length of stromal neovascularization was significantly shorter in OPN-null (KO) mice than in WT mice at day 7. $^{**}P < 0.01$.

volved in the phenomenon is still to be determined. Attenuated TGFβ-signaling might cause a reduction of angiogenic cytokine expression. Stimulation of VEGF expression by OPN was also reported in other cell types. Such reduction of expression of TGFβ1 and VEGF was not observed in KO peritoneal macrophages in the present experiments, though we previously showed that OPN is expressed in both myofibroblasts and macrophages in an alkali-burned mouse cornea.[26] However, OPN reportedly modulates the activities of various neovascularization-related cytokines in monocytes/macrophages. It was reported that OPN upregulates tumor necrosis factor, IL-1b, IL-6, and IL-8 in association with p38 phosphorylation in human monocytes.[35] The report further shows that supernatants of OPN-treated monocytes were highly angiogenic and that the angiogenic response by monocytes was completely abrogated by a neutralizing anti-IL-1 antibody.[35] Our preliminary in vitro coculture experiments showed that blocking OPN or adding OPN peptide stimulates the angiogenic behavior of HUVECs cultured on a fibroblast feeder layer, indicating that macrophages are not essential to an angiogenic effect of OPN. However, this does not exclude the possibility of the contribution of induction of IL-1b by OPN in macrophages to in vivo neovascularization formation in the cornea. It was also reported that OPN and FGF2 cooperate to recruit macrophages in the process of neovascularization.[36] These mechanisms may also be involved in the process of development of corneal neovascularization. In the present animal model of neovascularization, vessels became shortened after 7 days following injury. The angiogenic activity caused by cauterization in the central cornea is considered to depend on local inflammation. Although this mechanism of this phenomenon is to be clarified, any explanation must include that neovascularization may regress in association with the healing of a cauterization injury to the cornea.

Inflammation, neovascularization, and subsequent tissue fibrosis are the key phenomena in tissues after inflammatory diseases or healing processes following injury.[37,38] OPN reportedly modulates the behaviors of immune cells in inflammatory disorders (arthritis, inflammatory bowel disease). Loss of OPN attenuates tissue fibrosis in an incision injury in the corneal stroma and skin or a fibrotic reaction in an injured lens epithelium via epithelial-mesenchymal transition.[39–41] Both fibrogenic reaction and neovascularization are the main aspects of the unfavorable fibrovascular disorders of the autoimmune-based ocular surface (Stevens-Johnson syndrome, ocular pemphigoid).[42] To modify the role of endogenous OPN might be a strategy to prevent/treat such immuno-based ocular surface diseases.

In conclusion, endogenous OPN modulates the expression of angiogenic cytokines involved in the development of corneal neovascularization. Better understanding of the complex set of mechanisms in the modulation of neovascularization is required to establish strategies to overcome unfavorable neovascularization in cornea.

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