Mast cell hyperactivity underpins the development of oxygen-induced retinopathy

Kenshiro Matsuda,1 Noriko Okamoto,2 Masatoshi Kondo,3 Peter D. Arkwright,4 Kaoru Karasawa,1 Saori Ishizaka,1 Shinichi Yokota,2 Akira Matsuda,2 Kyungsook Jung,1 Kumiko Oida,1 Yosuke Amagai,1,5 Hyosun Jang,1 Eiichiro Noda,6 Ryota Kakinuma,7 Koujirou Yasui,7 Uiko Kaku,7 Yasuo Mori,8 Nobuyuki Onai,9 Toshiaki Ohteki,9 Akane Tanaka,1,7 and Hiroshi Matsuda1,2

1Cooperative Major in Advanced Health Science, Graduate School of Bio-Applications and System Engineering, Tokyo University of Agriculture and Technology, Tokyo, Japan. 2Laboratory of Veterinary Molecular Pathology and Therapeutics, and Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan. 3Department of Neonatology and Tokyo Metropolitan Children’s Medical Center, Tokyo, Japan. 4Institute of Inflammation and Repair, University of Manchester, Royal Manchester Children’s Hospital, Manchester, United Kingdom. 5Tokyo Biomarker Innovation Research Association, Tokyo, Japan. 6Department of Ophthalmology, Tokyo Metropolitan Children’s Medical Center, Tokyo, Japan. 7Laboratory of Comparative Animal Medicine, Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan. 8Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Japan. 9Department of Biodefense Research, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan.

Mast cells are classically thought to play an important role in protection against helminth infections and in the induction of allergic diseases; however, recent studies indicate that these cells also contribute to neovascularization, which is critical for tissue remodeling, chronic inflammation, and carcinogenesis. Here, we demonstrate that mast cells are essential for sprouting angiogenesis in a murine model of oxygen-induced retinopathy (OIR). Although mouse strains lacking mast cells did not exhibit retinal neovascularization following hypoxia, these mice developed OIR following infusion of mast cells or after injection of mast cell tryptase (MCT). Relative hypoxia stimulated mast cell degranulation via transient receptor potential ankyrin 1. Subsequent surges in MCT stimulated retinal endothelial cells to produce monocyte chemotactic protein-1 (MCP1) and angiogenic factors, leading to sprouting angiogenesis. Mast cell stabilizers as well as specific tryptase and MCP1 inhibitors prevented the development of OIR in WT mice. Preterm infants with early retinopathy of prematurity had markedly higher plasma MCT levels than age-matched infants without disease, suggesting mast cells contribute to human disease. Together, these results suggest therapies that suppress mast cell activity should be further explored as a potential option for preventing eye diseases and subsequent blindness induced by neovascularization.

Introduction
Mast cells are important for innate immune defense against microbes and parasites (1, 2). A number of toxins and chemicals can induce host-protective or pathogenic activation of mast cells (3, 4). Paul Ehrlich, in his 1878 doctoral thesis, was the first to describe mast cells (Mastzellen), noting that they often congregate around blood vessels of tissues (5). Mast cells accumulate around expanding neoplasms and correlate with the degree of tumor vascularization (6, 7). After activation, mast cells release a number of proinflammatory cytokines and angiogenic mediators as well as proteolytic enzymes, including tryptase and chymase (8, 9). Because of their multipotency, mast cell–derived factors can induce both tissue damage and remodeling.

The eye provides a window into the process of vascularization in both health and disease. Ocular neovascularization is the leading cause of blindness in adults, for instance, in age-related macular degeneration and diabetic retinopathy (10). In infants and children, retinopathy of prematurity (ROP) is also a leading cause of blindness, induced by oxygen-dependent retinal vascular injury and occlusion, and in severe cases (stage 3+), subsequent neovascularization, with a risk of retinal scarring and detachment (11–13). ROP only develops in preterm infants, particularly those at the extremes of survival (45% of infants born at 22–23 weeks gestation compared with 0.1% of infants born at 30–31 weeks gestation) and thus provides an insight at the interface between normal and abnormal vascular development. Excessive oxygen therapy given to extremely preterm infants is the key risk factor for ROP (14–16). It results in vascular occlusion and subsequent reactive neovascularization mediated by VEGF, which can be partly offset using anti-VEGF monoclonal therapy (bevacizumab) (17). However, some ROP patients still end up with visual impairment, even after treatment (18). The precise mechanism by which aberrations in infants’ oxygen tension triggers neovascularization is currently unclear.

Tissue vascularization begins with vasculogenesis, the creation of new vessels from angioblasts (19). Subsequent weaknesses in the supporting vascular basement membrane and extracellular matrix at intervals along these vessels result in secondary branches or sprouting angiogenesis (20, 21). Abnormal vascularization is recognized to be an integral part of chronic inflammatory response, tissue remodeling, and carcinogenesis (22, 23). Serine proteases, VEGF, and basic FGF, which are released by mast cells, have been reported to facilitate neovascular sprouts and promote
cells in the development of retinal neovascularization in Kit\textsuperscript{Wsh/Wsh} and Cpa3\textsuperscript{Cre/+} mast cell–deficient mice (25, 26) (Figure 1).

**Figure 1.** Mast cell deficiency prevented in the development of retinal neovascularization in an OIR mouse model. (A and B) Whole-mounted retinas revealed that pathological neovascularization, shown as tufts (white areas), was induced in mast cell–sufficient WT mice, but not in mast cell–deficient mice on P17. \( n = 8 \) in each group. **\( P < 0.01 \) versus WT mice, Dunnett’s test. (C) Retinal neovascularization on P17 was quantified by counting the number of neovascular cell nuclei at the retinal inner surface of eye sections after H&E staining. The number of neovascular nuclei was lower in Kit\textsuperscript{Wsh/Wsh}, Kit\textsuperscript{Wsh/Wsh}, and Cpa3\textsuperscript{Cre/+} mice than in WT mice. \( n = 8 \) in each group. **\( P < 0.01 \) versus WT mice, Dunnett’s test. (D–G) Cross-sectional analysis of retinas was performed by H&E (D), PECAM-1 (E), or toluidine blue (F) staining of formalin-fixed paraffin-embedded sections. Results are representative of 3 independent experiments. (E) Arrows indicate endothelial cells that have penetrated into the vitreous space. Toluidine blue staining showed mast cells in the dorsal skin (F) of WT and Kit\textsuperscript{Wsh/Wsh} mice, but not in the retina (G). Arrows and arrowheads indicate degranulated and nondegranulated mast cells, respectively (F). Scale bars: 500 \( \mu \)m (A); 100 \( \mu \)m (D–G). Results are shown as mean \( \pm \) SEM of values determined from 3 independent experiments (B and C).
Table 1. Number of mast cells in the skin of mice on P17

<table>
<thead>
<tr>
<th>Mice</th>
<th>Total number of cells/cm</th>
<th>Degranulated cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive WT</td>
<td>337.1 ± 8.4</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>WT</td>
<td>337.9 ± 6.0</td>
<td>43.3 ± 3.8</td>
</tr>
<tr>
<td>Kit+/Wsh</td>
<td>196.9 ± 4.2</td>
<td>40.5 ± 14</td>
</tr>
<tr>
<td>KitWsh/Wsh</td>
<td>78.0 ± 2.6</td>
<td>38.0 ± 2.3</td>
</tr>
<tr>
<td>Cpa3Cre/+</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Skin sections were stained with toluidine blue, and mast cells between epithelium and panniculus carnosus were counted. Results are shown as mean ± SEM of values obtained from 8 mice per group. *P < 0.01 versus WT mice, Dunnett’s test.

dependent mechanism. Whole-mount analysis showed that hyperoxic exposure for 5 days from P7 to P12 resulted in vascular occlusion in the central part of the retina in all mice on P12. In WT mice, after a further 5 days, neovascular sprouts and tufts developed, a hallmark of ROP in humans (27) (Figure 1, A–D). These neovascular sprouts and nuclei were markedly decreased in Kit+/Wsh/Wsh mice, Dunnett’s test. These mice, after a further 5 days, neovascular sprouts and tufts developed, a hallmark of ROP in humans (27) (Figure 1, A–D). These neovascular sprouts and nuclei were markedly decreased in Kit+/Wsh/Wsh mice, Dunnett’s test. Penetration of endothelial cells positive for PECAM-1 into the vitreous was also very low in mast cell–deficient mice (Figure 1E). No neovascularization was observed in any of the mice exposed to only room air (data not shown). In WT mice and Kit+/Wsh/Wsh mice, mast cells were observed in the dorsal skin on P17 and 40% of the skin mast cells had degranulated (Figure 1F and Table 1). In contrast, no or very few mast cells could be detected in the skin of mast cell–deficient mice (Figure 1F and Table 1). No mast cells were observed in the retina of all the mice (Figure 1G).

As more direct evidence that mast cells are involved in the pathogenesis of OIR, BM-derived cultured mast cells (BMCs) (28) were injected into the peritoneal cavity of Kit+/Wsh/Wsh and Cpa3Cre/+ mice on P1 or P2. Lp. injection of BMCMCs into mast cell–deficient mice resulted in neovascular tufts similar in extent to those observed in WT mice on P17 (Figure 2, A and B). H&E staining demonstrated that the numbers of neovascular nuclei were increased in Kit+/Wsh/Wsh and Cpa3Cre/+ mice injected with BMCs compared with those of mice injected with saline alone (Figure 2, C and D). In addition, PECAM-1–positive endothelial cells were found to extend into the vitreous after the injection of BMCs into mast cell–deficient mice (Figure 2E).

Retinal function is markedly impaired in mice with OIR, as measured by decreased b-wave amplitude (29). To assess the retinal function in our murine OIR model, we analyzed single-flash electroretinogram (ERG) patterns on P19. ERG analyses revealed that, while WT and BMC-MC–injected mast cell–deficient mice had complete loss of b-waves and oscillatory potential–waves (OP-waves), mast cell–deficient mice injected with saline had normal b- and OP-waves, comparable to those of naïve WT mice (Figure 2F and Table 2). Because there are some differences between the mouse and rat OIR models (30), we also performed experiments using mast cell–deficient Kit+/Wsh/Wsh rats (31). The results of the rat model were in keeping with those of the murine model (Figure 3 and Table 2).

Transient receptor potential ankyrin 1 is responsible for mast cell degranulation induced by relative hypoxia. To clarify the mechanism by which fluctuations in oxygen levels lead to mast cell degranulation, we measured β-hexosaminidase released from BMCs cultured in 75% oxygen for 5 days and then 20% oxygen for 12 hours in vitro. The protocol mimicked the in vivo experiments. Degranulation was induced after mast cells were moved from hyperoxic to normoxic conditions (Figure 4A), indicating that relative hypoxia triggered mast cell degranulation. As transient receptor potential ankyrin 1 (TRPA1) has been reported to act as an oxygen sensor in neural cells (32), we studied TRPA1 expression in mast cells. TRPA1 was expressed on BMCs, as demonstrated by flow cytometry (Figure 4B). TRPA1 from whole cell lysate of BMCs derived from C57BL/6 mice had a molecular weight of 110 kDa (Figure 4C). A TRPA1–specific inhibitor, HC-030031 (33), suppressed hypoxia–induced degranulation of mast cells in a dose-dependent manner (Figure 4A). To confirm the role of TRPA1 in relative hypoxia–induced degranulation of mast cells, we generated BMCs from BM cells isolated from TRPA1–deficient mice. Relative hypoxia–induced degranulation was markedly reduced in TRPA1–deficient BMCs (Figure 4A). These results indicate that TRPA1 mediates mast cell degranulation induced by relative hypoxia. To confirm the contribution of TRPA1 to oxygen–mediated mast cell degranulation in the OIR model, HC-030031 was administered daily to mice of the OIR model from P11 to P16, using the same protocol as for group 3, shown in Figure 4E. Neovascular nuclei were markedly suppressed in HC-030031–treated OIR mice (Figure 4D), indicating that TRPA1 was essential for the development of OIR.

The mast cell stabilizer cromolyn completely blocks retinal neovascularization induced by relative hypoxia. Mast cell granule components have been reported to be potent stimulators of angiogenesis. Therefore, we next examined whether inhibition of mast cell degranulation reduced retinal neovascularization in vivo. Murine pups were injected daily with a mast cell stabilizer, cromolyn, under conditions of varying oxygen tension (Figure 4E). Administration of cromolyn for 10 days (P6–P16) completely inhibited the formation of neovascular tufts in WT mice (Figure 4F). As expected, suppression of mast cell degranulation by cromolyn for 5 days from the day before mice were moved to conditions of relative hypoxia (P11–P16) significantly decreased the number of endothelial nuclei that extended into the vitreous space (Figure 4H). In contrast, no suppressive effects of cromolyn were observed when it was just administered during the period of exposure to hypoxia (P6–P11) (Figure 4G). Thus, the marked reduction in oxygen concentration is critical for mast cell activation and the development of OIR.

Neovascularization associated with OIR is induced by mast cell tryptase. Tryptase is the most abundant granule–derived serine protease in mast cells and has been reported to stimulate the proliferation of endothelial cells, promote tube formation, and degrade the connective tissue matrix to provide space for new vessel growth (8, 9). In mice, the major tryptases are mouse mast cell protease 6 (mMCP6) and mMCP7 (34). The C57BL/6 mouse strain lacks mMCP7 because of a spontaneous mutation within the Mcp7 gene (35). We therefore investigated whether mMCP6 promoted retinal angiogenesis in the OIR mouse model. Raised serum mMCP6 levels were found in the OIR model of WT and BMC-MC–injected Kit+/Wsh/Wsh pups compared with those of age–matched naïve WT or saline–injected Kit+/Wsh/Wsh pups after the expo-
Figure 2. Injection of mast cells induced the formation of neovascular tufts in mast cell–deficient mice. (A and B) BMCMC but not saline treatment induced the formation of new abnormal blood vessels (white arrows) in mast cell–deficient mice with OIR on P17. *P < 0.01 versus saline-injected mast cell–deficient mice. 1-way ANOVA with Tukey’s test. (C) Retinal neovascularization was quantified on P17 by counting the number of neovascular nuclei extending into the vitreous after H&E staining. The number of neovascular nuclei in BMCMC-injected mast cell–deficient pups was comparable to that in WT mice. **P < 0.01 versus saline-injected mast cell–deficient mice. 1-way ANOVA with Tukey’s test. (D and E) Cross-sectional analysis of retinas was performed by h& E (D) or PECAM-1 (E) staining of formalin-fixed paraffin-embedded sections. Arrows indicate endothelial cells that have penetrated into the vitreous space. Results are representative of 3 independent experiments. (F) Normal ERG responses on P19 were seen in age-matched naive WT and saline-injected mast cell–deficient mice, but not in WT or BMCMC-injected mast cell–deficient mice. Scale bars: 500 µm (A); 100 µm (D and E). Results are shown as mean ± SEM of values obtained from 8 mice per group. ANOVA significant difference when compared with saline-injected Cpa3Cre/+ pups on P17 (Figure 5E). To further confirm the role of mMCP6 in retinal neovascularization, we checked whether mmcp6-injected tube formation of murine retinal microvascular endothelial cells was independent of retinal infiltration of monocytes and other leukocytes. Addition of recombinant mmcp6 produced clear capillary tube formation in growth factor–reduced Matrigel, and pre-treatment with anti-MCP1 mAbs blocked the effect (Figure 6F). Recombinant MCP1 induced capillary tube formation comparable to that of mmcp6 (Figure 6G). Furthermore, as MCP1 is classically thought of as a chemotactic factor of monocytes/macrophages, infiltration of these cells was examined in the retina of WT mice. Few if any Ly6C−CD45 double-positive cells were observed within the retina neovascular sprouts and tufts (Supplemental Figure 1). These results indicated that mmcp6 released from relative hypoxia-stimulated mast cells activated MCP1 production in retinal endothelial cells to induce abnormal angiogenesis in the retina, resulting in OIR.

Table 2. Amplitude of b-waves and implicit times in ERG on P19

<table>
<thead>
<tr>
<th>Animals</th>
<th>Genotype</th>
<th>Injection</th>
<th>Amplitude (µV)</th>
<th>Implicit time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>Naive WT</td>
<td>−</td>
<td>337 ± 37a</td>
<td>60.1 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>−</td>
<td>Nonrecordable</td>
<td>Nonrecordable</td>
</tr>
<tr>
<td>Cpa3Cre/+</td>
<td>Saline</td>
<td></td>
<td>360 ± 110</td>
<td>66.4 ± 3.7</td>
</tr>
<tr>
<td>KitWsh/Wsh</td>
<td>BMCMC</td>
<td></td>
<td>Nonrecordable</td>
<td>Nonrecordable</td>
</tr>
<tr>
<td>Cpa3Cre/+</td>
<td>Saline</td>
<td></td>
<td>219 ± 39</td>
<td>60.2 ± 3.3</td>
</tr>
<tr>
<td>KitWsh/Wsh</td>
<td>BMCMC</td>
<td></td>
<td>Nonrecordable</td>
<td>Nonrecordable</td>
</tr>
<tr>
<td>Rats</td>
<td>Naive WT</td>
<td>−</td>
<td>852 ± 44</td>
<td>67.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>−</td>
<td>475 ± 21b</td>
<td>876 ± 6.1</td>
</tr>
<tr>
<td>Cpa3Cre/+</td>
<td>Saline</td>
<td></td>
<td>838 ± 25</td>
<td>65.5 ± 2.1</td>
</tr>
<tr>
<td>KitWsh/Wsh</td>
<td>PMC</td>
<td></td>
<td>461 ± 22</td>
<td>850 ± 6.7</td>
</tr>
</tbody>
</table>

Saline, BMCMCs, or PMCs were injected i.p. in pups on P1 or P2. Results are shown as mean ± SEM of values obtained from 8 mice per group. *No significant difference when compared with saline-injected KitWsh/Wsh mice, Dunnett’s test. **P < 0.01 versus saline-injected KitWsh/Wsh rats, Dunnett’s test.

MCT induces expression of angiogenic factors in the retina. To investigate the mechanism by which mast cells induce retinal neovascularization, we examined the expression of a number of angiogenic factors by real-time PCR. We found that monocyte chemotactic protein-1 (Mcp1) mRNA was highly expressed in the retina of the OIR model and that cromolyn completely blocked its expression (Figure 6A). Vegf and Fgf were also upregulated, but Hif1a and hepatocyte growth factor (Hgf) were not induced in the retina of OIR mice (data not shown). Cromolyn also decreased mRNA expression of Vegf and Fgf in the WT mice (Figure 6A).

Recombinant mMCP6 added to primary culture of murine retinal microvascular endothelial cells enhanced mRNA expression of Mcp1, Vegf, Fgf, and Hgf (Figure 6B). Since MCP1 has been proposed as a key angiogenic factor of microvascular endothelium (38), we examined the role of MCP1 in retinal angiogenesis in the OIR model. Intravitreal injection of siRNA against Mcp1 on P12 suppressed mRNA transcription of Mcp1 (Figure 6C). The development of OIR was markedly abrogated by the injection of siRNA against Mcp1 (Figure 6D). Since positive immunoreactions for CCR2 were observed at the tufts of the retina (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI89893DS1), CCR2-deficient mice were used to further clarify the involvement of CCR2 in this OIR model. In CCR2-deficient mice, we also found that retinal neovascularization was markedly reduced when compared with that in WT mice (Figure 6E). To clarify the role of mmcp6 and MCP1 in retinal neovascularization, we checked whether mmcp6-injected tube formation of murine retinal microvascular endothelial cells was independent of retinal infiltration of monocytes and other leukocytes. Addition of recombinant mmcp6 produced clear capillary tube formation in growth factor–reduced Matrigel, and pre-treatment with anti-MCP1 mAbs blocked the effect (Figure 6F). Recombinant MCP1 induced capillary tube formation comparable to that of mmcp6 (Figure 6G). Furthermore, as MCP1 is classically thought of as a chemotactic factor of monocytes/macrophages, infiltration of these cells was examined in the retina of WT mice. Few if any Ly6C−CD45 double-positive cells were observed within the retina neovascular sprouts and tufts (Supplemental Figure 1). These results indicated that mmcp6 released from relative hypoxia-stimulated mast cells activated MCP1 production in retinal endothelial cells to induce abnormal angiogenesis in the retina, resulting in OIR.

Since protease-activated receptor 2 (PAR2) is activated by tryptase (37), we examined the possible involvement of PAR2 in retinal neovascularization in the OIR model using PAR2-deficient mice. Unexpectedly, PAR2-deficient pups developed retinal neovascularization after exposure to relative hypoxia (Figure 5H). To investigate the possible involvement of PAR2 in retinal neovascularization, we examined the expression of a number of angiogenic factors by real-time PCR. We found that monocyte chemotactic protein-1 (Mcp1) mRNA was highly expressed in the retina of the OIR model and that cromolyn completely blocked its expression (Figure 6A). Vegf and Fgf were also upregulated, but Hif1a and hepatocyte growth factor (Hgf) were not induced in the retina of OIR mice (data not shown). Cromolyn also decreased mRNA expression of Vegf and Fgf in the WT mice (Figure 6A).

Recombinant mMCP6 added to primary culture of murine retinal microvascular endothelial cells enhanced mRNA expression of Mcp1, Vegf, Fgf, and Hgf (Figure 6B). Since MCP1 has been proposed as a key angiogenic factor of microvascular endothelium (38), we examined the role of MCP1 in retinal angiogenesis in the OIR model. Intravitreal injection of siRNA against Mcp1 on P12 suppressed mRNA transcription of Mcp1 (Figure 6C). The development of OIR was markedly abrogated by the injection of siRNA against Mcp1 (Figure 6D). Since positive immunoreactions for CCR2 were observed at the tufts of the retina (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI89893DS1), CCR2-deficient mice were used to further clarify the involvement of CCR2 in this OIR model. In CCR2-deficient mice, we also found that retinal neovascularization was markedly reduced when compared with that in WT mice (Figure 6E). To clarify the role of mmcp6 and MCP1 in retinal neovascularization, we checked whether mmcp6-injected tube formation of murine retinal microvascular endothelial cells was independent of retinal infiltration of monocytes and other leukocytes. Addition of recombinant mmcp6 produced clear capillary tube formation in growth factor–reduced Matrigel, and pre-treatment with anti-MCP1 mAbs blocked the effect (Figure 6F). Recombinant MCP1 induced capillary tube formation comparable to that of mmcp6 (Figure 6G). Furthermore, as MCP1 is classically thought of as a chemotactic factor of monocytes/macrophages, infiltration of these cells was examined in the retina of WT mice. Few if any Ly6C−CD45 double-positive cells were observed within the retina neovascular sprouts and tufts (Supplemental Figure 1). These results indicated that mmcp6 released from relative hypoxia-stimulated mast cells activated MCP1 production in retinal endothelial cells to induce abnormal angiogenesis in the retina, resulting in OIR.

Table 2. Amplitude of b-waves and implicit times in ERG on P19

<table>
<thead>
<tr>
<th>Animals</th>
<th>Genotype</th>
<th>Injection</th>
<th>Amplitude (µV)</th>
<th>Implicit time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>Naive WT</td>
<td>−</td>
<td>337 ± 37a</td>
<td>60.1 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>−</td>
<td>Nonrecordable</td>
<td>Nonrecordable</td>
</tr>
<tr>
<td>Cpa3Cre/+</td>
<td>Saline</td>
<td></td>
<td>360 ± 110</td>
<td>66.4 ± 3.7</td>
</tr>
<tr>
<td>KitWsh/Wsh</td>
<td>BMCMC</td>
<td></td>
<td>Nonrecordable</td>
<td>Nonrecordable</td>
</tr>
<tr>
<td>Cpa3Cre/+</td>
<td>Saline</td>
<td></td>
<td>219 ± 39</td>
<td>60.2 ± 3.3</td>
</tr>
<tr>
<td>KitWsh/Wsh</td>
<td>BMCMC</td>
<td></td>
<td>Nonrecordable</td>
<td>Nonrecordable</td>
</tr>
<tr>
<td>Rats</td>
<td>Naive WT</td>
<td>−</td>
<td>852 ± 44</td>
<td>67.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>−</td>
<td>475 ± 21b</td>
<td>876 ± 6.1</td>
</tr>
<tr>
<td>Cpa3Cre/+</td>
<td>Saline</td>
<td></td>
<td>838 ± 25</td>
<td>65.5 ± 2.1</td>
</tr>
<tr>
<td>KitWsh/Wsh</td>
<td>PMC</td>
<td></td>
<td>461 ± 22</td>
<td>850 ± 6.7</td>
</tr>
</tbody>
</table>

Saline, BMCMCs, or PMCs were injected i.p. in pups on P1 or P2. Results are shown as mean ± SEM of values obtained from 8 mice per group. *No significant difference when compared with saline-injected KitWsh/Wsh mice, Dunnett’s test. **P < 0.01 versus saline-injected KitWsh/Wsh rats, Dunnett’s test.
Mast cell distribution. We found mast cells in the connective tissue around the eyeball, but not in the retina of mice (Figure 7A). To investigate the mast cell distribution in BMCMC-injected KitWsh/Wsh recipients, we used BMCMCs derived from EGFP-transgenic mice. EGFP-positive cells were visualized by anti-GFP Abs and Alexa Fluor 594–conjugated anti-IgG Abs. EGFP-BMCMCs localized only to the peritoneal cavity on P11 (Figure 7B). On P17, 373 ± 97 BMCMCs were observed in the peritoneal cavity, and 42% of those had degranulated. These results indicate that mast cells were responsible for inducing the aberrant angiogenesis and retinal dysfunction in the OIR model. Furthermore, the data suggest that the angiogenic effect of mast cells did not require that they infiltrate into the retinal tissue, but indicate that they could be mediated by mMCP6 produced by mast cells residing in extraocular tissues. These results indicate that mMCP6 secreted from mast cells outside the eye must diffuse into the retina to induce neovascularization through upregulation of the angiogenic factors. It is well recognized that mast cell degranulation and release of chemical mediators, including tryptase, can cause a generalized increase in vascular permeability, fluid shifts out of the circulation, and subsequent anaphylactic shock. We therefore used i.v. injection of Evans blue to study vascular integrity in the OIR mouse model. Generalized leakage of dye out of the circulation and into the skin and brain was demonstrated in mice with OIR, but not in the controls (Supplemental Figure 2).

Human preterm neonates with ROP have higher plasma MCT than gestational age–matched controls. Finally, we measured plasma MCT levels of 23 preterm infants nursed in the neonatal intensive care unit until they achieved an oxygen saturation level of 90%. Examination of the fundi was performed weekly at the
equivalent of 28 to 35 weeks gestation. Blood samples were collected from 8 preterm infants delivered at 22 to 24 weeks gestation who all developed ROP (average stage 3) and also suffered from bronchopulmonary dysplasia. The control group consisted of 15 preterm infants delivered at 28 to 34 weeks gestation, none of whom developed ROP or bronchopulmonary dysplasia. Blood samples for measurement of MCT were taken from the 2 groups at comparable gestational equivalent ages (28, 30, 32, and 34 weeks) (Figure 8). Median (interquartile range) plasma MCT of the preterm newborns with ROP at the equivalent of 28–32 weeks gestational age was 61.3 (32.8–95.7) ng/ml, 5-fold higher than that of control infants at equivalent gestational age who did not develop ROP (12.4 [8.8–21.9] ng/ml) (P = 0.001). Mild to moderate extraretinal fibrovascular proliferation was observed in most ROP patients. Partial retinal detachment was identified in one of the patients with ROP. These data suggest that in humans, as in mice, relative hypoxia induces mast cell degranulation and subsequent MCT release.

Discussion

Our results prove for the first time, to our knowledge, that mast cells are indispensable for retinal neovascularization in both the mouse and rat models of OIR. Rodents lacking mast cells, either because of mutations in Kit (Kit<sup>Wsh/Wsh</sup>) or due to CPA3-driven Cre
It is interesting that it is not the initial change from low to high oxygen concentrations, but rather the subsequent relative hypoxia that triggers mast cell degranulation. Third, in these animal models of OIR, mMCP6 mediates spouting angiogenesis. The process can be inhibited by the mast cell stabilizer cromoly and the specific tryptase inhibitor NM, and it can be induced in mast cell–deficient mice with recombinant mMCP6. The presence of mast cells close to the blood vessels and their involvement in angiogenesis at sites of cancer, inflammation, and tissue repair through the activation of endothelial cells have been well documented. Tryptase is known to degrade connective tissue matrix and collagen IV in vascular basement membranes (40). PAR2 has toxicity (Cpa3Cre/+), do not develop retinal neovascularization. However, upon reconstitution with mast cells, severe OIR can also be induced in these inherently mast cell–deficient animals. Second, we also show that activation of mast cells by relative hypoxia but not hyperoxia is via TRPA1, as the process can be blocked using the TRPA1 inhibitor HC-030031 (33). TRPA1 can therefore be considered an oxygen sensor that induces mast cell degranulation. TRPA1 is a primarily ion channel sensor of noxious stimuli and is sensitive to changes in oxygen tension (32). Although it has previously been shown that relative hypoxia after exposure to hyperoxia altered the expression of a diverse set of hypoxia-regulated genes (39), we clearly demonstrate that mast cells can trigger an “angiogenic switch” in OIR. It is interesting that it is not the initial change from low to high oxygen concentrations, but rather the subsequent relative hypoxia that triggers mast cell degranulation. Third, in these animal models of OIR, mMCP6 mediates spouting angiogenesis. The process can be inhibited by the mast cell stabilizer cromoly and the specific tryptase inhibitor NM, and it can be induced in mast cell–deficient mice with recombinant mMCP6. The presence of mast cells close to the blood vessels and their involvement in angiogenesis at sites of cancer, inflammation, and tissue repair through the activation of endothelial cells have been well documented. Tryptase is known to degrade connective tissue matrix and collagen IV in vascular basement membranes (40). PAR2 has...
Figure 6. Tryptase directly induced the expression of angiogenic factors in retinal endothelial cells. (A) Mcp1 mRNA expression was significantly increased in OIR mice. This was suppressed by administration of cromolyn, but administration of PBS alone did not have any effect. Other angiogenic factors, Vegf and Fgf, were upregulated in OIR mice and decreased by cromolyn treatment. On P11 and P12, WT mice were injected with PBS or cromolyn, and eyes were collected at 6 hours after the second administration on P12. n = 8 in each group. **P < 0.01 versus PBS-injected mice, Dunnett’s test. (B) Addition of recombinant mMCP6 into the culture of primary retinal endothelial cells induced Mcp1, Vegf, Fgf, and Hgf gene expression. n = 8 in each group. *P < 0.05; **P < 0.01 versus vehicle treatment, Mann-Whitney U test. (C) Schematic of Mcp1 gene-silencing experiments. Intravitreal injection of MCP1 siRNA effectively suppressed retinal MCP1 expression. n = 8 in each group. **P < 0.01 versus control RNA-injected mice, Mann-Whitney U test. (D) Abnormal angiogenesis, following relative hypoxia, was suppressed by the specific inhibition of MCP1 in the retina. Neovascularization area (%) was quantified in whole-mount specimens. n = 8 in each group. **P < 0.01 versus PBS-injected mice, Mann-Whitney U test. (E) Relative hypoxia induced the formation of new abnormal blood vessels (white areas) in WT mice (n = 14), but not in CCR2-deficient mice (n = 8). **P < 0.01 versus WT mice, Mann-Whitney U test. (F) Addition of recombinant mMCP6 into the culture of primary retinal endothelial cells induced typical tube formation. The effect of mMCP6 was suppressed by addition of anti-MCP1 mAbs. n = 4 in each group. *P < 0.05 versus rat isotype mAb. Dunnett’s test. (G) Addition of recombinant mouse MCP1 (10 ng/ml) into the culture of primary retinal endothelial cells induced typical vascular tube formation. n = 4 in each group. **P < 0.01 versus vehicle control, Mann-Whitney U test. Scale bars: 500 μm (D, E); 100 μm (F, G). All results are shown as mean ± SEM of values determined from 3 to 4 independent experiments.
been proposed to mediate effects of MCT (41). However, in the present study, we could not demonstrate the necessity of PAR2 in OIR by using PAR2-deficient mice (42). It has been reported that pronounced intravitreal neovascularization develops in mice with conditional knockdown of PAR2 and that treatment with a PAR2 agonist accelerated normal revascularization (43). These findings suggest that PAR2 expressed in the retina functions as a modulator of oxygen-induced retinal inflammation. Therefore, MCT might not significantly affect the biological activity of PAR2 in the development of OIR. Further experiments using conditional PAR2 knockdown and treatment with PAR2 agonists may help to clarify whether this receptor has any modulatory role in mast cell–mediated retinal neovascularization in the murine model of OIR.

Weakening of the localized segments of vascular basement membrane by tryptase is consistent with the current concept of sprouting angiogenesis, which is initiated by pericyte detachment from the vessel wall and degradation of the solid support of the vascular basement membrane by enzymatic digestion. Pathological changes seen in OIR were associated with retinal dysfunction. We showed that mast cell–sufficient WT mice and mast cell–deficient mice injected with BMCMCs had lower or no amplitudes of b-waves and OP-waves in the ERG, suggesting functional damage to bipolar cells and amacrine/inner plexiform cells, but not to photoreceptor cells (44).

We also focused on the effect of MCP1 on retinal neovascularization in the OIR model. As previously reported, P17 retinal neovascularization induced in mice with embryonic disruption of the gene encoding MCP1 was comparable to that in WT mice (45). As embryonic gene disruption might lead to unexpected compensa-

Figure 7. Mast cell distribution. (A) Toluidine blue staining of eyes and their surrounding tissue in naive mice. Mast cells were distributed in the conjunctiva (arrowheads) in WT mice. R, retina; Cj, conjunctiva; C, cornea; I, iris; L, lens; Cil, ciliary body. Scale bar: 500 μm. (B) Mast cell distribution in Kit<sup>Wsh/Wsh</sup> mice reconstituted with EGFP-BMCMC was investigated by immunohistochemistry. Whole-body sections were stained with 0.05% toluidine blue (upper panels) and with anti-EGFP Abs (lower panels). Scale bars: 500 μm (A); 5 mm (B). Results shown are representative of 3 independent experiments (A and B).
with ROP examined in the current study were as high as those of adult subjects with systemic mastocytosis. Human neonates with ROP also had bronchopulmonary dysplasia, suggesting that the increased number of MCT-positive mast cells in the lung contributes to high levels of MCT in preterm neonates with ROP (50). In addition to ROP, very preterm newborns are also at increased risk of lung damage and intracranial hemorrhage (51). We demonstrated that there is a generalized disturbance in vascular integrity in mice with OIR. A question requiring further research is whether the same disturbances of mast cell activity may also contribute to overt vascular diseases in the brain and other organs in very premature neonates. Our results suggest the therapeutic potential of mast cell stabilizers in the treatment of ROP. Mechanistic studies are difficult in human neonates for ethical reasons, but a simple proof-of-principle study would be a randomized blinded placebo-controlled trial to evaluate the effectiveness of mast cell stabilizers in preventing the development of retinal neovascularization in a group of at-risk preterm neonates. This would provide further evidence that the biological basis of ROP in humans is the same as in rodents.

Mast cells with specific granules brimming with neutral serine proteases are ideally placed as providing an interface between physiological basis of ROP in humans is the same as in rodents.

Methods

Animals. C57BL/6-Kit<sup>Wsh/Wsh</sup> mice were obtained from the RIKEN BioResource Center. C57BL/6j mice were purchased from Japan SLC. PAR2-deficient mice were supplied by Kowa Co. mMCP6-deficient C.129S7-Tpaβ<sup>2m<sup>−</sup>Wsh</sup>/Mmnc mice were obtained from the Mutant Mouse Regional Resource Center (University of North Carolina, Chapel Hill, North Carolina, USA). EGFP-transgenic mice (C57BL/6 TgN(egfp)OsbiC14-YO1-FM131) were provided by M. Okabe (Genome Information Research Center, Osaka University, Osaka, Japan). TRPA1-deficient mice (B6;129P-Tpat<sup>−/−</sup>/j) and CCR2-deficient mice (B6;129S4-Ccr2<sup>−/−</sup>/j) were supplied by The Jackson Laboratory.

Animal model of OIR. OIR was induced as previously described (24). On P7, animals were exposed to 75% O<sub>2</sub> with their nursing dam in a sealed chamber. Pups remained in the chamber for 5 days (P7–P12) and were then placed in an ambient atmosphere for an additional 5 days (P12–P17).

Mast cell injection. As described previously (54), BM cells isolated from femora and tibiae were cultured in α-MEM with 10<sup>−4</sup> M 2-ME (Sigma-Aldrich), 10% FCS, and 10% pokeweed mitogen-stimulated spleen conditioned medium for 4 to 8 weeks. BMMCs (10<sup>6</sup> cells/20 μl) obtained from WT mice, EGFP-transgenic mice, or mMCP6-deficient mice were injected i.p. in pups on P1 or P2. For rat experiments, peritoneal mast cells (10<sup>6</sup> cells/20 μl) obtained from WT rats were injected following the same protocol.

Evaluation of retinal neovascularization. For whole-mount analysis, eyes were enucleated and fixed for 1 hour in 4% paraformaldehyde. Retinas were dissected and stained overnight in Alexa Fluor 488-conjugated Grifonia simplicifolia isoelectin B4 (Molecular Probes). Retinal flat mounts were generated, and images were obtained using a BIORÉVO system (Keyence). Total area, the number of clock hours of neovascularization, and avascular area were quantified with Photoshop (Adobe Systems).

Histology and immunohistochemistry. Eyes were enucleated from mice on P17 and fixed in Davidson’s fixative overnight. Serial 6-μm paraffin-embedded axial sections were stained with H&E. The nuclei on the vitreous side of the internal limiting membrane were counted.

To examine mast cell distribution in mice and rats, toluidine blue or chloroacetate esterase staining was performed on paraffin sections of the dorsal skin (4 μm) and the retina (6 μm). Mast cells between epithelium and panniculus carnosus were counted under a microscope. For immunohistochemistry, eye sections were incubated overnight at 4°C with anti-PECAM-1 Abs (Santa Cruz Biotechnology Inc., catalog sc-1506). To visualize the target cells, sections were treated with the second Ab conjugated with biotin (Jackson ImmunoResearch; catalog 705-065-147). Serial 5-μm frozen skin sections were incubated overnight at 4°C with anti-PECAM-1 Abs (Santa Cruz Biotechnology Inc., catalog sc-1506). To visualize the target cells, sections were treated with the second Ab conjugated with biotin (Jackson ImmunoResearch; catalog 705-065-147).

Histology and immunohistochemistry. Eyes were enucleated from mice on P17 and fixed in Davidson’s fixative overnight. Serial 6-μm paraffin-embedded axial sections were stained with H&E. The nuclei on the vitreous side of the internal limiting membrane were counted. To examine mast cell distribution in mice and rats, toluidine blue or chloroacetate esterase staining was performed on paraffin sections of the dorsal skin (4 μm) and the retina (6 μm). Mast cells between epithelium and panniculus carnosus were counted under a microscope. For immunohistochemistry, eye sections were incubated overnight at 4°C with anti-PECAM-1 Abs (Santa Cruz Biotechnology Inc., catalog sc-1506). To visualize the target cells, sections were treated with the second Ab conjugated with biotin (Jackson ImmunoResearch; catalog 705-065-147).
C57BL/6 mice that were treated with 0.1% Triton X-100 for permeabilization of cell membrane. Rabbit serum was applied instead of mouse serum. Rat IgG (Abcam, catalog ab18450, clone RTK2758) was used as an isotype control.

Drug administration. We injected 20 μl of a mast cell stabilizer, cromolyn (57) (50 mg/kg, Sigma-Aldrich), a specific inhibitor of tryptase in newborns. KM and N. Okamoto wrote the draft. PDA and AT contributed to experiments with TRPA1-deficient mice. N. Onai and TO contributed to experiments with CCR2-deficient mice. MK, KA, YM, KK, SI, SY, AM, KJ, KO, YA, and HJ performed the experiments, evaluated data, and applied statistical analysis. YM contributed to experiments with TRPA1-deficient mice. N. Onai and TO contributed to experiments with CCR2-deficient mice. MK, RK, KY, and UK took care of premature infants and collected blood samples. EN performed ophthalmologic examinations on premature newborns. KM and N. Okamoto wrote the draft. PDA and AT collected data and extensively reviewed and revised the paper. HM conceived and directed the project. All the authors had the opportunity to discuss the results and commented on the manuscript.

Acknowledgments We thank Y. Kitamura (Shionogi & Co. Ltd.) and A. Nishida (Tokyo Metropolitan Children’s Medical Center) for valuable suggestions and comments, N. Kajiwara (Tokyo Metropolitan Institute of Medical Science) for help with degranulation assays, and S. Nakamura, M. Koshiha, and Y. Shirakawa (Tokyo University of Agriculture and Technology) for help in taking images of whole-mount retinas. We thank T.B. Feyerabend and H.R. Rodewald (German Cancer Research Center, Heidelberg, Germany), who kindly provided Cpa3Cre+/- mice for this study. We are grateful to M. Okabe (Osaka University) for providing EGFP transgenic mice. We also thank J.
38. Lyle RE, Tryka AF, Griffin WS, Taylor BJ. Tryptase immunoreactive mast cell hyperplasia in