Homocysteine Facilitates Prominent Polygonal Angiogenetic Networks of a Choroidal Capillary Sprouting Model

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H omocysteine (hcy), a sulfur-containing amino acid and metabolite of methionine, is located at a crucial point in the methionine cycle. Disruption of the methionine cycle may cause biochemical or cellular disorders.¹ High levels of hcy in the blood act as a warning for the disruption of this cycle. This condition, termed “hyperhomocysteinemia,” is profoundly associated with coronary artery diseases due to an increased risk of atherosclerosis² and cardiovascular ischemic events.³-⁴ Hyperhomocysteinemia is also believed to be related to cerebral arterial diseases,⁵ including small-vessel disease and atherosclerotic large-vessel disease in stroke patients. All these diseases are thought to occur due to atherosclerosis in different parts of the human body.

In addition to atherosclerotic diseases, high levels of hcy may also be involved in neural degenerative diseases,⁶ including Parkinson’s disease⁷ and Alzheimer’s dementia⁸,⁹. The prime causes of hcy imbalance in the human body are related to genetic, nutritional, and hormonal factors.⁹ Petras et al.¹⁰ also indicated that hyperhomocysteinemia increases oxidative stress in experimental animals, and the consequently produced free radicals lead to the development of neural degenerative disorders. Similar to neural degeneration, several retinal disorders have been associated with high levels of hcy. Elevated plasma hcy is a risk factor for retinal artery or vein occlusion.¹¹-¹³ and this condition is possibly improved by the intake of folate, vitamin B₆, and vitamin B₁₂ supplements, according to Martin et al.¹² Hyperhomocysteinemia is also a main factor in the pathogenesis of retinal neuronal death,¹⁴,¹⁵ diabetic retinopathy,¹⁶ and even glaucoma.¹⁷,¹⁸ However, the association between choroidal diseases and hcy levels has not yet been elucidated. We had previously reported that plasma hcy levels were significantly higher in certain patients with choroidal vasculopathy than in those without vasculopathy.¹⁹ Hcy is very likely to play a key role in the vascular changes of the choroid. We therefore established a choroid capillary sprouting model with hcy enrichment to investigate the possible role of hcy in the angiogenesis of choroid vessels. Furthermore, we investigated the expression of several possibly related growth factors in this model, including VEGF, placental growth factor (PIGF), platelet-derived growth factor B (PDGF-B), platelet-derived growth factor C (PDGF-C), angiopoietin 1 (Ang1), and Ang2.

MATERIALS AND METHODS

Choroidal Capillary Sprouting Assay

C57BL/6 mice (of both sexes; age, 8–12 weeks) from BioLASCO Technology (Taipei, Taiwan) were used for the capillary...
sprouting model of choroidal explant preparations. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Institutional Animal Care and Use Committee of Fu-Jen Catholic University approved animal protocols. The experiment protocol, a modified version of that given by Shao et al.\textsuperscript{20} was as follows: soon after the animals were euthanized, the eyes were immediately enucleated; the choroidal fragments (containing the retinal pigment epithelium [RPE] layer and choroid) were isolated, mounted onto cover slips, embedded with Matrigel (Matrigel Matrix Growth Factor Reduced; BD Biosciences, Erembodegem, Belgium), and placed in 24-well culture plates. After the placement of the choroidal explants, the culture plates were incubated at 37°C and 5% CO\textsubscript{2}. Subsequently, 500 \mu L of medium with different concentrations of hcy (0.1, 0.5, 1, 2, and 4 mM; D,L-homocysteine, H\textsubscript{4}H28; Sigma-Aldrich, Merck Millipore, Billerica, MA, USA), or without hcy, was added to each well. The hcy solution was freshly prepared before use, dissolved in autoclaved water, and diluted with culture medium. The culture media with or without hcy were changed every day for 5 consecutive days. The area of choroidal capillary sprouting (the area within the circle connecting the outer ends of the sprouts) was measured every 24 hours for each sample over 5 days (120 hours), using the method described by Shao et al.\textsuperscript{20}

**Immunofluorescence Staining**

The expression of vascular cell markers and different growth factors was studied using immunofluorescence staining. The choroidal explants were fixed with paraformaldehyde before immunofluorescent staining. Anti-VEGF antibody (1:400, sc-152; Santa Cruz Biotechnology, Dallas, TX, USA), anti-PDGF antibody (1:200, sc-1880; Santa Cruz Biotechnology), anti-Ang1 (1:400, ab95230; Abcam, Cambridge, MA, USA), anti-Ang2 (1:200, ab56301; Abcam), anti-PDGF-B (1:400, ab23914; Abcam), anti-PDGF-C (1:400, sc-18228; Santa Cruz Biotechnology), and anti-chondroitin sulfate proteoglycan neuron-glial antigen 2 (NG2; 1:200, AB5320; Chemicon, Merck Millipore, Billerica, MA, USA) were used as the primary antibodies. Alexa 488 (1:500, A11055; Molecular Probes, Life Technologies, Eugene, OR, USA) and Alexa 555 (1:500, A31572; Molecular Probes, Life Technologies) were used as the secondary antibodies. Isolectin IB4 (isolectin) conjugated with Alexa 594 (1:500, I21413; Molecular Probes, Life Technologies) was used to label the endothelial cells. Cell nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI; 1:1000, sc-5986; Santa Cruz Biotechnology). An upright fluorescent microscope (DM2500; Leica Wetzlar, Germany) with a CCD camera system (CoolSNAP EZ; Roper Scientific, Martinsried, Germany) was used to observe the epi-fluorescence expression of the retinal sections.

**Immunofluorescence-Positive Cell Counting**

For this capillary sprouting model it was unsuitable to use Western blotting to examine protein expression; therefore, we use immunofluorescence to quantify cells expressing various proteins. Dual-colored immunofluorescent-labeled cells positive for VEGF, PI GF, PDGF-B, PDGF-C, Ang1, Ang2, NG2, and isolectin were counted to investigate the percentages of these cells in the sprouting capillaries between the different groups. Microscopic images of the immunofluorescent staining of the aforementioned growth factors were taken from different sprouting areas of the explants, and different fluorescent color-labeled (green for VEGF, PI GF, Ang1, Ang2, PDGF-B, PDGF-C, and NG2, red for isolectin) cells were counted in every image. Moreover, DAPI was used to stain the nucleus of every cell, and the number of DAPI-labeled cells (blue) in each image was considered as the denominator (100%). The green- and red-labeled cell counts in the experimental group were divided by their own denominators for each image and presented as the “percentages of cell count.” Image analyzing software was used to subtract the color signals while counting the cells.

**Statistical Analysis**

All data from choroidal explants were analyzed using IBM SPSS Statistics 20 software (IBM, Armonk, NY, USA). Statistical analyses were performed to determine the differences in capillary sprouting areas and protein expression in the retinas. One-way ANOVA with Dunnett’s test for multiple comparisons was used to determine the significance of the differences in the sprouting areas between the groups. Student’s t-test was used to determine the significance of the differences in the cell count experiment between the control and hcy-treated groups. Differences were considered statistically significant when P was less than 0.05.

**RESULTS**

The images depicted the angiogenetic effects of hcy on the choroidal explants (Fig. 1). Microimages of the hcy-treated and untreated choroid capillary sprouting explants were photographed using a stereomicroscope. According to these photomicrographs, the explants exhibited a continuously increasing capillary sprouting area after 24, 48, 72, 96, and 120 hours of treatment (Fig. 1). Quantitative analysis of the capillary sprouting areas is also presented in Figure 1K. The average area of capillary sprouting in the control group was 14.1 mm\textsuperscript{2} after 120 hours of culture. The largest capillary sprouting area evoked by hcy was 21.3 mm\textsuperscript{2} (on average) at 1-mM hcy concentration, and the second-largest effect was an area of 19.9 mm\textsuperscript{2} at 2-mM hcy concentration, after 120 hours of culture. Both of these results were statistically significant. At concentrations of 0.5 and 4 mM, hcy treatment led to increase capillary sprouting; however, statistical significance was only achieved at 48, 72, and 96 hours compared with the control group. The detailed data of this experiment are listed in Table 1. The results suggest that choroidal angiogenesis could be evoked by hcy within a certain concentration range.

The distribution of the sprouting capillaries from the choroidal explants is presented in Figure 2. The NG2-positive cells labeled with green fluorescence were pericytes, isolectin IB4 (isolectin)-positive cells labeled with red fluorescence were endothelial cells, and the cell nuclei were labeled with blue fluorescence (DAPI) in the images illustrated in Figure 2. Staining for isolectin revealed many capillaries growing from the cultured explant treated with 1-mM hcy (Figs. 2E, 2F), and these capillaries regularly formed a polygonal shape in the upper layer of the sprouting area. Conversely, fewer capillaries grew from the explant of the control group compared with the hcy-treated group (Figs. 2B, 2C). In high-power microscopic images, the polygonal-shaped choroidal capillaries in the upper layer were regularly arranged in both the proximal and distal areas of the sprouting capillaries (arrows in Figs. 2H, 2J). Because there were multiple layers of the sprouting capillaries in the explants, the layers other than focused capillaries would be blurred in the same view field of the images. These polygonal tubes were not observed in the control group (Figs. 2G, 2I). This regular polygonal shape is usually noted in the mature capillary vasculature of the choroid.\textsuperscript{21} In addition, a greater number endothelial (isolectin-positive) cells were observed in the hcy-treated explants than in the control
explant (Fig. 3). Figure 3 shows high-power images of the capillaries in the control and 1-mM hcy-treated choroidal explants. The upper layer of the sprouting capillaries presents the shape of the vasculature in the hcy-treated group (Figs. 3C, 3D). In some areas of the upper layer of sprouting capillaries, vascular lumen structures were observed in the hcy-treated preparations, indicating that vessel formation was in progress (Figs. 3E–G).

Figure 1. Capillary sprouting from the retinal explants treated with different concentrations of hcy or not treated with hcy. Images (A) to (E) are of retinal explants at different time points, from 24 to 120 hours. Images (F) to (J) are of another retinal explant treated with 1-mM hcy; the images were taken at different treatment times, from 24 to 120 hours. Image (K) shows the capillary sprouting area from retinal explants treated with 0.1- to 4-mM hcy and from those without hcy (control) at 24, 48, 72, 96, and 120 hours. These images show that the capillaries exhibited better outgrowth in the hcy-treated group than in the control group over time. Scale bar: 1 mm. These results indicate that the capillary sprouting areas were significantly increased in the retinal explants treated with 1- and 2-mM hcy for 120 hours. The data are expressed as mean values, with the error bars representing the standard error of the mean. One-way ANOVA with Dunnett’s test for multiple comparisons was used for the data analysis, N = 5–8, *P < 0.05 compared with the control group. Detailed data and P values are shown in Table 1.
The expressions of VEGF and PlGF and isocitrate (the vascular endothelial cell marker) were observed in the choroidal capillary sprouting explants (Fig. 4). Vascular endothelial growth factor expression was noted in the outgrowing endothelial cells both in the control and hcy-treated explants, which was reasonable because these capillaries were under outgrowth conditions (Figs. 4C, 4D). In addition, PlGF-positive cells were also detected both in the control and hcy-treated explants. Placental growth factor expression in some cells was colocalized with isocitrate, which is a vascular endothelial cell marker. However, a major proportion of the PlGF-positive cells were nonendothelial cells (Figs. 4A, 4B), which indicated that PlGF might be expressed in both endothelial cells and pericytes. The results reveal that both VEGF and PlGF are expressed in the control and hcy-treated sprouting capillaries; VEGF was mainly expressed in the endothelial cells, and PlGF was mainly seen in the nonendothelial cells (which may have been pericytes).

In addition, the expressions of PDGF-B and PDGF-C were also examined. Figure 5 illustrates the immunofluorescent staining of PDGF-B and PDGF-C in the control and the hcy-treated choroidal explants. Minimal PDGF-B expression was noted in the sprouting capillaries of the control and hcy-treated explants, and no obvious difference was detected between these two groups (Figs. 5A, 5B). By contrast, the expression of PDGF-C was upregulated in the hcy-treated choroidal explants (Fig. 5D), but this upregulation was not detected in the control group (Fig. 5C). Moreover, PDGF-C staining was observed in both the endothelial cells and pericytes, especially the tip cells of the sprouting capillaries (arrows in Fig. 5D). In addition, the expressions of Ang1 and Ang2 were examined using immunofluorescent staining (Fig. 6). A greater number of Ang1-positive cells were observed in the hcy-treated choroidal explants than in the control explant (Figs. 6A, 6B). Staining for Ang1 was mostly noted in the endothelial cells (Fig. 6B). In addition, no significant differences were observed in the distribution of Ang2-positive cells between the hcy-treated and control choroidal explants (Figs. 6C, 6D). The results suggest that Ang1 and Ang2 may act differently in the process of angiogenesis evoked by hcy. Furthermore, PDGF-C and Ang1 were both involved in the angiogenesis of choroidal capillaries evoked by hcy.

To investigate the possible growth factors mediating the sprouting of capillaries and formation of polygonal networks, the proportions of different growth factor–expressing cells were determined. Figure 7 presents the percentages of cells expressing VEGF, PlGF, Ang1, Ang2, PDGF-B, PDGF-C, NG2, and isocitrate. The statistical details of positive-cell counting are presented in Table 2. It was noticed that the percentage of isocitrate and NG2 remained similar in the hcy and control groups. This indicates that although the capillary sprouting area increases in the hcy group rather than control group, the proportion of endothelial cells and parietal cells remains unchanged. The results of the statistical analysis for the percentages of cell counts suggest that only PDGF-C and Ang1 were upregulated in the hcy-treated group.

**DISCUSSION**

We established a choroidal capillary sprouting model and studied the effects of hcy on capillary sprouting. Our results revealed that the area of choroidal capillary sprouting was significantly increased when we treated the choroidal capillary explants with 1 mM hcy for 24, 48, 72, 96, and 120 hours. This model provided an ideal tool for investigating choroidal capillary sprouting, which was not observed in the animal model in vivo.22 This effect was also observed when the concentration of hcy was increased to 2 mM, but the sprouting area was not as large as that obtained with 1 mM hcy after 120 hours of culture. Hcy has been reported to be cytotoxic through the upregulation of Fas receptor expression, which leads to cell apoptosis in human umbilical vein endothelial cells cultured with 5 mM hcy.23 This Fas-mediated apoptosis pathway was proven to activate the Akt pathway and to downregulate a caspase-8 inhibitor, FLICE inhibitory protein.23, 24 Hence, high-dose hcy may cause cell apoptosis via the Fas-mediated pathway. Curro and colleagues25 tested the cytotoxicity of hcy in a neuroblastoma cell line and reported that the highest toxicity of hcy was at 80 μM, which caused 80% cell death after 5 days of incubation. They later found that increased reactive oxygen species levels and expressions of Bax and Bcl-2 were involved in the cell death caused by hcy, although the concentrations of hcy were only 40 to 80 μM in the culture medium.25 However, our results...
FIGURE 2. Immunofluorescent staining of the capillary sprouting areas treated with or without hcy (control) for 120 hours. NG2-positive cells (green) present pericytes, and isolectin IB4-labeled cells (red) present endothelial cells. Cell nuclei were stained with DAPI (blue). (A–C) are images of an explant from the control group, and (D–F) are images of an explant from the 1-mM hcy-treated group. (A) and (D) show cell nuclei labeled with DAPI. (B) and (C) show isolectin-labeled endothelial cells. (C) and (F) are DAPI and isolectin-merged images. A greater number of capillaries were formed in the hcy-treated group than in the control group, and many polygonal-shaped capillaries were formed in the hcy-treated preparation. (G) and (H) are high-power images of the peripheral areas of the sprouting capillaries in the control and 1-mM hcy-treated choroidal
revealed that hcy caused choroidal capillary sprouting at a certain range (1–2 mM) of concentrations. A similar range of concentration was used by Cui et al.\textsuperscript{26} to determine the hcy-related endothelial effect. According to our results, hyperhomocysteinemia may induce the angiogenesis of choroidal capillaries at a certain range of concentration. Hyperhomocysteinemia is related to atherosclerosis.\textsuperscript{2} The possible hypothesis for hcy causing atherosclerosis is due to

![Image](https://example.com/image1)

**FIGURE 3.** Immunofluorescent staining of NG2 and isolectin in the choroidal capillaries treated with or without hcy (control) for 120 hours. Immunoreactions of NG2 (green, for pericytes) and isolectin IB4 (red, for endothelial cells) were labeled with different colors of fluorescence. The cell nuclei were stained with DAPI (blue). Images (A) and (B) are taken from the control and 1-mM hcy-treated choroidal explants to illustrate the distribution of pericytes and endothelial cells. (C) and (D) are high-power images showing the distribution of pericytes and endothelial cells in the upper (D) and lower (C) layers at the same area in the 1-mM hcy-treated choroidal explant. (E-G) are high-power images showing vascular lumens (arrowheads) in the upper layer of the sprouting capillaries. Scale bar: 50 μm.
the deficiencies of cystathionine-beta-synthase (CBS), methionine synthase, or methylene tetrahydrofolate reductase (MTHFR). High plasma levels of homocysteine were detected in CBS (-/-) mutant mice, which experienced a retinal structure and vasculopathy disorder. In another study of a hyperhomocysteinemia mouse model with deficiency of CBS, abnormal RPE cell morphology, and even choroidal neovascularization were noticed. In addition, a transgenic mouse model with MTHFR (+/-) exhibiting hyperhomocysteinemia demonstrated reduced ganglion cell function and mild vasculopathy by 24 weeks. The retinal phenotype of MTHFR (+/-) transgenic mice was similar to that of the hyper-
homocysteinemic mice with a deficiency of CBS. Considering that MTHFR and CBS are important key factors for the formation of Hcy, these reports support the hypothesis that high levels of Hcy may cause retinal vasculopathy.

Vascular endothelial growth factor is a primary mediator of angiogenesis in normal physiology or the pathology of diseases. There are many members in the VEGF family, including VEGF and PlGF. Vascular endothelial growth factor is widely known for playing a major role in angiogenesis during development, but PlGF appears to be crucial for pathological angiogenesis in some diseases. The most widely used therapeutic agents for treating ocular neovascularization, including exudative AMD, are VEGF-A antagonists. Currently, a combination of VEGF and PlGF antagonists have been suggested to improve the therapeutic effects on ocular neovascularization and neovascular AMD. In some cases, VEGF may also induce the
reduction of fluid absorption and be pathogenetic to edema. This phenomenon suggests that VEGF plays a key role in regulating RPE barrier properties and leading to pathological retinal edema. However, instead of VEGF and PlGF, we observed the involvement of PDGF-C in the hcy-facilitating activity for capillary angiogenesis in our model.

In addition to the VEGF family, PDGFs are important regulators of angiogenesis. There are four types of PDGFs (PDGF-A, PDGF-B, PDGF-C, and PDGF-D), which regulate the development of connective tissue cells. Earlier studies have revealed that PDGF/PDGFR-receptor signaling has provided a possible therapeutic target in proliferative diseases and cancer. A study on PDGF-evoked cellular response in human RPE cells reported that PDGF-A was the main factor involved, and the signal transduction of ERK1/2, p38, and Akt was activated. Another study indicated that PDGF-C and PDGF-D cause cell proliferation and migration of RPE as well as choroidal cells. Given that PDGFs have exhibited considerable involvement and numerous mechanisms for the development of proliferative and chronic diseases, an investigation of...
their role in regulating choroidal capillary angiogenesis is warranted. In our study, PDGF-B was not upregulated by hcy in the capillaries outgrown from the explant; however, PDGF-C was upregulated in both the pericytes and endothelial cells of the hcy-treated explant. The expression of PDGF-C was particularly noted in the tip cells of the sprouting capillaries. Tip cells have a unique function in regulating the angiogenesis of capillaries.41 Vascular endothelial growth factor-A and -C are involved in the filopodia extension of the endothelial tip cells41 and then in regulating angiogenic sprouting via the formation of heterodimers VEGFR2/3.42 In addition to VEGF, PDGF-C was upregulated in the hcy-treated endothelial tip cells in our study. Studies from different groups have reported that endothelial tip cells secrete PDGF-B, resulting in the proliferation and migration of pericytes during vessel maturation,43,44 but thus far, no report is available regarding PDGF-C expression in endothelial tip cells. Our new finding of the upregulation of PDGF-C in hcy-treated preparations suggests a different function of PDGF-C involvement in the growth direction of the capillaries during angiogenesis.

In angiogenesis, angiopoietin and VEGF play important roles in vessel tube formation.45 The angiopoietin family includes four members: Ang1, Ang2, Ang3, and Ang4.46 Given that Ang1 and Ang2 control vascular permeability, inflammation, and remodeling in mature vessels,47,48 the possible involvement of Ang1 and Ang2 in our choroidal capillary sprouting model was investigated. In this study, we observed capillaries sprouting from the choroidal explants and forming a regular polygonal shape in the upper layer of the sprouting capillaries. Ninomiya and his colleagues21 studied the retinal and choroidal structures using scanning electron microscopy and reported that a regular honeycomb hexagonal shape is the mature capillary arrangement in horses. A similar polygonal network was also seen in our hcy-treated choroidal capillary sprouting explants, and some capillaries with lumens were clearly observed in the hcy-treated preparations; we therefore assumed that the angiogenesis induced by hcy leads to the maturation of the capillary vasculature in the choroid. In addition, VEGF and Ang1 have been reported to guide vessel tube formation in angiogenesis.45 Ang1 promotes endothelial cell survival, sprouting, and tube formation.46 All Angs bind to Tie2 receptor.46 Some studies have indicated that Ang1 and Ang4 activate Tie2 receptor, whereas Ang2 and Ang3 inhibit Ang1-induced Tie2 phosphorylation.46 Therefore, Ang1 and Ang2 are considered antagonists in their physiological func-

**TABLE 2.** Statistical Details of the Positive-Cell Counting Study (see Fig. 7)

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<th>SEM %</th>
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In our results, no significant changes in VEGF expression of the control and hcy-treated groups were noted. However, we observed the upregulated expression of Ang1 in the hcy-treated explants. Because Ang1 plays an important role in tube formation in angiogenesis, and because greater capillary network and lumen formation was observed in the hcy-treated group, we assumed that Ang1 was involved in the angiogenesis and capillary network formation facilitated by hcy in our choroidal capillary sprouting model.

Although the mechanism by which hyperhomocysteinemia can lead to atherosclerosis and thrombosis has not been completely confirmed, the VEGF family has been thought to play an important role in the progression of atherosclerosis. Maeda and colleagues revealed that hyperhomocysteinemia could promote the development of atherosclerotic lesions through VEGF induction in macrophages. Because VEGF is believed to control angiogenesis in many pathological conditions, antibodies to VEGF (anti-VEGFs) would probably be useful in the treatment of angiogenetic diseases. Anti-VEGFs have been used for treating several retinal diseases such as AMD and diabetic macular edema. Placental growth factor is a member of the VEGF family, but it binds to different VEGF receptor subtypes. They advised that PlGF might synergize with VEGF in angiogenesis. They proposed that PlGF contributed to both angiogenesis and collateral capillary growth in ischemic tissues, with a comparable effect to that of VEGF; hence, the therapeutic potential of PlGF might be significant. Carmeliet et al. also indicated that PlGF might synergize with VEGF under some conditions, with key roles in pathological angiogenesis. The mRNA expression of PlGF has been demonstrated in the intact choroid, however, the exact roles of PlGF in regulating endothelial cells require further studies. In our immunofluorescent staining study, neither VEGF nor PlGF expression significantly differed between the hcy-treated and control choroidal capillary explants. However, VEGF expression was colocalized with isocititin, a vascular endothelial cell marker, and PlGF expression was observed in both endothelial cells and pericytes. We therefore suggest that PlGF regulates angiogenesis via the activation of pericytes and endothelial cells in the choroid, whereas VEGF might also activate pericyte function in angiogenesis.

Our capillary sprouting explant model provides a simple and useful method for evaluating the effect of hcy directly on vessel growth in vitro to mimic the angiogenesis in the choroid. We conclude that Ang1 and PDGF-C were involved in the angiogenesis of the choroidal capillaries evoked by hcy. Ang1 may regulate the vessel tube formation in angiogenesis, and PDGF-C may mediate the capillary outgrowth direction. Both VEGF and PlGF were expressed during angiogenesis: VEGF was expressed in endothelial cells, whereas PlGF was expressed in both endothelial cells and pericytes.

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