Predilection of the Macular Region to High Incidence of Choroidal Neovascularization After Intense Laser Photocoagulation in the Monkey

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Objective: To determine the key factors for creating a high incidence model of choroidal neovascularization (CNV) in the monkey.

Methods: Intense laser photocoagulation was performed in 8 eyes of 4 monkeys using krypton red and green-yellow and Alcon frequency-doubled diode ophthalmic lasers. Eight to 13 lesions were delivered to an area between the temporal vascular arcades in each eye. Development of CNV was monitored by fluorescein angiography at 2 and 4 weeks after laser treatment, and the results were correlated with histological analysis.

Results: A much higher incidence of CNV occurred in the macular region, which refers to an anatomic area equivalent to a mean±SD 2.5±0.4 times the horizontal diameter of the optic disc in the fundus. Regardless of the type of ophthalmic laser used, 72% of lesions developed fluorescein leakage within the macula, compared with 12% outside the macula (P<.001). By histological analysis, 89% of lesions developed microscopic CNV within the macula vs 22% outside the macula (P<.001).

Conclusion: The macular region is predisposed to creation of laser-induced CNV in the monkey.

Clinical Relevance: The predilection of the macular region to a high incidence of laser-induced CNV may account for the high recurrence rate of subfoveal CNV after laser treatment in humans.


Choroidal neovascularization (CNV), the invasion of newly formed blood vessels from the choroid through a break in Bruch membrane, is a feature of many eye diseases, including age-related macular degeneration, ocular histoplasmosis, angiod streaks, high myopia, chronic uveitis, and choroidal rupture.1,2 Choroidal neovascularization develops underneath the retina and disrupts the retinal pigment epithelium (RPE) and neurosensory retina, resulting in severe visual loss in most cases.1,2 The conventional management of CNV by laser photocoagulation has inherent drawbacks, including less dramatic effect on subfoveal CNV, a high rate of CNV persistence and recurrence, and irreversible damage to the RPE and normal retina.3,4 Several new therapeutic strategies, including photodynamic therapy,5 viral vector–mediated gene therapy,6,7 and new antiangiogenic drugs,8,9 have been introduced or are being investigated, and all of these need a reproducible and clinically related model of CNV.

Induction of CNV in animals can be achieved in 2 ways. One method is to create acute breaks in Bruch membrane traumatically9,10 or enzymatically,11 while the other is to induce CNV without immediate breaking of Bruch membrane. The disease process induced by the latter method is slow, and the permeability of the newly formed blood vessels is uncertain.12,13 Intense laser photocoagulation has been used to break Bruch membrane to create CNV in the rat,14,15 rabbit,16 cat,17 and monkey.9,10 The rat and monkey models have been the most commonly used in eye research. Previous success rates of laser-induced CNV of 50% to 80% have been reported in the rat.14,15 The incidence of laser-induced CNV in the monkey, however, is only 30% to 40%, and results vary markedly.9,10

Previously, clinical and experimental investigations have overlooked the importance of the macular region in CNV development.1,2,9,10 Ryan10 reported that 39% of laser-induced lesions developed clinical CNV (fluorescein leakage) in the macular region, with 3% in the area nasal to the optic nerve head and 0.3% in the peripheral retina of the rhesus monkey. However, no
information is available to clearly define the anatomic territory of the macula; thus, no landmark index can be used to precisely deliver laser lesions and achieve a high incidence of CNV in the primate model. As the retinal-choroidal circulation and macular anatomy are similar between humans and primates, the information obtained from the monkey model of CNV would be particularly valuable and crucial for assessment of new antiangiogenic therapies before clinical application. In a previous study,\(^9\) however, the low incidence of CNV in monkeys limited statistical evaluation of an antiangiogenic therapy. Considering the valuable information for clinical application and the high cost of experimentation on monkeys, it is critical to find key factors that would improve the success rate of laser-induced CNV in the monkey model. In this study, we measured the anatomic size of the macular region and analyzed the incidence of laser-induced CNV within this region, compared with that outside the macular region. Our results show that the macular region is predisposed to the creation of laser-induced CNV in the monkey.

### METHODS

#### ANIMAL PREPARATION AND ANESTHESIA

Eight eyes from 4 Macaca fascicularis monkeys were used in accord with the guidelines of the Association for Research in Vision and Ophthalmology on the use of animals in research and following the guidelines of the Animal Care Committee at National University of Singapore. The animals were anesthetized with ketamine hydrochloride (20 mg/kg of body weight), acepromazine maleate (0.25 mg/kg), and atropine sulfate (0.125 mg/kg). Pupils were dilated with 2.5% phenylephrine hydrochloride and 1% tropicamide drops. A trained veterinarian monitored the airway, respiration, and pulse during all procedures.

#### LASER PHOTOCOAGULATION

Laser photocoagulation was performed as previously described,\(^9,10\) with modifications. Three ophthalmic lasers, a krypton red (647 nm) (Coherent Radiation System, Salt Lake City, Utah), krypton green-yellow (528-568 nm) (Coherent Radiation System), and an Alcon frequency-doubled diode (532 nm) (Alcon 532 Ophthalas EyeLite Photocoagulator; Alcon, Inc, Houston, Tex), were used in this study (Table 1). With each ophthalmic laser, the levels of energy were initially assessed in an area away from the macula to test the ability to produce a blister without subretinal hemorrhage. The final protocols used were 1.0-W power density, 50-µm spot size, and 0.2-second duration for the krypton red; 1.5-W power density, 50-µm spot size, and 0.2-second duration for the krypton green-yellow; and 1.5-W power density, 50-µm spot size, and 0.1-second duration for the Alcon frequency-doubled diode lasers. Eight to 13 lesions were delivered in an area between the temporal vascular arcades in each eye using a slitlamp and fundus contact laser lens.

#### FUNDUS PHOTOGRAPHY AND FLUORESCEIN ANGIOGRAPHY

Fundus photographs were taken immediately after laser photocoagulation and at 2 and 4 weeks after laser treatment. Fundus fluorescein angiography (FFA) was performed to monitor the development of CNV at 2 and 4 weeks after laser treatment. Angiography was performed by intravenous injection of 10% fluorescein sodium (0.1 mL/kg of body weight). Identification of CNV was based on fluorescein behavior during the phases of angiography from 10 seconds to 10 minutes after dye injection. The status of the lesions was graded in a masked fashion by 2 examiners (W.-Y.S. and C.-M.L.) using reference angiograms. The scores were recorded as follows: 0, no hyperfluorescence; +, slight hyperfluorescence staining; ++, moderate fluorescein leakage; and ++++, prominent fluorescein leakage or pooling.

### HISTOLOGICAL ANALYSIS

All monkeys were humanely killed 4 weeks after laser treatment. The eyes were enucleated and fixed in 2.5% glutaraldehyde plus 2% paraformaldehyde in 0.1M phosphate-buffered saline.

### Table 1. Development of Fluorescein Leakage After Intense Laser Photocoagulation With Krypton Red and Green-Yellow and Alcon Frequency-Doubled Diode (AFDD) Ophthalmic Lasers in the Macaca fascicularis Monkey

<table>
<thead>
<tr>
<th>Ophthalmic Laser Used and Eye No.</th>
<th>Total Lesions Delivered</th>
<th>Leaky Lesions on Angiography</th>
<th>Inside Macula</th>
<th>Outside Macula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krypton red (647 nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13 (T₅ + 8)</td>
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<td>0/8</td>
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<td>2</td>
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</tr>
<tr>
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<td>8</td>
<td>2 (+++, ++)</td>
<td>2/3</td>
<td>0/5</td>
</tr>
<tr>
<td>Krypton green-yellow (528-568 nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>17 (T₅ + 11)</td>
<td>3 (+++, ++, ++)</td>
<td>3/3</td>
<td>0/8</td>
</tr>
<tr>
<td>AFDD (532 nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>11 (T₂ + 9)</td>
<td>3 (+++, ++, ++)</td>
<td>0</td>
<td>3/9</td>
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<tr>
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<tr>
<td>8</td>
<td>13</td>
<td>9 (6: ++, 3: ++)</td>
<td>6/6</td>
<td>3/7</td>
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</table>

Abbreviations: T, test lesions; ++, moderate fluorescein leakage; and +++, prominent fluorescein leakage or pooling.
saline for a minimum of 24 hours, then processed for paraffin embedding. Paraffin sections of 5-µm thickness were cut, and serial sections were collected when the first lesion was identified. Hematoxylin-eosin staining was performed every 4 slides. The primary goals of histological analysis were to: (1) correlate the incidence of leaky lesions with the number of microscopic CNV lesions detected by histological analysis, (2) determine the association of broken Bruch membrane with CNV development, and (3) orient histological CNV inside or outside the macular region (Table 2).

RESULTS

To evaluate the imbalance of CNV development between the macular and nonmacular regions, the size of the macula was initially determined by histological analysis (Figure 1). The ratio of the macula to the horizontal optic disc was measured in 4 samples from 4 monkeys. The ratio was used as an index to further determine the number of lesions within and outside the macular region on angiography.

With maximum levels of laser energy, photocoagulation using the krypton red and green-yellow and the Alcon frequency-doubled diode lasers did not induce obvious hemorrhage in most lesions, except for 2 testing spots showing minor hemorrhage where the lesions were delivered on small retinal vessels (data not shown). Four eyes were treated with the krypton red laser (Table 1). Of 34 lesions delivered, 5 (15%) developed fluorescein leakage on angiography. In 1 eye in which all 8 lesions were placed outside the macular region, none showed clinical CNV by FFA (Figure 2A). In the remaining 3 eyes in which 26 lesions were delivered, 5 (38%) of 13 lesions developed clinical CNV inside the macular region, compared with none showing leakage outside the macula (Figure 2B). Using the krypton red laser, none of the 21 lesions outside the macular region developed CNV-related fluorescein leakage at 2 and 4 weeks after laser treatment. Eleven lesions were delivered into 1 eye using the krypton green-yellow laser, 3 (27%) of which developed fluorescein leakage within the macular region. In contrast, none of the 8 lesions outside the macular region developed CNV-related fluorescein leakage (Figure 2C). Using the frequency-doubled diode laser,

Table 2. Development of Microscopic Choroidal Neovascularization (CNV) After Intense Laser Photocoagulation With Krypton Red and Green-Yellow and Alcon Frequency-Doubled Diode (AFDD) Ophthalmic Lasers in the Macaca fascicularis Monkey

<table>
<thead>
<tr>
<th>Ophthalmic Laser Used and Eye No.</th>
<th>Total Lesions Delivered</th>
<th>Lesions Detected by Histological Analysis</th>
<th>Broken BM</th>
<th>CNV</th>
<th>Lesions Detected by Histological Analysis</th>
<th>Broken BM</th>
<th>CNV</th>
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<tr>
<td>Krypton red (647 nm)</td>
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<td></td>
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<tr>
<td>1</td>
<td>13 (T1 + B)</td>
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<td>1</td>
<td>1</td>
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<td>3</td>
<td>3</td>
<td>2</td>
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<td>0</td>
</tr>
<tr>
<td>Krypton green-yellow (528-568 nm)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>17 (T6 + 11)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>AFDD (532 nm)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>11 (T1 + B)</td>
<td>3</td>
<td>3</td>
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<td>2</td>
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</table>

Abbreviations: BM, Bruch membrane; T, test lesions.

Figure 1. A, The mean ± SD macular diameter–optic disc (OD) ratio was 2.5 ± 0.4 times the horizontal diameter of the OD in the fundus. The short arrow points to a lesion located next to the parafoveal retina. The macula and OD were detected in serial sections. B, The relative size of the OD to the macula in histological analysis. GCL indicates ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; and CRV, central retinal vessels. Hematoxylin-eosin staining, original magnification ×38.

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33 lesions were delivered into 3 eyes, 19 (58%) of which developed CNV-related leakage (Figure 2D-F). Of 13 lesions delivered inside the macular region, all developed CNV-related fluorescein leakage (Figure 2D and F). Of 20 lesions that were delivered in the boundary or outside the macula, however, only 6 (30%) showed CNV-related fluorescein leakage (Figure 2D and F).
related fluorescein leakage (Figure 2D and F). In general, regardless of the type of ophthalmic laser used, 21 (72%) of 29 lesions developed CNV-related leakage inside the macular region, compared with 6 (12%) of 49 lesions outside the macula (P < .001, unpaired t test). In all cases, CNV-related fluorescein leakage was observed at 2 weeks and at 4 weeks after laser treatment. Based on the criteria of the Macular Photocoagulation Study Group,18 most CNV lesions in the laser-induced model showed features of classic CNV, characterized by well-demarcated boundaries discernible at an early stage of angiography, with progressive pooling or leakage in later phases of angiography (Figure 2E and F). However, occult CNV lesions were also observed, characterized by poorly defined areas of hyperfluorescence in the early phase and progressive pooling or leakage in later phases of angiography (white arrow, Figure 2E and F).

Histological analysis of 10 lesions outside the macular region in 4 eyes treated by the krypton red laser showed broken Bruch membrane in 5 lesions, but all failed to develop histological CNV (Table 2 and Figure 3A and B). Inside the macular region, however, 9 of 10 lesions showed broken Bruch membrane, and all developed microscopic CNV (Figure 3C and D). The development of CNV was initiated with disruption of Bruch membrane, and the new vessels originated from the choriocapillaries and extended into the subretinal space, forming neovascular membrane networks. Histological analysis of the macular region in the eye treated by the krypton green-yellow laser was not available, because of unexpected tissue destruction. Two lesions outside the macular region showed broken Bruch membrane, but none developed microscopic CNV. Among the eyes treated by the Alcon frequency-doubled diode laser, all 29 lesions showed broken Bruch membrane, regardless of their geographic location. Within the macular region, 16 (89%) of 18 lesions showed microscopic CNV (Figure 4A and B). In contrast, 5 (45%) of 11 lesions outside the macular region developed histological CNV, and most of them formed fibrous tissue. Among the few lesions showing microscopic CNV in the extramacular region, the number of new vessels was limited, and most of them were embedded in firmly packed fibrous tissue (Figure 4C-F). Among some lesions, several elongated fibroblast-like cells and pigment-laden cells enveloped the new vessels, apparently limiting their further development (Figure 4E and F). In general, regardless of the type of ophthalmic laser used, 25 (89%) of 28 lesions developed histological CNV inside the macular region vs 5 (22%) of 23 outside the macular region (P < .001, unpaired t test).

Our results demonstrate that the macular region was predisposed to laser-induced CNV. Fundus fluorescein angiography showed that 72% of lesions developed CNV-related fluorescein leakage inside the macula, compared with 12% outside the macula. Furthermore, histological analysis showed that 89% of lesions developed microscopic CNV inside the macula vs 22% outside the macula. Previous investigations have demonstrated that the presence of fluorescein leakage or pooling on angiography correlated with microscopic CNV, but that not all microscopic CNV developed fluorescein leakage on an-
In the present study, 27 (35%) of 78 lesions developed fluorescein leakage on angiography, but 30 (59%) of 51 lesions on histological analysis showed microscopic CNV. This observation is similar to findings in a previous study in a rat model showing that 28% of lesions developed fluorescein leakage, with subsequent histological analysis revealing microscopic CNV in 60% of lesions. In primate models, Ryan and Miller reported that the incidence of lesions showing fluorescein leakage on angiography was much lower than that of microscopic CNV. In humans, Gass reported that, while FFA failed to show fluorescein leakage, subsequent postmortem examination demonstrated pathologic CNV development. The reason why some histological CNV membranes remained inactive during FFA is unclear. In the present study, most lesions inside the macula developed classic CNV. However, we observed that certain lesions inside the macula developed occult CNV. By histological analysis, most CNV membranes outside the macula contained fewer new vessels, embedded in firmly packed fibrous tissue, compared with the microscopic CNV inside the macula, and some new vessels were enveloped by elongated fibroblast-like cells and RPE-like cells. The limited number of microscopic CNV lesions and the absence of a fluid-filled space may account for the inactivation of fluorescein leakage from lesions outside the macula. It could be possible that the CNV lesions within the macular region mainly developed classic CNV, while the lesions outside the macular region predominantly formed occult CNV, which is difficult to interpret on angiography. In addition, the differences in endothelial cell fenestration and pericyte maturation between leaky and nonleaky new vessels—and the local molecular mechanisms, such as the level of vascular endothelial growth factor that is known to initiate and maintain angiogenesis and to increase vascular permeability, may also affect the activity of lesions during angiography.

Figure 4. Histological choroidal neovascularization (CNV) after intense laser photocoagulation treated by the Alcon frequency-doubled diode laser. The figures on the right show high magnifications of the insets on the left. Arrows in B indicate well-formed new vessels in a CNV membrane inside the macula. Arrows in D indicate new vessels in a CNV membrane outside the macula. In F, a new vessel (NV) was enveloped by several elongated fibroblast-like cells and pigment laden cells (white arrows). RPE indicates retinal pigment epithelium. Hematoxylin-eosin staining, original magnification ×198 (A and E), ×376 (B, D, and F), and ×94 (C).
Previous studies9,10 have shown that the occurrence of hemorrhage at the time of laser photocoagulation does not affect CNV development. In contrast, massive subretinal hemorrhage usually leads to scar tissue formation.9,10 Because the intensity of laser burns varies depending on treatment protocols (power density, spot size, and duration), properties and conditions of ophthalmic lasers, fundus pigmentation, and magnification factors of laser lens used, we initially delivered laser spots far away from the macular region for energy testing. The final laser protocols were designed to promote laser burns with heating blasters but without obvious hemorrhage. McAllister and colleagues25 showed that an ophthalmic laser with a green wavelength spectrum requires at least a 1.5-W power density to reliably break Bruch membrane in humans when a 50-µm spot size and a 0.1-second duration were applied. In this study, we used the most powerful laser burns to break Bruch membrane. For the krypton red laser, a 1.0-W power density was used because of the evidence that rupture of Bruch membrane requires less power density compared with the green spectrum lasers.24 In the present study, of 91 lesions delivered in 8 eyes, only 2 showed minor hemorrhage in which the lesions hit small vessels. With the krypton red laser, rupture of Bruch membrane occurred much more frequently inside the macular region compared with outside the macular region.

Based on the histological analysis, CNV only occurred in lesions in which Bruch membrane was broken. However, a break in Bruch membrane was not always sufficient to induce CNV. In the laser-induced CNV model, factors affecting the success rate of CNV may include the wavelengths and energy levels of ophthalmic lasers and the locations where lesions are delivered. Moreover, a recent study25 demonstrated age to be an independent risk factor for severity of laser-induced CNV in rodents. With the 3 different types of laser used, the success rate of CNV ranged from 15% to 58% in the present study. Given the fact that different power densities and durations were used for different types of lasers, it is hard to draw any conclusions about the relative efficiency of these lasers in terms of their production of CNV, although it appeared that the Alcon frequency-doubled diode laser produced the highest incidence of CNV when the highest energy level was used.

The mechanisms of laser-induced CNV and the imbalance of CNV development between the macula and extramacula are unclear. It has been proposed that a balance between factors that stimulate or inhibit vessel growth controls neovascularization.26-27 In most normal tissues, inhibitory factors are active and vessels remain quiescent.28 In contrast, in different pathologic states, such as tumor growth and neovascular forms of age-related macular degeneration, neovascularization occurs because of the disruption of the balance between angiogenic factors and angiogenic inhibitors.29-30 Recent investigations have shown that sole overexpression of vascular endothelial growth factor, one of the most potent angiogenic growth factors, is sufficient to induce ocular neovascularization.22,28 Furthermore, vascular endothelial growth factor is involved in the maturation and stabilization of newly formed vessels.29 With a disruption of Bruch membrane, the effect of laser photocoagulation on the development of CNV is often attributed to the up-regulation of angiogenic factors. Previous studies35,36-37 have demonstrated that expression of several angiogenic factors, including vascular endothelial growth factor, basic fibroblast growth factor, and matrix metalloproteinase 2, is up-regulated in activated RPE and infiltrating cells in laser-induced CNV. Most recently, Renno et al38 demonstrated that down-regulation of pigment epithelium–derived factor, a potent endogenous inhibitor for vascular endothelial cell proliferation and migration, also enhances laser-induced CNV. In the normal retina, pigment epithelium–derived factor is expressed most intensely in the outer nuclear layer (photoreceptor nuclei).39 After laser treatment, however, immunostaining for the factor within the outer nuclear layer was absent or decreased for up to 3 weeks, which seemed to parallel the up-regulation of vascular endothelial growth factor.35,36,37,38 In the present study, the macular region was predisposed to the incidence of laser-induced CNV. Anatomically and physiologically, the macula is different from the extramacula by its denser distribution of photoreceptors and RPE cells and more abundant blood supply from the choroidal circulation.34,35 It is possible that these differences lead to the imbalance between the macula and extramacula in overexpression of angiogenic factors and in down-regulation of angiogenic inhibitors after laser photocoagulation. Immunostaining for angiogenic factors and angiogenic inhibitors on macular and extramacular new vessels could clarify these speculations. However, all enucleated eyes in the present study were fixed in 2.5% glutaraldehyde plus 2% paraformaldehyde for a minimum of 24 hours, which challenges the immunostaining techniques. Our study was initially designed to increase the incidence of CNV in the primate model for preclinical evaluation of antiangiogenic therapies, rather than to investigate the mechanisms regulating CNV formation. When fresh tissue is available in the future, further investigations will be directed to elucidate the molecular mechanisms that could explain the imbalance of laser-induced CNV between the macular and extramacular regions.

The predilection of the macular region to a high incidence of CNV is also of clinical significance. Iatrogenic CNV is a common complication of laser photocoagulation for treatment of subfoveal CNV.34 Previous studies12,37,38 demonstrated that chronic cellular processes following moderate-intensity laser treatment resulted in gradual dissolution of Bruch membrane. Clinically, comparable laser intensities unable to immediately break Bruch membrane induce choriocapillary budding, with subsequent digestion of Bruch membrane and histological CNV formation.12,37,38 The current observation, the predilection of the macular region to a high incidence of laser-induced CNV, may account for the high recurrence rate of subfoveal CNV after laser treatment in humans.

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