Glial Cell and Inflammatory Responses to Retinal Laser Treatment: Comparison of a Conventional Photocoagulator and a Novel, 3-Nanosecond Pulse Laser

Glyn Chidlow,1–3 O'Sam Shibeeb,1,2 Malcolm Plunkett,4 Robert J. Casson,1,2 and John P. M. Wood1–5

PURPOSE. Retinal laser photocoagulation represents a major treatment strategy for the management of diabetic macular edema (DME). However, the thermal nature of this procedure defines that collateral tissue injury result, meaning that it cannot be used near the fovea centralis. We studied inflammatory and glial responses resulting from treatment of rats with a conventional laser and with a novel short-duration, nonthermal laser (retinal regeneration therapy [2RT]) at clinically relevant energy levels.

METHODS. Pigmented Dark Agouti rats were treated with either a conventional thermal continuous wave (CW; 532-nm, 100-ms pulse duration) or a short-pulse (2RT; 532-nm, Q-switched, 3-ns pulse) laser. Settings were at visible threshold for the CW laser (12.7 J/cm²/pulse) and at supra- and subvisible thresholds for the 2RT laser (“high,” 2RT-H, 163 mJ/cm²/pulse; “low,” 2RT-L, 109 mJ/cm²/pulse). Rats were killed at various subsequent time points. Samples were processed for histology, immunohistochemistry, RT-PCR, and Western blotting.

RESULTS. The CW laser caused outer retinal lesions that were associated with photoreceptor death, astrocyte and Müller cell activation, and infiltration of macrophages and neutrophils. Furthermore, inflammatory cytokines, heat shock proteins, endogenous trophic factors, and matrix metalloproteinases were induced. In comparison, all of these changes were drastically attenuated when the 2RT laser was used, particularly at the subthreshold setting.

CONCLUSIONS. The conventional laser produced marked retinal damage and cellular responses consistent with an inflammatory response to thermal injury. In contrast, the 2RT laser produced negligible retinal damage and cellular responses at clinically relevant settings. These results may have important implications for the treatment of retinal disease. (Invest Ophthalmol Vis Sci. 2013;54:2319–2332) DOI:10.1167/iovs.12-11204

Along with anti-VEGF therapy,1–3 retinal laser photocoagulation represents a common therapeutic strategy for treatment and management of diabetic macular edema (DME).4–6 During retinal photocoagulation, laser energy is converted to heat energy in the RPE and choroid, resulting in the focal death of these cells.7–9 It is believed that this focal RPE destruction underlies the therapeutic benefit of laser photocoagulation.5,6 Because of the inherent characteristics of the lasers used for photocoagulation, however, it is intrinsically impossible to confine the generated thermal energy to the RPE. This necessarily means that surrounding cells, notably overlying photoreceptors, suffer collateral damage at clinically relevant energy settings.5,6,8,10,11 Thus, retinal laser photoagulation always produces visible retinal burns and, therefore, is itself responsible for the formation of visual scotomas.

One way to prevent collateral damage is to reduce the amount of applied laser energy. Ongoing studies are investigating the therapeutic efficacy of this strategy.12–15 To achieve this objective, we have developed a novel laser, the retinal regeneration therapy (2RT) laser—which has similar characteristics to conventional 532-nm continuous wave lasers, except that the pulse duration is only 3 ns.16 Studies to date have shown that the 2RT laser specifically ablates RPE cells at a much lower fluence, and over a greater range of energy settings than the conventional continuous wave (CW) system,16 and has a similar effectiveness to conventional photocoagulation for treating retinal thickness in DME patients.17

Since the current favored retinal laser protocol in the clinic still involves the formation of visible retinal burns, then we believe that it is prudent to more fully reveal the alterations that occur in the retina/RPE following this procedure. This will enable us to better understand the therapeutic benefit of laser treatment. In a companion study to the present one, we described the retinal damage profiles and neuronal effects of both a conventional CW laser regime and our novel 2RT laser.18 We demonstrated that both lasers ablated the RPE, the CW laser and the higher energy setting of the 2RT laser caused photoreceptor damage, but neither laser produced any significant effects to any of the inner retinal neuronal classes, either overlying or adjacent to irradiated regions. Damage to photoreceptors is known to affect retinal glial cells: Müller cells are in close contact with photoreceptors and loss or damage to the latter can cause activation, proliferation, and/or dedifferentiation of this class of cell.19,20 Moreover, resident microglia will also be stimulated in response to tissue injury.
and can release a variety of factors and cytokines to influence bloodborne and other in situ retinal cells. Indeed, alterations in glial production of “stress-related proteins”—such as glial fibrillary associated protein (GFAP), nestin, inflammatory cytokines, and growth factors—have been described in retinas subsequent to laser irradiation.

In the current study, we aimed to more fully elucidate glial and inflammatory cell changes that occur subsequent to retinal laser irradiation in an attempt to relate such tissue plasticity to the known therapeutic potential of this treatment. Furthermore, we compared these effects for both the conventional CW laser and the novel 2RT laser.

MATERIALS AND METHODS

Lasers

Two lasers were used for treatment of animals: both were frequency-doubled, neodymium:yttrium-aluminum-garnet (Nd:YAG) lasers with 532-nm wavelengths and both were provided by Ellex R&D Pty Ltd. (Adelaide, South Australia). A 5.4-mm fundus laser contact lens was used to focus the light beam onto the retina (Ocular Instruments, Bellevue, WA) in each case. The first laser had a single 3-ns pulse duration with a 380-μm diameter spot size in air and a 285-μm diameter spot size on the rat retina, and a fine speckle beam profile (the nanosecond laser; 2RT). The 2RT laser was used at two different energy settings: the first was at the point at which the operator was able to discern a clear visual effect on the eye at the time of laser application; this was identified as a subtle blanching and was defined as the “low” energy setting (2RT-L; 106 mJ/cm²/pulse). A similar energy setting was used in the 2RT clinical trials to “range find” for each patient. The second energy setting was similar to that used in clinical trials for DME, and which equated to exactly 2/3 of the 2RT-F energy level; this was defined as the “low” energy setting (2RT-L; 106 mJ/cm²/pulse). Previous in vitro experiments had established that this lower energy setting was within the therapeutic range of the laser. The second laser used in the study was a CW laser, with a 400-μm diameter spot in air and 300-μm diameter spot on the rat retina, a flat top beam profile and which had a total beam exposure duration of 100 ms. This laser was used at the 90-mW setting, which equated to a radiant exposure level of 1.27 J/cm²/pulse, and produces light laser burns, defined as blanching of the fundus pigmentation (but without candid whitening) and devoid of adjacent edema, subretinal, or retinal hemorrhage.

Treatment of Animals

This study was approved by the Animal Ethics Committees of SA Pathology and the University of Adelaide. The study conformed to the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult Dark Agouti rats (approximately 150 g) were housed in a temperature- and humidity-controlled room with a 12-hour light, 12-hour dark cycle and were provided with food and water ad libitum.

Prior to induction of laser treatment, rats were anesthetized with an intraperitoneal injection of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine. When general anesthesia had been obtained, the pupils were dilated by topical application of tropicamide, allowing visualization of the optimum area of retina through the eye. Animals were then placed on a custom-designed platform attached to the slit-lamp laser delivery system. Rats were randomly assigned to one of three treatment groups: CW, 2RT-H, or 2RT-L. For animals that were subsequently used for Western immunoblotting or RT-PCR, approximately 100 laser spots were applied randomly to each retina around the optic nerve head, taking care to avoid the macula region and major blood vessels; all spots were applied in the posterior hemisphere of the eye. For animals that were subsequently used for histology/immuno-
was synthesized from DNase-treated RNA. Real-time PCR reactions were carried out in 96-well optical reaction plates using the cDNA equivalent of 20 ng total RNA for each sample in a total volume of 25 \mu L containing 3 SYBR Green PCR master mix (Bio-Rad Laboratories, Hercules, CA), forward, and reverse primers. The thermal cycling conditions were 95°C for 3 minutes and 40 cycles of amplification comprising 95°C for 12 seconds, annealing temperature for 30 seconds, and 72°C for 30 seconds. Primer sets used are detailed in Supplementary Table S1 (see Supplementary Material and Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11204/-/DSSupplemental). In order to allow a comparison to be made between the levels of expression of target mRNAs in the retinas of sham and laser-irradiated animals, results were quantified using the Relative Expression Software Tool (REST) and statistical significance was determined using the Pair-Wise Fixed Reallocation Randomization Test. Threshold cycles were calculated using IQ5 iCycler Software (Bio-Rad Laboratories), all values were normalized using two endogenous reference genes GAPDH and cyclophilin and results are expressed as mean ± SEM.

RESULTS

As shown in the accompanying paper, use of the CW laser, the higher setting of the 2RT laser (2RT-H), and the lower setting of the 2RT laser (2RT-L), all resulted in the ablation of RPE cells in the irradiated zone when compared with neighboring nonirradiated areas. In conjunction with RPE cell destruction, the CW laser precipitated substantial destruction of the outer nuclear layer (ONL) and photoreceptor segments; 2RT-H-treated retinas displayed minor loss of cells in the ONL together with disruption to the photoreceptor segments; in 2RT-L-treated animals, there was no apparent disruption to the ONL, although disorganization of the photoreceptor outer segments was evident.

Expression of Intermediate Filaments

To ascertain the effect of CW and 2RT laser treatment on macroglial reactivity in the retina, we investigated expression of three intermediate filament proteins: glial fibrillary acidic protein (GFAP), vimentin, and nestin. In the normal retina, GFAP was expressed by astrocytes, but was largely absent from Müller cells (Fig. 1). At 1 day after CW laser treatment, Müller cells throughout the retina, with the exception of the actual lesion sites, were GFAP-positive (data not shown). By day 3, there was a dramatic increase in GFAP labeling in astrocytes and Müller cell processes within lesioned areas (Fig. 1). Of particular note was the formation of a GFAP scar at the lesion core. A similar pattern of elevated GFAP expression persisted at 7 days after treatment (data not shown). The pattern of GFAP expression in 2RT-H–treated animals was broadly similar to that observed in CW retinas, but without an obvious scar in the outer retina (Fig. 1). In 2RT-L retinas, induction of GFAP expression, while evident, was markedly less pronounced, both in Müller cell processes and astrocytes.

![Induction of GFAP expression following treatment with CW or 2RT laser, used at 2RT-H and 2RT-L energy settings. Representative images of GFAP immunolabeling at 3 days after laser treatment. The CW retina features intensely labeled astrocytes and Müller cell processes with the formation of a GFAP scar at the lesion core. A similar response is observed in the 2RT-H retina, without the presence of a scar; while in the 2RT-L retina, induction of GFAP expression, while evident, is markedly less pronounced. Scale bar: 50 \mu m. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.](image-url)

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**TABLE. Antibodies Used in the Study**

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DSHB, Developmental Studies Hybridoma Bank.

* Antibody clones.
† 2-step immunofluorescence.
Western blotting confirmed that the CW laser induces GFAP expression to a markedly greater extent than the 2RT laser (see Supplementary Material and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11204/-/DCSupplemental).

The patterns of vimentin immunolabeling in CW- and 2RT-lasered retinas were strikingly similar to those of GFAP; however, as both Müller cells and astrocytes in the normal retina constitutively express vimentin, data obtained with vimentin are, in practice, less informative than those of GFAP.

In the normal retina, nestin expression was negligible, as evidenced by quantitative PCR (qPCR; Fig. 2A), Western blotting (Fig. 2B) and immunolabeling of retinal wholemounts and transverse sections (Fig. 2C). Following CW treatment, the nestin mRNA level was upregulated approximately 12-fold and 24-fold at 6 hours and 1 day, respectively, then returned to normal by 7 days. Protein expression, however, was longer-lived with nestin immunoreactivity at 7 days indistinguishable from that of 3 days. The pattern of nestin immunolabeling was broadly similar to that of GFAP, but with noteworthy differences: nestin was chiefly limited to the lesions rather than expressed throughout the retina; nestin expression by astrocytes was negligible; and nestin was induced de novo in RPE cells at the lesion. As with GFAP, nestin forms a scar at the lesion core in the CW, but not in the 2RT-H or -L, retina. The wholemount images reveal that nestin expression is largely restricted to the lesions rather than upregulated throughout the retina. Scale bar: 30 μm.

To determine the extent of cell proliferation/migration following CW and 2RT laser treatment, transverse sections of the retina were immunolabeled for proliferating cell nuclear antigen (PCNA). In the normal retina, there was an absence of PCNA-positive cells. Following CW and 2RT laser treatment, PCNA-positive cells were evident only in lesioned areas of the retina, the peak timing of which occurred at 3 days (Fig. 3). In the CW group, PCNA-positive cells were scarce in the nerve fiber layer, but abundant in the inner nuclear layer (INL), and throughout the damaged outer retina including the RPE cell layer. Double-labeling of PCNA with the Müller cell marker glutamine synthetase (data not shown) identified Müller cells as the predominant cell type expressing PCNA in the INL and ONL, a result indicative of Müller cell proliferation and migration. Infiltrating leukocytes and RPE cells likely comprised the remaining PCNA-positive cell types. In 2RT-H-treated rats, proportionally fewer cells were PCNA-positive, reflecting
the more limited photoreceptor death that occurred in this group, while in 2RT-L–treated retinas, PCNA-labeled cells were typically only observed in the RPE layer.

Expression of Trophic Factors

It is well-known that various insults to the rat retina lead to an upregulation of certain trophic factors. We examined whether CW and 2RT laser treatment altered expression of the two factors most commonly associated with this phenomenon, fibroblast growth factor-2 (FGF-2; Fig. 4) and ciliary neurotrophic factor (CNTF; Fig. 4).

At 6 hours after laser treatment, FGF-2 mRNA was significantly upregulated in all three groups. By day 1, FGF-2 mRNA expression continued to increase in the CW group, was approximately the same as at 6 hours in the 2RT-H group, and was lower than at 6 hours in the 2RT-L group. Between 3 days and 7 days, FGF-2 level was not different from the controls in any of the three treatment groups (see Supplementary Material and Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11204/-/DCSupplemental). Immunohistochemistry showed that in normal retinas, FGF-2 was associated exclusively with Müller cell nuclei. CW treatment resulted in an upregulation of FGF-2 by photoreceptors immediately adjacent to the lesion, a response not observed in 2RT-treated retinas. 2RT laser treatment caused a modest increase in FGF-2 labeling intensity in Müller cell nuclei in lasered regions.

CW-treated retinas displayed robust CNTF immunolabeling in Müller cells and astrocytes in regions of the retina overlying laser spots (Fig. 4). Labeling was considerably more intense than in surrounding areas or nontreated samples. Interestingly, CNTF expression was also upregulated in irradiated areas of 2RT-H and 2RT-L retinas, despite limited (2RT-H) and negligible (2RT-L) neuronal death occurring in these treatment groups.

Expression of Heat Shock Proteins (HSPs)

aB-Crystallin. Unlike the lens (Fig. 5A), the normal retina RPE was devoid of aB-crystallin immunoreactivity, with the exception of weakly labeled astrocytes (Figs. 5B, 5E). In response to CW and 2RT laser treatment, there was a rapid upregulation of aB-crystallin, the extent of which was related to the laser power. Thus, aB-crystallin was detectable in Müller cells, astrocytes, and RPE cells immediately adjacent to the lesion at 1 day after CW treatment (Figs. 5C, 5F), in astrocytes and RPE cells, but not Müller cells in the 2RT-H group (Figs. 5D, 5G), and only in RPE cells in the low energy 2RT-L group (Fig. 5H). By 7 days after treatment, the patterns of aB-crystallin immunoreactivity in glia were similar to those seen at 1 day, while proliferating/migrating RPE cells at the lesion were also positively labeled (data not shown).

Hsp27. In the normal retina, Hsp27 expression was low, as evidenced by qPCR (Fig. 6A), Western blotting (Fig. 6B), and immunolabeling of retinal wholemounts and transverse sec-

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**Figure 3.** Representative images of PCNA immunolabeling following treatment with CW or 2RT laser, used at 2RT-H and 2RT-L energy settings. In the normal eye, expression of PCNA is confined to dividing cells, such as the germinative zone of the lens epithelium (arrow). At 3 days after laser treatment, numerous PCNA-positive cells, corresponding to migrating Müller cells, infiltrating leukocytes and dividing RPE cells, are detectable at the lesion in the CW retina. A similar, but considerably weaker response, is apparent after 2RT-H lasering, while in the 2RT-L retina, PCNA-positive cells are only evident at the level of the RPE (arrow). Scale bar: 30 μm.

**Figure 4.** Expression of trophic factors following treatment with CW or 2RT laser, used at 2RT-H and 2RT-L energy settings. Representative images of immunohistochemistry for CNTF and FGF-2. In unlasered areas of retina, CNTF immunoreactivity is weakly associated with Müller cells (long arrow) and astrocytes (short arrow). At 7 days after CW, 2RT-H, or 2RT-L treatment, upregulated CNTF expression is evident in Müller cells and astrocytes in lasered regions of the retina. In unlasered areas of retina, FGF-2 immunoreactivity is associated with Müller cell nuclei (arrow). At 7 days after treatment with the CW laser, photoreceptors adjacent to the lesion (arrowhead) can be observed to upregulate FGF-2. At 7 days after treatment with the 2RT laser, few photoreceptors are FGF-2-positive, but Müller cell nuclei adjacent to the lesion (arrows) are more robustly labeled than in nonlasered areas. Scale bar: 50 μm. CNTF, ciliary neurotrophic factor.
and proliferating/dividing RPE cells (Fig. 7). Unlike a
As expected, labeling of Hsp32 by Müller cells in 2RT-H and

representation characteristic of movement (polarized) or activa-

tions (Fig. 6C). As early as 6 hours after laser treatment, the
Hsp27 mRNA level was significantly upregulated in all three
experiment groups, but expression returned to baseline by 1
day in the 2RT-L group and by 7 days in the 2RT-H group.
Nevertheless, Hsp27 protein expression remained elevated for
at least 7 days in all three groups. In general, patterns of Hsp27
expression before and after laser treatment were similar to
those of β-crystallin, namely an upregulation in astrocytes and
a de novo induction in some proliferating/migrating RPE cells.
Unlike β-crystallin, Hsp27 was not associated with Müller
cells. Interestingly, in the CW group, occasional retinal
ganglion cells directly overlying lesions were Hsp27-positive (data not shown).

Hsp32. The normal retina was devoid of Hsp32 immuno-
labeling. At 3 days after laser treatment, Hsp32 immunoreac-
tivity was associated with Müller cells, infiltrating macrophages
and proliferating/dividing RPE cells (Fig. 7). Unlike β-
crystallin and Hsp27, Hsp32 was not expressed by astrocytes.
As expected, labeling of Hsp32 by Müller cells in 2RT-H and
2RT-L retinas, was proportionally weaker than in CW retinas. In
all three groups, Hsp32 immunoreactivity was less intense at 7
days than at 3 days (data not shown).

Early Activation of Microglia and Expression of
Proinflammatory Cytokines

Microglia are highly susceptible to any disturbance of neuronal
homeostasis, reacting in a well-defined manner. To delineate
early microglial changes, we immunolabeled for iba1, a well-
characterized, specific marker of quiescent and activated
microglia. In the normal rat retina, microglia were restricted
to the inner retina (Figs. 8A, 8D, 8E). By 6 hours after CW laser
treatment, iba1-positive microglia were seen to have migrated
from the inner to the outer retina at lesion sites and to have
adopted a less-ramified, polarized or amoeboid morphological
appearance characteristic of movement (polarized) or activa-
tion (amoeboid; Figs. 8B, 8C, 8F). Similar responses were
observed in 2RT-H and -L retinas (Figs. 8G, 8H), but proportionally fewer microglia were affected.

The onset of acute neuroinflammatory injury is typically
characterized by the release of proinflammatory cytokines. We
investigated the timing and nature of any changes in
expression of three proinflammatory gene products, namely
interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα),
and inducible nitric oxide synthetase (iNOS), following laser
treatment using qPCR and immunohistochemistry. For IL-1β
and TNFα an abundance pattern of CW > 2RT-H > 2RT-L was
found. Thus, at 6 hours, the peak time-point, there were
approximately 28-fold (CW); 9-fold (2RT-H); and 4-fold (2RT-L)
increases in IL-1β mRNA expression and 17-fold (CW); 5-fold
(2RT-H); and 3.5-fold (2RT-L) increases in TNFα mRNA
expression compared with the nontreated group (Figs. 9A,
9B). Both cytokines remained elevated for longer in the CW
than in the 2RT groups. In the normal rat retina, IL-1β-positive
immunoreactivity was not detected (Figs. 9D, 9G). At 6 hours
after CW treatment, IL-1β-positive cells were visible in the
inner (Figs. 9E, 9H) and outer (Figs. 9F, 9I) retina in lesioned
areas. Double-labeling with iba1 indicated that expression
of each of the cytokines was exclusive to microglia. IL-1β-positive
microglia were also evident in the 2RT groups (Figs. 9J, 9K);
but, again, proportionally fewer cells were detectable. Inter-
estingly, neither CW nor 2RT laser treatment had any effect on
the level of iNOS mRNA synthesis (Fig. 9C).

Infiltration of Leukocytes

The healthy CNS contains few leukocytes, but in response
to various injuries, including laser photocoagulation, there can be
a significant recruitment of macrophages, neutrophils and T
cells. The process, which is driven via proinflammatory
cytokines, involves upregulation of leukocyte adhesion
molecules, notably intercellular adhesion molecule (ICAM)-1 and
P-selectin, and activation of matrix metalloproteinase-9 (MMP-9).
We examined whether the CW and 2RT lasers stimulated these
pathways in the retina. Following laser treatment, there were

FIGURE 5 Representative images of β-crystallin immunolabeling at 1 day following treatment with CW or 2RT laser, used at 2RT-H and 2RT-L
energy settings. In the normal eye, there is a robust presence of β-crystallin in the lens (A), a weak association with astrocytes (B, short arrow),
but negligible labeling in the other layers of the retina or the RPE (B, E). In the CW retina (C, F), upregulated β-crystallin expression is detectable
in Müller cell somas (white arrow) and their processes, astrocytes (short arrow), and RPE cells immediately adjacent to the lesion (black arrow).
In the 2RT-H retina (D, G), upregulated β-crystallin expression is discernible in astrocytes (D, short arrow) and RPE cells (G, black arrow), but
not Müller cells, while in the 2RT-L retina (H), upregulated β-crystallin expression is only evident in the RPE cell layer (black arrow). Note: Gold
asterisks indicate laser lesions. Scale bar: 50 μm (A), 15 μm (B–H).
approximately 9-fold (CW); 4-fold (2RT-H); and 3-fold (2RT-L) increases in ICAM-1 mRNA expression and 18-fold (CW); 4-fold (2RT-H); and 2-fold (2RT-L) increases in P-Selectin mRNA expression compared with the unlasered group (Figs. 10A, 10B). MMP-9 mRNA was highly upregulated (7-fold) in the CW group, but was unaffected in the 2RT groups (Fig. 10C).

Immunolabeling for myeloperoxidase, a specific marker of neutrophils, revealed numerous positively labeled cells in CW retinas (Fig. 10D). These were typically observed in the vicinity of inner retinal blood vessels overlying lesions and in the subretinal space at the lesion. Myeloperoxidase-labeled cells were very rarely observed in the retina of 2RT-H- or -L-treated rats, but were sometimes detectable in the choroidal vasculature at lesion sites (Fig. 10E). Immunolabeling for CD3, a pan marker of T cells, highlighted occasional positive cells in CW-treated retinas (Fig. 10F), but not in either 2RT group.

**Microglia/Macrophage Responses at Later Time Points**

Alongside iba1, we examined labeling for two other microglial markers: ED1 and OX6. The presence of ED1 is considered indicative of phagocytosis, while OX6 recognizes major histocompatibility (MHC) class II. Under normal physiological conditions, both markers are undetectable. At 1 day after CW treatment, a considerable proportion of iba1-positive microglia at lesion sites had migrated from the inner retina to the ONL and the subretinal space (Fig. 11A). At this time point, there was little evidence of macrophage-like cells (large, round, with a foamy appearance); however, many microglia had adopted an activated, amoeboid morphology and a proportion was ED1-positive (data not shown). By 3 days, numerous iba1- and ED1-positive cells with a macrophage-like appearance were present in the ONL and subretinal space of CW retinas (Figs. 11B, 11D,
Many of these cells were labeled by OX6 (Fig. 11J). The patterns of iba1 (Fig. 11C), ED1, and OX6 (data not shown) at 7 days were very similar to those seen at 3 days.

In the 2RT-treated groups, a proportion of iba1-positive microglia at lesion sites migrated from the inner retina to the outer retina and subretinal space, but there was little evidence of a macrophage presence at 1 day, 3 days, or 7 days after treatments. In the 2RT-H group, microglia frequently adopted an activated morphology and expressed ED1, both in the ONL and at the level of the RPE (Figs. 11E, 11H). In the 2RT-L group, microglia typically retained a ramified appearance in the ONL, but assumed an amoeboid morphology and were positive for ED1 at the RPE cell layer (Figs. 11F, 11I). MHC II expression was rarely evident after 2RT laser treatment (Figs. 11K, 11L).

The secreted protein osteopontin (OPN) is expressed by macrophages in multiple pathological situations and is implicated in various processes, such as phagocytosis and expression of cytokines. We examined OPN expression after CW and 2RT laser treatment (see Supplementary Material and Supplementary Fig. S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11204/-/DCSupplemental). In the CW group, the level of OPN mRNA was markedly elevated, peaking (14-fold) at 3 days after laser treatment. At this time point, OPN immunoreactivity was associated with macrophage-like cells. Although the OPN mRNA level was significantly (2-fold) upregulated in the 2RT-H group, no OPN-positive cells were noted in the retina. OPN mRNA expression was unaltered in the 2RT-L group.

**DISCUSSION**

The traditional laser-based method for treating visual impairment caused by DME is focal/grid laser photocoagulation. The mechanism(s) by which this technique stabilizes vision has, however, not been definitively elucidated. In the current study, we have employed a combination of qPCR, immunohistochemistry, and Western blotting to investigate early glial cell and inflammatory effects resulting from treatment of normal pigmented rats with a standard CW photocoagulation laser set at a clinically relevant energy level. In addition, we have performed parallel analyses on rats treated with a novel 3-ns pulse laser, the 2RT laser, which has recently been shown to approximate the clinical efficacy of conventional photocoagulation in a randomized, noninferiority, clinical trial. The CW laser caused RPE cell ablation in the irradiated zone, histological damage to the ONL, and cellular responses that were consistent with an inflammatory response to thermal injury. Cellular responses that were detected included rapid activation and migration of microglia together with expression of OPN.

**FIGURE 7.** Representative images of heat shock protein Hsp32 immunolabeling following treatment with CW or 2RT laser, used at 2RT-H and 2RT-L energy settings. At 3 days after laser treatment, Hsp32 immunoreactivity in the CW retina is associated with Müller cell processes (black arrows), together with infiltrating macrophages and proliferating/dividing RPE cells (white arrows). Similar but markedly weaker responses are evident in the 2RT-H and 2RT-L retinas. Scale bars: 30 μm.

**FIGURE 8.** Representative images of early microglial activation following treatment with CW or 2RT laser, used at 2RT-H and 2RT-L energy settings, as shown by immunolabeling for iba1 in transverse sections and wholemounts. In the normal retina (A, D, E), iba1 labels a population of quiescent, ramified microglia located in the inner retina. At 6 hours after CW treatment, there is evidence, firstly, of a migration of iba1-positive microglia from the inner (B) to the outer (C, F) retina at the lesion (demarcated by asterisk), and secondly, of a change in morphological appearance to one characteristic of activation (arrows). Similar but considerably less marked responses are apparent in the 2RT-H (G, arrow) and 2RT-L (H, arrow) retinas. Scale bars: 30 μm. PS, photoreceptor segments.
of proinflammatory cytokines, induction of leukocyte adhesion molecules, infiltration of macrophages and neutrophils, activation, dedifferentiation, proliferation, and migration of macroglia, accompanied by synthesis of trophic factors. Upregulation of inducible HSPs was observed in Müller cells, astrocytes, and RPE cells. In contrast, the lower energy level of the 2RT laser caused focal RPE cell ablation in the irradiated zone without accompanying retinal histological damage. As expected, proinflammatory and glial cell responses were substantially attenuated relative to the CW laser. The systematic character-
The impact of the CW laser on the RPE cell layer and on the different classes of retinal neurons is considered in detail in the accompanying article. In brief, the results show that 1 day after treatment, the CW laser caused ablation of RPE cells and the appearance of numerous pyknotic nuclei in the ONL, which by 3 to 7 days was manifest as an almost complete loss of nuclei within the irradiated zone. These findings are in good agreement with previous histological studies. The best-characterized response of the retina to laser photocoagulation is upregulation of the intermediate filament GFAP. Induction of GFAP by Müller cells and enhanced expression by astrocytes occurs pursuant to any pathological situation and is considered the signature event in reactive gliosis. Our results, which correspond to the findings of these earlier studies, show a dramatic upregulation of GFAP within irradiated zones, notably the presence of a GFAP scar, alongside a retina-wide induction by Müller cells. Induction of another intermediate filament, nestin, by Müller cells has also previously been reported following laser photocoagulation. Nestin is expressed in neural precursor and proliferating cells during development but is largely absent from the mature healthy CNS. Nevertheless, following various types of injury, glia have been shown to re-express nestin, an event considered to reflect dedifferentiation of these cells. Our results substantiate and extend earlier findings. In confirmation of previous work, we noted a pattern of nestin immunolabeling by Müller cells, which paralleled that of GFAP within irradiated zones. Through the use of retinal whole-

**Figure 10.** Effect of CW and 2RT laser, used at 2RT-H and 2RT-L energy settings on expression of mRNAs encoding leukocyte adhesion molecules and MMP-9 and on infiltration of leukocytes. (A–C) Quantification of ICAM-1, P-selectin, and MMP-9 mRNA levels at 6 hours, 1 day, and 3 days after laser treatment. Values (represented as mean ± SEM) are normalized for housekeeping genes and expressed relative to the control group. **P < 0.01, by Pair-wise Fixed Reallocation Randomization Test (treated versus control). (D–F) Representative images of neutrophil infiltration (arrows) in CW and 2RT-H retina at 1 day after laser treatment, as shown by immunohistochemistry for myeloperoxidase. Neutrophils are frequently observed in the retina in the vicinity of CW lesions, but not after 2RT-H lasering. (E) Representative image of T cell infiltration (arrow) in CW retina at 1 day after laser treatment, as shown by immunohistochemistry for CD3. Scale bar: 30 μm.
mounts, we were additionally able to demonstrate that nestin expression was predominantly restricted to laser-irradiated zones rather than expressed throughout the retina as was GFAP. This was the case even when laser spots were placed close together. Finally, we showed that nestin was induced de novo in dividing RPE cells at the lesion. The conclusion that Müller cells within the irradiated zone are dedifferentiating is supported by their positive labeling for PCNA, a marker of proliferation, 3 days after laser treatment. At this time point, PCNA-positive Müller cells were evident not only in the INL, but also within the demuded ONL, denoting migration toward the site of injury. The overall results suggest that the retina has instigated an endogenous response in an attempt to produce (rudimentary) new photoreceptors by dedifferentiation of Müller cells. Similar responses have been shown in other models of photoreceptor injury.

Following acute injury, the retina activates endogenous protective mechanisms in an attempt to both limit neuronal loss and increase resistance to subsequent potential stress. This phenomenon encompasses multiple pathways and is the rationale behind the concept of preconditioning therapy. We therefore investigated whether CW laser induces expression of key mediators that have been shown to be involved in preconditioning (e.g., the trophic factors, FGF-2 and CNTF, and the small heat shock proteins, aB-crystallin, Hsp27, and Hsp32), which also function as molecular chaperones. Of these molecules, only FGF-2 has previously been studied with regard to retinal laser injury, but a lack of consistency in the published literature invited further study. Our results corresponded with the unambiguous findings of Xiao et al. who showed FGF-2 presence within Müller cells in normal retinas, and de novo expression by photoreceptors flanking lesions.

**Figure 11.** Representative images of microglial activation and macrophage infiltration at later time points following treatment with CW or 2RT laser, used at 2RT-H and 2RT-L energy settings. (A-C) Immunolabeling for iba1 in transverse sections of the CW retina at 1 day, 3 days, and 7 days. At 1 day, microglia migrated to the lesion, but macrophages were absent. By 3 days, iba1-positive macrophages were abundant within the lesion (arrow), which persisted to at least 7 days. (D-F) Comparison of iba1 immunolabeling in CW-, 2RT-H-, and 2RT-L-treated wholemounts at 3 days after laser treatment. Iba1-positive macrophages are numerous at the lesion in the CW retina, but not in the 2RT-H or 2RT-L retinas. (G-I) Immunolabeling for the phagocytic marker ED1 at 3 days after CW, 2RT-H, and 2RT-L laser treatment at 1 day, 3 days, and 7 days. ED1-positive cells are distributed throughout the CW retina, are present in low numbers in the outer retina after 2RT-H treatment (H, black arrow), and are restricted to the RPE cell layer in the 2RT-L retina (I, black arrow). (J-L) MHC II expression at 3 days after CW, 2RT-H, and 2RT-L laser treatment. MHC II-positive cells are distributed throughout the CW retina (J, black arrow), but are largely absent after 2RT-H (K, black arrow) or -L laser treatment. Scale bars: 30 μm.
following laser treatment. Regarding CNTF, we noted a clear upregulation of the neurotrophin by Müller cells and astrocytes, but not photoreceptors, in irradiated areas, a cell-type response analogous to that observed after excito-retinal injury. Laser treatment also caused an upregulation of all three inducible small HSPs (Hsp27, Hsp32, and Hsp34) by glial cells, with each molecule displaying a distinct pattern of expression. αB-crystallin was detectable in astrocytes and Müller cells, Hsp32 by Müller cells and infiltrating macrophages, and Hsp27 solely by astrocytes. αB-crystallin and Hsp34 appeared to be restricted to irradiated zones, but Hsp27 was upregulated more widely. Interestingly, induction of HSPs was also evident in RPE cells adjacent to, and subsequently migrating into, the lesioned area. Astrocytes and Müller cells are in intimate contact with, and contribute to the proper functioning of, the inner blood-retinal barrier, while RPE cells are responsible for the outer blood-retinal barrier. Since small HSPs play important roles in stabilizing the cytoskeleton under conditions of physiological stress, in preventing denaturation of proteins and in facilitating refolding of abnormal proteins, it is conceivable that their induction after laser treatment plays a role in improved vascular integrity.

Selected reports have documented microglia/immune responses to scatter and focal laser photoacoagulation, but the high density of burns administered in the former studies and the excessive radiant exposures used in the latter studies, potentially render the data of limited relevance to DME treatment. In spite of differences in experimental regimes, however, common responses are evident when comparing the results of these studies with our own findings. These include rapid morphological transition/migration of microglia into the lesion site, together with expression of immunological markers, upregulation of leukocyte adhesion molecules, and infiltration of macrophages and leukocytes. It is likely these events are caused predominantly by injury to photoreceptors rather than to RPE cells, since vastly diminished outcomes were observed with the 2RT laser, which exclusively targets RPE cells (see below). The responses described above are characteristic of neuroinflammation, which is triggered primarily by microglia (the archetypal innate immune cell of the CNS), mediated via release of proinflammatory cytokines, and typically results in exacerbation of primary neuronal damage.

To shed more light on the inflammatory response to CW laser, we performed qPCR and immunohistochemistry for proinflammatory cytokines, with particular attention paid to IL-1β, “the master regulator of neuroinflammation.” The results showed a striking upregulation of IL-1β, as well as TNFα and a cellular pattern of IL-1β expression that was exclusive to microglia. It is well-documented that TNFα and IL-1β can stimulate trafficking of neutrophils/monocytes to sites of injury via upregulation of leukocyte adhesion molecules, chemokines, and MMP-9 on vascular endothelia. Thus, our observations of these latter-named events after CW laser treatment are entirely consistent. By 3 days after laser expression of proinflammatory cytokines had returned to baseline, while phagocytic microglia/macrophages were abundant in the subretinal space and denuded ONL. Interestingly, expression of osteopontin, a secreted glycoprotein protein with pleiotropic properties, was maximal at this time point. The function of osteopontin after laser treatment is unknown, but may involve promotion of photoreceptor survival and remodeling of the extracellular matrix.

2RT Laser

It is increasingly recognized that the beneficial effects of laser treatment for DME are elicited through mechanisms of action that may be unrelated to damage inflicted to the neuroretina, instead being mediated through targeting of the RPE cell layer. This viewpoint originated with the adoption of the modified ETDRS protocol and has been advanced by the evolution of shorter pulse duration lasers (for example, micropulse lasers with low duty cycles and, by inference, a greater resting period between pulses), either of conventional wavelength (514–532 nm) or longer wavelength (810 nm), that largely confine energy absorption to the RPE. Micropulse lasers are proving successful in clinical trials for DME. Here we investigated early glial cell and inflammatory effects to 2RT laser using two energy settings: the first energy setting (high; 2RT-H) was suprathreshold; the second energy setting (low; 2RT-L) was subthreshold, approximating that used in the clinical trial. The Dark-Agouti rats used in the present study featured uniform pigmentation, hence titration of the “high” and “low” energy setting between individuals was not necessary. In the clinic, variations in transparency and pigmentation between patients necessitate range finding, whereupon the “high” setting is first established, then the energy titrated down for treatment purposes.

2RT-H. At suprathreshold energy levels, the 2RT laser will cause collateral damage via expanding and collapsing vapor bubbles that affect neighboring photoreceptors. In contrast, longer pulse duration lasers (≥50 ms) produce collateral damage via thermal coagulation. Comparison of the 2RT-H and CW results offers a perspective on the effects of thermal versus mechanical damage on glial/inflammatory events in the retina. Our results show that 2RT-H retinas suffered some loss of photoreceptors, but damage was minor compared with CW animals. The moderate level of injury was reflected in a reduced inflammatory/glial profile. Thus, a statistically significant upregulation of mRNAs encoding proinflammatory mediators and leukocyte adhesion molecules was observed, but the responses were seemingly inadequate robust to precipitate neutrophil/monocyte infiltration. Müller cell and astrocyte gliosis was demonstrated together with induction of survival pathways, but again the responses were less dynamic than in CW retinas. The results show that mechanical damage elicits identical cellular responses to thermal damage, but owing to the differential in photoreceptor injury between the 2RT-H and CW lasers, it is unclear whether thermal damage per se induces a more efficacious inflammatory/glial profile than mechanical damage.

2RT-L. Histologically, 2RT-L retinas showed ablation of RPE cells in the irradiated zone without accompanying neuroretinal damage. This lack of collateral injury likely accounts for the very muted inflammatory/immune responses that were measured after 2RT-L treatment. For example, induction of IL-1β mRNA was 7-fold and P-selectin mRNA 9-fold less pronounced than after CW treatment, while MMP-9 mRNA was not significantly elevated above controls. No evidence was found of neutrophil, T cell, or macrophage infiltration in 2RT retinas or for expression of MHC class II by microglia. Nevertheless, a limited presence of ED1-positive microglia was observed in the subretinal space, presumably to phagocytose RPE cell debris. Interestingly, despite the specificity of the 2RT-L setting for the RPE cell layer, Müller cells and astrocytes displayed activated phenotypes, characterized by upregulated intermediate filaments, trophic factors, and small HSPs, albeit these responses were less striking than in the other treatment groups. The significance of this result with respect to DME is unknown at
present, but it is conceivable that activated macroglia play a role in improving vascular integrity. In conjunction with RPE cell ablation, 2RT-L stimulated a “wound healing” response in neighboring RPE cells, characterized by dedifferentiation, proliferation, migration, and upregulation of small HSPs. It is this response that many researchers believe is beneficial in DME.14,65 and this response that is in all probability common to the 2RT laser and to subthreshold micropulse laser treatment. Indeed, the micropulse laser is used clinically at energy settings designed to produce a biological response in targeted RPE cells without killing the cells, owing to the narrow range of energies at which RPE ablation can occur without accompanying retinal damage.10,66 The 2RT laser, in contrast, has a relatively wide range of energies over which RPE treatment can be performed without collateral damage occurring.16 It is presently unknown whether lethal or sublethal injury to RPE cells gives an optimal healing response. Further clinical trials are needed to address this issue.

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