Platelet-Derived Growth Factor Promotes Repair of Chronically Demyelinated White Matter

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Abstract
In multiple sclerosis, remyelination becomes limited after repeated or prolonged episodes of demyelination. To test the effect of platelet-derived growth factor-A (PDGF-A) in recovery from chronic demyelination we induced corpus callosum demyelination using cuprizone treatment in hPDGF-A transgenic (tg) mice with the human PDGF-A gene under control of an astrocyte-specific promoter. After chronic demyelination and removal of cuprizone from the diet, remyelination and oligodendrogiole density improved significantly in hPDGF-A tg mice compared with wild-type mice. In hPDGF-A tg mice, oligodendrocyte progenitor density and proliferation values were increased in the corpus callosum during acute demyelination but not during chronic demyelination or the subsequent recovery period, compared with hPDGF-A tg mice without cuprizone or to treatment-matched wild-type mice. Proliferation within the subventricular zone and subcallosal zone was elevated throughout cuprizone treatment but was not different between hPDGF-A tg and wild-type mice. Importantly, hPDGF-A tg mice had reduced apoptosis in the corpus callosum during the recovery period after chronic demyelination. Therefore, PDGF-A may support oligodendrocyte generation and survival to promote remyelination of chronic lesions. Furthermore, preventing oligodendrocyte apoptosis may be important not only during active demyelination but also for supporting the generation of new oligodendrocytes to remyelinate chronic lesions.

Key Words: Apoptosis, Cuprizone, Demyelinating disease, Oligodendrocyte, Platelet-derived growth factor (PDGF), Remyelination, Subventricular zone.

INTRODUCTION
CNS demyelination results from multiple sclerosis (MS), toxic insults, leukoencephalopathies, vascular lesions, and traumatic injury. Loss of myelin impairs action potential conduction and increases the vulnerability of axons to atrophy and transection. In MS, the most prevalent demyelinating disease, remyelination becomes limited with repeated or chronic episodes of demyelination (1). Factors leading to the eventual inability to remyelinate chronic MS lesions are not well understood.

The pathology of MS lesions is heterogeneous, and the effect of MS on the oligodendrocyte lineage population varies dramatically (2, 3). Oligodendrocyte progenitor (OP) cells and premyelinating oligodendrocytes can persist in MS lesions yet fail to efficiently remyelinate denuded axons (4–7). With prolonged disease duration, depletion of the OP and premyelinating oligodendrocyte populations may limit the capacity for remyelination of chronic lesions (4, 5, 8, 9). OP cells in MS lesions and in experimental demyelination have been identified by expression of NG2 proteoglycan and platelet-derived growth factor-alpha receptor (PDGFαR) (7, 10–12). PDGF-A ligand activation of PDGFαR signaling can stimulate OP proliferation in response to acute experimental demyelination (13–15), but this signaling pathway has not yet been tested in the context of chronic demyelination.

In the current study we examine the effect of PDGF-A transgene expression on the potential of endogenous OP cells to generate remyelinating oligodendrocytes in chronically demyelinated lesions. Cuprizone ingestion in mice is used to induce chronic demyelination of the corpus callosum with limited remyelination, even after removal of cuprizone from the diet (16, 17). After cuprizone-induced chronic demyelination, axons remain viable and can be remyelinated by transplanted OP cells (9). We analyzed this chronic lesion model using hPDGF-A transgenic (tg) mice with the human PDGF-A gene under control of the astrocytic glial fibrillary acidic protein (GFAP) promoter (18). Because reactive astrocytes are a major source of endogenous PDGF-A in demyelinated lesions (10), regulated overexpression of PDGF-A was exhibited in reactive astrocytes of hPDGF-A tg mice. Mice were examined throughout acute and chronic cuprizone demyelination followed by a recovery period with consumption of a normal diet. Surprisingly, we show that apoptosis continues in chronic lesion areas during the recovery period with normal chow. This cell death may contribute to the limited capacity of OP cells to generate remyelinating oligodendrocytes in a chronic lesion environment. In hPDGF-A tg mice, apoptosis was reduced.
during the recovery period after chronic demyelination and the extent of remyelination was increased relative to that in wild-type mice. Therefore, a significant effect of PDGF-A in vivo may be as a survival factor in the context of generating oligodendrocytes in chronic lesions. Furthermore, preventing apoptosis may be important not only during active demyelination but also during the subsequent recovery phase to enhance remyelination of chronic lesions.

**MATERIALS AND METHODS**

**Mice**

Mice were bred and maintained in the Uniformed Services University of the Health Sciences animal housing facility in accordance with guidelines of the National Institutes of Health and the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee. The hPDGF-A tg line of mice has been characterized previously (14, 18, 19). Homozygous hPDGF-A tg mice, which do not have any overt CNS abnormalities. The hPDGF-A gene encodes a 318-base pair “short” diffusible alternative-splice isoform of the human PDGF-A gene (20), along with a myc epitope tag fused to the carboxy terminus, under the control of the mouse GFAP promoter (21). Expression of the hPDGF-A transgene construct does not appear to cause a compensatory response in endogenous mouse PDGF-A gene expression (19). Secretion of the hPDGF-A transgene product has been indicated in previous studies comparing this transgene construct driven from a neuronal promoter (18). With neuronal expression of the hPDGF-A transgene, OP numbers were increased near neuron cell bodies, where PDGF-A could be secreted, but not along axons that do not have a mechanism for PDGF-A secretion (18). Furthermore, compared with this hPDGF-A construct, expression of a transgene encoding a variant of hPDGF-A that is retained in the endoplasmic reticulum did not increase the OP number (19). Expression of the hPDGF-A transgene from the GFAP promoter in astrocytes has been demonstrated in hemizygous hPDGF-A tg mice in developing and in adult CNS, with increased expression in reactive astrocytes during acute (6 weeks) cuprizone-induced demyelination (14, 18).

**Cuprizone Experimental Demyelination**

Cuprizone ingestion in mice results in a highly reproducible model of corpus callosum demyelination (22, 23). Acute cuprizone administration (6 weeks) is followed by spontaneous remyelination during subsequent weeks of normal chow, whereas after chronic cuprizone administration (12 weeks) remyelination is limited (9, 17, 24). Male mice at 8 weeks of age were started on a 0.2% (w/w) cuprizone diet [finely powdered oxalic bis(cyclohexylidenedihydrazide); Sigma-Aldrich, Milwaukee, WI] mixed into milled chow (Certified LM-485 code 7012CM; Harlan Teklad, Madison, WI), which was available ad libitum. Mice were killed after 3, 6, 9, or 12 weeks of consumption of the cuprizone diet to examine the acute and chronic phases of disease progression. To examine recovery from chronic demyelination, additional mice were fed cuprizone for 12 weeks and then allowed a subsequent 6 weeks of consumption of a normal chow diet (17).

**Tissue Preparation**

Mice were intracardially perfused with 4% paraformaldehyde (Sigma, St. Louis, MO), and brains were dissected before post fixation in 4% paraformaldehyde at 4°C overnight (10). Brain tissue was then cryoprotected in 30% sucrose (Sigma) at 4°C overnight, embedded in Tissue Tek OCT (Sakura, Torrance, CA), and stored at −80°C. Coronal sections were cut at 15 μm on a cryostat (Bright Instruments, Cambridge, England) and thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) for in situ hybridization and immunohistochemistry.

**Immunohistochemistry**

Myelin was immunostained with 8-18C5 mouse monoclonal antibody, which recognizes myelin oligodendrocyte glycoprotein (MOG) (hybridoma cells provided by Dr. Minetta Gardinier; University of Iowa, Iowa City, IA [25]). The hPDGF-A transgene fusion protein was detected by immunostaining for the myc epitope tag with a rabbit polyclonal antibody (MBL International, Woburn, MA). MOG and myc immunolabeling were detected, respectively, with donkey anti-mouse or anti-rabbit IgG F(ab′)2 conjugated with Cy3 (Jackson ImmunoResearch, West Grove, PA).

**In Situ Hybridization**

In situ hybridization was performed with methods detailed previously (23, 26) using riboprobes for proteolipid protein (PLP) (10), PDGRαR (10), and hPDGF-A (27). Digoxigenin-labeled riboprobes were hybridized to coronal brain sections, and digoxigenin was detected with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche, Indianapolis, IN), followed by reaction with NBT/BCIP substrate (DAKO, Carpinteria, CA).

**Cell Proliferation**

Cell proliferation was estimated by incorporation of bromodeoxyuridine (BrdU). At 4 and 2 hours before perfusion, mice were injected intraperitoneally with 200 mg/kg BrdU (Sigma) (10). After in situ hybridization detection for PDGFαR mRNA (see above), sections were processed for immunostaining with a monoclonal anti-BrdU antibody directly conjugated with horseradish peroxidase (mouse monoclonal IgG Fab fragment; Roche). Peroxidase activity was detected by incubation with 3,3′-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA). Cell proliferation was also evaluated by immunostaining for Ki67 antigen, which is expressed in the nuclei of actively dividing cells but absent at G0 (28). Ki67 was recognized with a rat...
anti-mouse monoclonal antibody (DAKO) followed by detection with the ABC elite kit using DAB as a substrate.

**Apoptosis**

Apoptosis was assessed with a modified terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (ApopTag in situ apoptosis detection kit; Intergen, Purchase, NY) performed in hPDGF-A tg and wild-type mice generated from the hPDGF-A heterozygous mice as well as in male C57BL/6 mice purchased from The Jackson Laboratory (Bar Harbor, MA). The 3'-OH DNA ends, generated by DNA fragmentation typically observed in cells undergoing apoptosis, were labeled with digoxigenin-dUTP using terminal deoxynucleotidyl transferase. The digoxigenin tag was then detected with an anti-digoxigenin antibody conjugated with peroxidase or fluorescein. Peroxidase was detected with DAB substrate to yield a dark brown reaction product, and nuclei were counterstained with methyl green (Vector Laboratories). In situ hybridization for PDGF<sup>R</sup> (see above) was performed before use of ApopTag with DAB. Immunostaining for Olig2 (1:200; Chemicon International, Temecula, CA) was used to identify cells at stages throughout the oligodendrocyte lineage (29) in combination with ApopTag using fluorescein detection. The combined protocols were not appropriate for quantitative analysis because of either fewer detectable TUNEL-labeled nuclei with the in situ protocol or autofluorescence causing potential false-positive results in the tissues from chronic but not acute cuprizone treatment.

**Imaging, Quantification, and Statistical Analysis**

All in situ hybridization and immunohistochemistry images were acquired with Spot2 software using a digital camera combined with an IX-70 inverted microscope (Olympus, Melville, NY). Images were prepared as panels using Adobe Photoshop (Adobe Systems, Mountain View, CA). Quantitative analysis was performed on the white matter of the body of the corpus callosum area between the midline and extending to below the apex of the cingulum on each side. Quantitative analysis was also performed in proliferative zones that can continue to generate oligodendrocytes in the adult brain, the subventricular zone (SVZ) (extending dorsolaterally from the lateral ventricle and along the lateral wall of the ventricle) and the subcallosal zone (SCZ) (extension of the germinal zone rostral to the lateral ventricle located between the corpus callosum and hippocampus).

For comparing cell densities, cells expressing PLP mRNA were quantified using unbiased stereologic morphometric analysis (Stereologer System; Systems Planning and Analysis, Inc., Alexandria, VA) (17, 23). Positively labeled cells were identified with cytoplasmic signal delineating the nucleus. Using the Stereologer System, the thickness was sampled as part of the definition of each “dissector” volume so that density measurements reflect cells/mm<sup>3</sup>. Unbiased

**FIGURE 1.** Detection of human platelet-derived growth factor-A (hPDGF-A) transgene expression in cuprizone-demyelinated corpus callosum. (A–F) In situ hybridization to detect hPDGF-A transgene mRNA transcripts in hPDGF-A transgenic (tg) mice (A–E) and wild-type mice (F). (E) Quantification of cells expressing hPDGF-A transgene mRNA transcripts in corpus callosum sections of hPDGF-A tg mice ± cuprizone (cup) for the time period indicated. (G–K) Immunofluorescence detection of the myc epitope tag of hPDGF-A transgene fusion protein in wild-type (G) and hPDGF-A tg mice (H–K). All panels are coronal brain sections aligned as in F with the corpus callosum midline along the left border (indicated by double-headed arrow) and showing the corpus callosum (cc) laterally to below the cingulum (cg). Mice were fed normal chow (A, H) or chow with 0.2% cuprizone with analysis after acute treatment (B, F, G, I; 6 wk cup), chronic treatment (C, J; 12 wk cup), or chronic treatment followed by a 6-week recovery period with consumption of normal chow (D, K; 12 wk cup 6 off). Scale bar (A–F as shown in D; G–K as shown in K) = 250 μm.
stereology cannot be used appropriately for conditions with relatively low cell densities. Therefore, quantification of cells expressing PDGFαR mRNA, BrdU, ApopTag, and/or Ki67 required counting of all labeled cells and use of the Spot2 camera and software to measure the area sampled (17, 23). Without use of the Stereologer System, section thickness could not be sampled in the mounted section, and the density of units is reported as cells/mm².

Quantification of corpus callosum myelination was estimated from MOG immunofluorescence, detected with a narrow band pass filter for Cy3 (Chroma Technologies, Brattleboro, VT). With the use of Metamorph software...
hPDGF-A Transgene Is Expressed in Acute and Chronic Cuprizone Lesions

Chronic cuprizone demyelination of the corpus callosum is followed by limited remyelination and serves as a reproducible model to test the ability of PDGF-A overexpression to promote remyelination in vivo. To determine whether the GFAP promoter maintained hPDGF-A transgene expression in chronically demyelinated lesions, we used in situ hybridization to detect hPDGF-A mRNA transcripts and immunostaining for the myc epitope tag of the transgene fusion protein (Fig. 1). In hPDGF-A tg mice, the transgene was most strongly expressed in the corpus callosum during the acute phase of cuprizone treatment (i.e. 6 weeks [Fig 1B, I]; see also 4 and 6 weeks after cuprizone in Reference 14). After 12 weeks of cuprizone, mRNA transcripts (Fig. 1C) for the transgene were readily detectable in corpus callosum lesions and the fusion protein was detected more diffusely (Fig. 1J), as expected for a secreted ligand (see Materials and Methods section). Both immunostaining and in situ hybridization indicated reduced transgene expression after removal of cuprizone from the diet to allow recovery after chronic demyelination (Fig. 1D, K). In hPDGF-A tg mice, hPDGF-A transgene expression is detected in 3.7 times more cells at 6 weeks of cuprizone with elevated levels continuing during the chronic stage as 2.0 times at 12 weeks of cuprizone and 1.7 times still present after 6 weeks of recovery. This analysis reveals the temporal and spatial patterns of the hPDGF-A transgene mRNA and fusion protein expression throughout the disease course for correlation with the genotype differences observed in each of the cellular responses examined (see below). At each time point, wild-type mice bred from heterozygote crosses were examined as controls, and a lack of signal above background levels demonstrated specificity of the hPDGF-A in situ hybridization (Fig. 1F) and the myc immunostaining (Fig. 1G).

After Chronic Demyelination, Remyelination Is Enhanced in hPDGF-A tg Mice Compared With Wild-Type Mice

Overexpression of PDGF-A in hPDGF-A tg mice did not alter remyelination after acute demyelination (14). However, robust remyelination already occurs in acute

FIGURE 2. After chronic demyelination, spontaneous remyelination is increased in human platelet-derived growth factor-A (hPDGF-A) transgenic (tg) mice compared with wild-type mice. (A, B) Quantification of corpus callosum myelination estimated by immunofluorescence for myelin oligodendrocyte glycoprotein (MOG) in wild-type mice (A) and hPDGF-A tg mice (B). Black bars indicate perfusion after 3, 6, 9, or 12 weeks of continuous 0.2% cuprizone (cup) feeding, or 12 weeks of cuprizone followed by 6 weeks of consumption of normal chow. White bars indicate no cuprizone treatment. Pixel intensity values were normalized between tissue sections by thresholding to exclude values below the level of immunoreactivity in the dorsal fornix, which is not demyelinated by cuprizone. In mice of both genotypes, significant demyelination occurs with cuprizone treatment (*, p < 0.05 compared with no cup; 1-way analysis of variance [ANOVA] within each genotype). The hPDGF-A tg mice show significant remyelination of the corpus callosum during the recovery period with consumption of normal chow relative to the 12-week cuprizone chronic treatment (#, p < 0.05 for hPDGF-A tg 12 wk cup 6 off compared with hPDGF-A tg 12 wk cup 1; 1-way ANOVA within genotype). Further, myelination in the hPDGF-A tg mice is significantly increased from the wild-type mice only during the recovery after chronic cuprizone treatment (+, p < 0.05 for hPDGF-A tg 12 wk cup 6 off compared with hPDGF-A wild-type 12 wk cup 6 off; 2-way ANOVA for genotype and treatment). With this pattern during the recovery period, the values for hPDGF-A tg mice have returned to within normal levels (p > 0.05 for 12 wk cup 6 off compared with no cup; 1-way ANOVA within genotype), whereas wild-type values remain significantly below normal levels (p < 0.001 for 12 wk cup 6 off compared with no cup; 1-way ANOVA within genotype). All statistical analyses are based on a sample size of 3 to 5 mice per condition. (C–H) Representative images of MOG immunostaining of coronal sections through the corpus callosum (areas shown as noted in Fig. 1; cc, corpus callosum; df, dorsal fornix; cg, cingulum, double-headed arrow along cc midline). (C, F) Wild-type and hPDGF-A tg mice, respectively, maintained on normal chow, without cuprizone treatment. (D, G) Wild-type and hPDGF-A tg mice, respectively, fed cuprizone continuously for 12 weeks. (E, H) Wild-type and hPDGF-A tg (H) mice, respectively, fed cuprizone continuously for 12 weeks, followed by 6 weeks of recovery. Scale bar (C–H shown in H) = 250 μm.
demyelination models, including cuprizone treatment (17, 24). Therefore, we tested the capacity of PDGF-A overexpression to positively influence the limited remyelination that follows chronic cuprizone demyelination in the corpus callosum (Fig. 2). Mice of \textit{hPDGF-A} \textit{tg} and wild-type genotypes were killed at intervals throughout a period of continuous cuprizone ingestion for up to 12 weeks followed by a return to normal chow for a 6-week recovery period. During the cuprizone treatment period, mice of both genotypes exhibited a similar disease progression with a similar extent of demyelination at 12 weeks of cuprizone ingestion (Fig. 2A, B, D, G). During the recovery period with normal chow for 6 weeks, corpus callosum myelination in the \textit{hPDGF-A} \textit{tg} mice improved significantly compared with the 12-week cuprizone values (Fig. 2B, H). In contrast, extensive demyelination persisted in wild-type mice during the recovery period (Fig. 2A, E), as observed in C57BL/6 mice (17).

\textbf{hPDGF-A tg Mice Have Increased OP Proliferation in the Corpus Callosum During Acute Demyelination but Not During Chronic Demyelination}

PDGF-A signaling can regulate proliferation and amplification of OP cells in response to acute demyelination (14, 15). The OP response in \textit{hPDGF-A} \textit{tg} and wild-type mice was examined in acute and chronic cuprizone models to determine whether PDGF-A overexpression might improve remyelination in \textit{hPDGF-A} \textit{tg} mice by increasing

\textbf{FIGURE 3.} Human platelet-derived growth factor-A (hPDGF-A) transgenic (tg) mice have increased oligodendrocyte progenitor (OP) amplification in the corpus callosum only during the acute stage of cuprizone (cup) demyelination. (A–D) Quantification of the OP response in the corpus callosum of wild-type (A, C) and \textit{hPDGF-A} \textit{tg} (B, D) mice. Black bars indicate perfusion after 3, 6, 9, or 12 weeks of continuous 0.2% cuprizone feeding or 12 weeks of cuprizone followed by 6 weeks of normal chow. White bars indicate no cuprizone treatment. (A, B) Density of the total OP population, identified by PDGF$\alpha$R mRNA expression. (C, D) Density of proliferating OP cells identified by PDGF$\alpha$R mRNA and incorporation of BrdU during a 4-hour terminal pulse. Before the start of cuprizone treatment, \textit{hPDGF-A} mice of both genotypes have similar densities of total OP cells (A, B) and similar extents of proliferation among the OP pool (C, D). After 6 weeks of cuprizone treatment in \textit{hPDGF-A} \textit{tg} mice, significant increases are observed in the total OP cell density (B, *p < 0.001) and the extent of OP proliferation (D, *p < 0.001) compared with \textit{hPDGF-A} \textit{tg} mice without cuprizone treatment. All statistical analyses are based on a sample size of 3 to 5 mice per condition. (E–J) Representative coronal sections showing PDGF$\alpha$R mRNA in situ hybridization (blue/black cytoplasm) and BrdU incorporation (brown nuclei) in the corpus callosum (E: cc, corpus callosum; df, dorsal fornix, arrow along cc midline). (E, F) Wild-type and \textit{hPDGF-A} \textit{tg} mice, respectively, maintained on normal chow without cuprizone treatment. (G, H) Wild-type and \textit{hPDGF-A} \textit{tg} mice, respectively, fed cuprizone continuously for 6 weeks. (I, J) Wild-type and \textit{hPDGF-A} \textit{tg} mice, respectively, fed cuprizone continuously for 12 weeks followed by a 6-week recovery period of normal chow. Scale bar (E–J shown in H) = 250 \textmu m.
FIGURE 4. Subventricular zone (SVZ) activation continues throughout acute and chronic demyelination. (A–C) Representative coronal sections through the corpus callosum (cc, double arrow along midline) and SVZ showing platelet-derived growth factor-α receptor (PDGFRα) mRNA in situ hybridization (blue/black cytoplasm) to identify oligodendrocyte progenitor (OP) cells and bromodeoxyuridine (BrdU) incorporation (brown nuclei) during a 4-hour terminal pulse. (A) Within the SVZ, BrdU labeling is evident in nontreated adult (8 weeks of age) human platelet-derived growth factor-A (hPDGF-A) transgenic (tg) (shown) and wild-type mice (not shown). (B) After 5 weeks of cuprizone (cup), hPDGF-A tg mice demonstrate robust proliferation and OP amplification in the lesioned area of the corpus callosum relative to the extent of response in the SVZ. (C) After cuprizone for 12 weeks with a 6-week recovery period, proliferation is marked within the SVZ relative to the attenuated response in the corpus callosum in both wild-type (shown) and hPDGF-A tg mice (not shown). Scale bars (D–I) = 200 μm. Quantification within germinal zones of overall proliferative index (BrdU labeling in D, G), total OP density (E, H), and density of proliferating OP cells (F, I) in wild-type (D–F) and hPDGF-A tg (G–I) mice. White bars indicate no cuprizone treatment. Gray bars indicate acute cuprizone treatment (3 or 6 weeks of cuprizone or 6 weeks of cuprizone with 3 weeks for recovery). Black bars indicate 12 weeks of continuous cuprizone feeding (12 weeks of cuprizone or 12 weeks of cuprizone with 3 or 6 weeks for recovery). *, p < 0.05 for 1-way analysis of variance within each genotype.
the pool of OP cells available for generating oligodendrocytes after chronic demyelination (Fig. 3). Before the start of cuprizone treatment, wild-type and \( hPDGF-A \) tg mice had a similar density of OP cells, which were identified by PDGFRα mRNA transcripts. Also, among nontreated mice, mice of both genotypes exhibited a similar level of OP proliferation, as estimated by BrdU incorporation among PDGFRα+ cells (Fig. 3A–F). In response to acute (6-week) cuprizone demyelination, the densities of total OP cells (Fig. 3A, B) and proliferating OP cells (Fig. 3C, D) were significantly increased in \( hPDGF-A \) tg mice, which was not observed in wild-type mice (Fig. 3A–D, G, H). Immunostaining for Ki67 antigen also confirmed increased proliferation in corpus callosum lesions of \( hPDGF-A \) tg mice compared with wild-type mice during acute demyelination after 6 weeks of cuprizone (40.8 ± 9.6 cells/mm² for wild-type mice, \( n = 5 \); 114.1 ± 29.0 cells/mm² for \( hPDGF-A \) tg mice, \( n = 4 \); \( p = 0.0332 \)). This increased OP density and proliferation in the \( hPDGF-A \) tg mice indicates a functional effect of the transgene expression during the acute cuprizone demyelination, consistent with a previous report (14). Interestingly, this effect of PDGF-A overexpression on OP proliferation during the acute disease phase does not continue in the chronic phase. During chronic demyelination (12 weeks of cuprizone) and recovery (12 weeks of cuprizone with 6 weeks of recovery), the OP population...
and proliferative capacity are similar to nontreated values and are not different between mice of hPDGF-A \(^\text{tg}\) and wild-type genotypes (Fig. 3A–D, I, J).

**SVZ and SCZ Activation Continues Throughout Acute and Chronic Demyelination**

The secreted hPDGF-A fusion protein from reactive astrocytes in corpus callosum lesions could influence cells within the SVZ and SCZ proliferative areas from which OP cells can migrate into the corpus callosum and potentially contribute to the pool of newly generated remyelinating oligodendrocytes. Activation of these germinal zones was examined using BrdU incorporation to estimate the proliferative population and in situ hybridization for PDGF\(\alpha R\) mRNA transcripts to identify OP cells (Fig. 4). An increased density of BrdU-labeled cells was observed throughout the cuprizone treatment period in both hPDGF-A \(^\text{tg}\) and wild-type mice (Fig. 4D, G). This proliferative response during the acute stages (3 and 6 weeks of cuprizone treatment) is similar to the SVZ response observed in other models of acute demyelination of the corpus callosum (30). The current findings with 12 weeks of cuprizone treatment demonstrate that activation of the SVZ continues with chronic demyelination of the corpus callosum.

Within these proliferative areas, the PDGF\(\alpha R\)+ OP population was examined relative to BrdU incorporation during the 4-hour terminal pulse (Fig. 4E, F, H, I). Surprisingly, the OP densities were not different during cuprizone treatment compared with those for nontreated mice. Yet, during recovery from the 6 weeks of acute cuprizone treatment, the OP response was significantly increased. This OP response during the recovery period was not observed after a chronic (12-week) course of cuprizone treatment, indicating a potential depletion of this proliferative capacity.

Importantly, this analysis of proliferative zones associated with the corpus callosum does not reveal any differences between mice of each genotype that could account for the enhanced remyelination after chronic cuprizone treatment in the hPDGF-A \(^\text{tg}\) mice compared with the wild-type mice.

**Oligodendrocyte Repopulation of Chronic Lesions Is Enhanced in hPDGF-A \(^\text{tg}\) Mice**

PDGF\(\alpha R\) expression is associated mainly with the OP stage of the oligodendrocyte lineage, but newly generated oligodendrocytes maintain specific responses to PDGF (31, 32). Therefore, oligodendrocyte repopulation of chronic lesions was quantified to further examine the cellular responses associated with improved remyelination in hPDGF-A \(^\text{tg}\) mice (Fig. 5). An effect of the hPDGF-A genotype was evident among nontreated adult control mice. The density of oligodendrocytes identified by PLP mRNA in situ hybridization was significantly higher in hPDGF-A \(^\text{tg}\) mice than in wild-type mice at 8 weeks of age before cuprizone treatment was begun (Fig. 5A–C, F). Cuprizone administration resulted in significant loss of oligodendrocytes in the corpus callosum at each time point examined, indicating that the disease severity was not different in hPDGF-A \(^\text{tg}\) versus wild-type mice (Fig. 5A, B, D, G). In mice of both genotypes, oligodendrocyte densities increased significantly from the 12 week chronic disease level during the 6-week recovery period. However, the hPDGF-A \(^\text{tg}\) mice exhibited significantly greater improvement of oligodendrocyte densities during the recovery period compared with the wild-type mice (Fig. 5A, B, E, H).

**During Recovery From Chronic Demyelination, Apoptosis Is Reduced in hPDGF-A \(^\text{tg}\) Mice**

Differences in the extent of cell death were examined as potentially contributing to the improved oligodendrocyte repopulation and remyelination that was observed in hPDGF-A \(^\text{tg}\) mice relative to wild-type mice (Fig. 6). Relatively low levels of TUNEL-positive cells were present in 8-week-old mice of either genotype in the absence of cuprizone treatment. With cuprizone treatment, active

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**FIGURE 5.** Oligodendrocyte repopulation of chronic lesions is enhanced in human platelet-derived growth factor-A (hPDGF-A) transgenic (tg) mice. (A, B) Quantification of the density of oligodendrocytes, identified by in situ hybridization for PLP mRNA, in the corpus callosum (cc) of wild-type (A) and hPDGF-A \(^\text{tg}\) (B) mice. Black bars indicate perfusion after 3, 6, 9, or 12 weeks of continuous 0.2% cuprizone (cup) feeding or 12 weeks of cuprizone followed by 6 weeks of normal chow. White bars indicate no cuprizone treatment. At least 3 sections were quantified per mouse from at least 3 mice per condition. Before the start of cuprizone treatment, hPDGF-A \(^\text{tg}\) mice have a higher density of oligodendrocytes than wild-type mice at 8 weeks of age (*, \(p = 0.0009\); Student’s t-test between genotypes of mice without cuprizone treatment). During cuprizone treatment of both wild-type and hPDGF-A \(^\text{tg}\) mice, the density of oligodendrocytes is significantly decreased from normal levels (*, \(p < 0.0001\) compared with no cup; 1-way analysis of variance (ANOVA) within each genotype). Mice of both genotypes show significant oligodendroglial repopulation of the corpus callosum during the recovery period (\#, \(p < 0.05\) for 12 wk cup 6 off compared with 12 wk cup; 1-way ANOVA within genotype). However, the oligodendroglial repopulation in the hPDGF-A \(^\text{tg}\) mice is significantly increased from that in the wild-type mice (+, \(p < 0.05\) for hPDGF-A \(^\text{tg}\) 12 wk cup 6 off compared with wild-type 12 wk cup 6 off; 2-way ANOVA for genotype and treatment). With this pattern during the recovery period, the values for hPDGF-A \(^\text{tg}\) mice have returned to within normal levels (p > 0.05 for 12 wk cup 6 off compared with no cup; 1-way ANOVA within genotype), whereas wild-type values remain significantly below normal levels (*, \(p < 0.05\) for 12 wks cup 6 off compared with no cup; 1-way ANOVA within genotype). All statistical analyses are based on a sample size of 3 to 5 mice per condition. (C–H) Representative coronal sections showing PLP mRNA in situ hybridization in the corpus callosum (C, cc, corpus callosum; df, dorsal fornix, double headed arrow along cc midline). (C, F) Wild-type and hPDGF-A \(^\text{tg}\) mice, respectively, maintained on normal chow, without cuprizone treatment. (D, G) Wild-type and hPDGF-A \(^\text{tg}\) mice, respectively, fed cuprizone continuously for 12 weeks. (E, H) Wild-type and hPDGF-A \(^\text{tg}\) mice, respectively, fed cuprizone continuously for 12 weeks, followed by 6 weeks of recovery. Scale bar (C–H shown in H) = 250 \(\mu\)m.
demyelination (i.e. 2–4 weeks of treatment) is associated with increased TUNEL in mature oligodendrocytes (33). As expected from the similar oligodendrocyte loss in hPDGF-A tg and wild-type mice after 3 weeks of cuprizone ingestion (Fig. 5), the extent of apoptosis was similar between mice of both genotypes at 3 weeks of cuprizone treatment (Fig. 6A, B). After 6 weeks of cuprizone, the hPDGF-A tg mice had a higher density of TUNEL+ cells compared with the wild-type mice (Fig. 6A, B). However, by 12 weeks of cuprizone treatment, the values were not different between mice of each genotype (Fig. 6A, B). Surprisingly, after 12 weeks of cuprizone administration there was continued apoptosis during the 6-week recovery period on a normal chow diet. During this recovery period, significantly fewer apoptotic cells were detected in the corpus callosum of hPDGF-A tg mice relative to wild-type mice (Fig. 6A, B). Apoptotic cells included those within rows of cells aligned longitudinally between axons, a characteristic of interfascicular

**FIGURE 6.** During recovery from chronic demyelination, apoptosis is reduced in human platelet-derived growth factor-A (hPDGF-A) transgenic (tg) mice. (A, B) Quantification of the density of apoptotic cells, identified by a modified terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, in the corpus callosum of wild-type (A) and hPDGF-A tg mice (B). White bars indicate no cuprizone (cup) treatment. Gray bars indicate perfusion after 3 or 6 weeks of cuprizone treatment. Black bars indicate perfusion after 12 weeks of continuous cuprizone feeding or 12 weeks of cuprizone followed by 6 weeks of normal chow. During cuprizone treatment of both wild-type and hPDGF-A tg mice, the density of apoptotic cells is significantly increased from normal levels (*, p < 0.05 compared with no cup; 1-way analysis of variance (ANOVA) within each genotype). With 6 weeks of cuprizone treatment, TUNEL labeling in the hPDGF-A tg mice is significantly increased from that in the wild-type mice (#, p < 0.05 for hPDGF-A tg 6 wk cup compared with wild-type 6 wk cup; 2-way ANOVA for genotype and treatment). Elevated apoptosis values continued after removal of cuprizone from the diet of wild-type mice (+, p < 0.05 compared with no cup). Although apoptosis remains elevated somewhat during recovery in hPDGF-A tg mice, the values are no longer significantly above those in nontreated hPDGF-A tg mice. Further, apoptosis in the hPDGF-A tg mice is significantly decreased from that in the wild-type mice (+, p < 0.05 for hPDGF-A tg 12 wk cup 6 wk off compared with wild-type 12 wk cup 6 wk off; 2-way ANOVA for genotype and treatment). All statistical analyses are based on a sample size of 3 to 5 mice per condition. (C) Representative coronal section showing the corpus callosum (cc, double headed arrow along midline; cg, cingulum) in a wild-type mouse treated with cuprizone for 12 weeks followed by a 6-week period for recovery. TUNEL (brown) signal is present in individual cells distributed throughout the corpus callosum and in groups of cells aligned as is characteristic of interfascicular oligodendrocytes (single arrows and inset). Nuclei stained with methyl green. Scale bars = 250 and 10 μm. (D–F) Immunostaining for Olig2 (D, and red in F) to identify oligodendrocyte lineage cells in combination with TUNEL (E, and green in F) within the corpus callosum in a wild-type mouse treated with cuprizone for 12 weeks followed by a 6-week period for recovery. Scale bar (D–F) shown in F = 10 μm. (G–H) Representative coronal sections showing PDGFrα mRNA in situ hybridization (blue/black cytoplasm) and TUNEL labeling (brown nuclei) in the corpus callosum (G: cc, corpus callosum, double arrow along cc midline). (G) After 6 weeks of cuprizone treatment, hPDGF-A tg mice show OP amplification (as in Fig. 3). TUNEL labeling is high but does not colocalize with PDGFrα expression, as expected for apoptosis of oligodendrocytes from ongoing cuprizone treatment. Scale bar = 200 μm. (H, I) During recovery after 12 weeks of cuprizone treatment, TUNEL and PDGFrα mRNA transcripts can be detected separately (H; wild-type mouse) but colocalization was also observed (I; wild-type mouse). Scale bars = (H, I) 10 μm.
oligodendrocytes (Fig. 6C). Some TUNEL+ cells could be identified with Olig2 immunostaining (Fig. 6D–F) to detect immature through mature stages of the oligodendrocyte lineage. In addition, some TUNEL+ cells expressed PDGFRα (Fig. 6I), indicating an OP phenotype and potential regulation by PDGF-A ligand levels.

To test whether the continued level of apoptosis in wild-type mice of the hPDGF-A genetic background may have been an effect of this C57BL6/cba hybrid background, we performed TUNEL analysis in C57BL/6 mice from The Jackson Laboratory as this is the most commonly used strain for cuprizone treatment. A relatively high level of TUNEL+ cells was observed in the C57BL/6 mice during the recovery phase after the 12-week chronic cuprizone treatment (Fig. 7). Therefore, continued apoptosis during recovery from chronic demyelination is not a genetic background effect in the wild-type mice and may contribute to limited repair of chronic lesions. Furthermore, PDGF-A may promote cell survival during recovery from chronic demyelination, which correlates well with a previously demonstrated role for PDGF-A in preventing oligodendrocyte apoptosis during myelination in development (31).

**DISCUSSION**

Remyelination of MS lesions becomes limited after repeated or prolonged episodes of demyelination. Factors influencing this progression to a chronic disease state are important to identify because denuded axons have impaired neurotrophic support and increased vulnerability to damage. Studies using cuprizone to induce chronic demyelination have demonstrated limited remyelination that is associated with depletion of OP cells and inhibition of OP differentiation in lesions (9, 17). Our current results show that apoptosis continues after removal of cuprizone from the diet and is an additional factor contributing to the limited remyelination observed after chronic demyelination. Furthermore, PDGF-A transgene expression reduces apoptosis after chronic demyelination, increases repopulation of the lesion areas with oligodendrocytes, and significantly promotes remyelination of corpus callosum lesions.

The chronic cuprizone model of demyelination is advantageous for evaluating potential improvements in conditions of poor remyelination. Acute demyelination models with robust OP amplification and complete remyelination are well suited for examining how successful remyelination can be accomplished. However, strategies to improve remyelination can be difficult to test in the context of extensive spontaneous remyelination. Indeed, analysis of hPDGF-A tg mice using acute toxin-induced demyelination did not detect an effect on remyelination (14). The chronic cuprizone model in C57BL/6 mice results in apoptosis of newly generated oligodendrocytes upon maturation, which leads to chronic demyelination with eventual depletion of the OP population and limited remyelination even after removal of cuprizone from the diet (17, 24). In the current study, this chronic disease scenario revealed significant improvement in the extent of remyelination in hPDGF-A tg mice (Fig. 2).

Expression of the hPDGF-A transgene under control of the astrocyte GFAP promoter is especially appropriate for analyses in the context of demyelination. Endogenous PDGF-A is synthesized in reactive astrocytes associated with demyelinated lesions (10, 13, 34). In hPDGF-A tg mice, the GFAP promoter upregulates hPDGF-A expression in correlation with an astroglial response to demyelination, as observed using in situ hybridization for hPDGF-A mRNA transcripts (Fig. 1) (14) or the myc tag to localize the transgene product in lesion areas (Fig. 1). Overexpression of PDGF in the adult CNS can induce abnormal hyperplasias and tumor formation in immature cells of the corpus callosum or the SVZ (35, 36). However, regulated expression of the hPDGF-A transgene from the GFAP promoter in heterozygous mice did not result in hyperplasias or tumors in the current study of mice up to 26 weeks of age examined using BrdU incorporation (Fig. 3) or immunostaining for Ki67 antigen (see Results). Driving PDGF-A overexpression in lesions via astrocytes may also facilitate signaling with appropriate extracellular matrix molecule interactions, such as...
as tenasin-C modulation of PDGF-A induced OP proliferation and survival (37).

We initially predicted that PDGF-A overexpression in hPDGF-A tg mice would improve remyelination by acting as an OP mitogen to counter depletion of OP cells during chronic demyelination. Indeed, OP density increased dramatically during acute demyelination in hPDGF-A tg mice (Fig. 3), consistent with a previous report (14). This OP response in hPDGF-A tg mice is even more notable given the poor OP response in the wild-type littermates (Fig. 3), which indicates that this genetic background may be less favorable for OP proliferation than other lines we have examined (17). Surprisingly, the increased OP response to acute demyelination in hPDGF-A tg mice did not result in subsequent elevation of OP cell density or proliferation during the chronic disease stage. OP densities were similar after chronic cuprizone demyelination in hPDGF-A tg and wild-type (Fig. 3), and these values were similar to those measured after chronic cuprizone treatment in C57BL/6 mice (17). We also observed that proliferation within the SVZ/SCZ germinal zones adjacent to the corpus callosum was elevated throughout the cuprizone treatment period (Fig. 4). However, a genotype effect was not evident in these germinal zones. Thus, improved remyelination after chronic demyelination in hPDGF-A tg mice cannot be directly attributed to stimulation of proliferation of PDGFR expressing OP cells. Furthermore, at the end of the 12-week chronic cuprizone treatment period, hPDGF-A tg and wild-type mice had equivalent densities of OP cells, detected by PDGFRα, and oligodendrocytes, detected by PLP. This finding led us to focus further on specific effects occurring during the recovery phase. However, it is possible that a population of OP cells may be generated during the acute phase in response to PDGF-A yet may not be detected by our PDGFRα or PLP in situ hybridization at later disease stages.

PDGF-A can also act as a survival factor during oligodendrocyte development (31, 38, 39). Relatively low concentrations of PDGF-A can promote survival as developing OP cells exit the cell cycle and generate new oligodendrocytes before downregulation of PDGFRα expression (31, 32). During remyelination, newly generated oligodendrocytes may be particularly vulnerable before they establish effective interactions with axons that can provide survival signals for myelinating oligodendrocytes (40, 41). An effect of PDGF-A in promoting survival of newly generated oligodendrocytes (31) is consistent with our results in hPDGF-A tg mice during the chronic stages of cuprizone demyelination and recovery. OP cells continue to generate new oligodendrocytes during the course of chronic cuprizone treatment (17, 24). Upon maturation, the newly generated cells become susceptible to cuprizone toxicity and undergo apoptosis. After cuprizone is removed from the diet, we show that apoptosis continues in the chronic lesion environment (Fig. 6). During this recovery period, the reduced level of apoptosis in hPDGF-A tg mice may allow survival of a greater number of newly generated oligodendrocytes (Fig. 5) and result in improved remyelination (Fig. 2) compared with wild-type mice. The potential signaling pathway for PDGF-A to promote survival in this context is not yet clear but could possibly occur directly through PDGFRα activation in oligodendrocyte lineage cells or might potentially involve indirect effects of PDGFRα activation of other cell types in the lesion environment. However, the PDGF-A ligand should act through the PDGFRα on OP cells and would not be expected to activate the PDGFRα that is expressed by other CNS cell types.

Our finding of continued apoptosis during the recovery period after chronic demyelination (Figs. 6, 7) has important implications for promoting remyelination in demyelinating diseases. Multiple mechanisms of cell death are associated with active demyelination, such as immune and inflammatory responses and glutamate excitotoxicity (42, 43). In addition to these pathogenic mechanisms, surviving and newly generated oligodendrocytes may die during attempts to repopulate and remyelinate chronic lesions. After acute cuprizone demyelination, apoptosis is extremely rare several weeks after mice are returned to a normal diet (9, 23) (Figs. 6, 7). In contrast, after chronic cuprizone demyelination we found that apoptosis continues after removal of cuprizone from the diet. Further studies to determine whether levels of potentially detrimental factors, such as cytokines, may be active in the chronic lesion environment (44, 45) would be of interest. An alternative possibility is that the environment of chronically demyelinated lesions may not provide adequate support for survival of oligodendrocytes and so may contribute to the limited capacity for remyelination of chronic lesions. PDGF-A transgene expression appears to improve cell survival during the period for recovery after chronic demyelination. An effect of PDGF-A on survival of newly generated oligodendrocytes rather than mature cells is indicated by the lack of protection of mature myelinating oligodendrocytes during acute cuprizone toxicity in hPDGF-A tg mice (3 weeks cuprizone: Fig. 5). Our results in hPDGF-A tg mice clearly differ from effects attributed to insulin-like growth factor 1 or leukemia inhibitory factor, which each promote survival of mature myelinating oligodendrocytes during active demyelination induced by acute cuprizone ingestion (33, 46).

A remarkable capacity of endogenous OP cells to remyelinate areas of chronic demyelination can be recognized from analyses of mouse models that may attenuate detrimental characteristics of the chronic lesion environment. In addition to the current findings of improved remyelination with decreased apoptosis in hPDGF-A tg mice, a similar study demonstrated almost complete remyelination after chronic cuprizone demyelination in fibroblast growth factor 2 (FGF2) null mice (17). FGF2 is upregulated in demyelinated lesions and can inhibit OP differentiation into oligodendrocytes (15, 17, 23). After chronic demyelination, the depleted OP population more effectively generated remyelinating oligodendrocytes in the absence of FGF2 (17). Thus, the ability of endogenous cells to remyelinate chronic lesions may be controlled not only by the number of available OP cells but also by the permissiveness of the environment for those OP cells to differentiate and survive to efficiently generate remyelinating oligodendrocytes. In light of the improved remyelination observed by
promoting differentiation in FGF2 null mice, it is possible that the improved remyelination from hPDGF-A transgene expression could also involve effects on OP differentiation. However, reduced expression of PDGFRα in acute cuprizone demyelination did not alter OP differentiation during remyelination (15).

Immature oligodendrocyte lineage cells can persist in MS lesions (6, 7, 12, 47). However, MS lesions may have increased expression of signals that inhibit differentiation (48, 49) or variable expression of molecules that support newly generated oligodendrocytes to survive and myelinate viable axons (50, 51). Such conditions may have detrimental effects on endogenous cells and may also compromise the potential of transplanted cells to remyelinate throughout large areas of demyelination (52). Therefore, treatments for chronic demyelinating diseases may require attenuation of active demyelination as well as alteration of the lesion environment to optimize remyelination.

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