Metachromatic leukodystrophy and transplantation: remyelination, no cross-correction

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Abstract

Objective: In metachromatic leukodystrophy, a lysosomal storage disorder due to decreased arylsulfatase A activity, hematopoietic stem cell transplantation may stop brain demyelination and allow remyelination, thereby halting white matter degeneration. This is the first study to define the effects and therapeutic mechanisms of hematopoietic stem cell transplantation on brain tissue of transplanted metachromatic leukodystrophy patients.

Methods: Autopsy brain tissue was obtained from eight (two transplanted and six nontransplanted) metachromatic leukodystrophy patients, and two age-matched controls. We examined the presence of donor cells by immunohistochemistry and microscopy. In addition, we assessed myelin content, oligodendrocyte numbers, and macrophage phenotypes. An unpaired t-test, linear regression or the nonparametric Mann–Whitney U-test was performed to evaluate differences between the transplanted, nontransplanted, and control group.

Results: In brain tissue of transplanted patients, we found metabolically competent donor macrophages expressing arylsulfatase A distributed throughout the entire white matter. Compared to nontransplanted patients, these macrophages preferentially expressed markers of alternatively activated, anti-inflammatory cells that may support oligodendrocyte survival and differentiation. Additionally, transplanted patients showed higher numbers of oligodendrocytes and evidence for remyelination. Contrary to the current hypothesis on therapeutic mechanism of hematopoietic cell transplantation in metachromatic leukodystrophy, we detected no enzymatic cross-correction to resident astrocytes and oligodendrocytes.

Interpretation: In conclusion, donor macrophages are able to digest accumulated sulfatides and may play a neuroprotective role for resident oligodendrocytes, thereby enabling remyelination, albeit without evidence of cross-correction of oligo- and astroglia. These results emphasize the importance of immunomodulation in addition to the metabolic correction, which might be exploited for improved outcomes.
Introduction

Metachromatic leukodystrophy (MLD, MIM 250100) is caused by biallelic mutations in ARSA, leading to deficient activity of arylsulfatase A (ASA), a lysosomal enzyme digesting sulfatides.1 Sulfatides are enriched in myelin sheaths; their accumulation in MLD causes demyelination and inhibits oligodendrocyte differentiation from precursor cells, thereby preventing remyelination.7 Depending on age at onset, MLD is divided into late-infantile (onset before 30 months), juvenile (onset between 30 months and 16 years), and adult (onset after 16 years) forms. If diagnosed early, later-onset MLD forms (juvenile and adult) can be treated by allogenic hematopoietic stem cell transplantation (HSCT).3,4 HSCT may halt further demyelination, and in some transplanted patients brain white matter abnormalities even improve on MRI.5,6

Hematopoietic stem cell transplantation is believed to provide donor myeloid cells that migrate into the brain and engraf as macrophages secreting ASA, thereby restoring sulfatide degradation and cross-correcting ASA-deficient resident cells.7 Ex vivo gene therapy also relies on the migration of gene-corrected myeloid cells into the brain where these cells produce ASA in a supraphysiological amount supposed to cross-correct other cell types.8,9 However, the exact mechanism of HSCT remains elusive.8

We therefore investigated brain tissue of two transplanted4 and six nontransplanted MLD patients, obtained at autopsy, and assessed the presence of donor cells, ASA, sulfatide storage, and digestion and macrophage phenotypes. We also explored myelin content, oligodendrocyte numbers and remyelination to better understand the improvement of white matter changes after transplantation.

Methods

The study was approved by the IRB of the VU University Medical Center, with informed parental consent. The study was performed according to the Declaration of Helsinki. We included patients with pathology-proven diagnosis of MLD for whom brain tissue was available. Brain autopsies of patients 1, 2, and 3 and the two controls were performed within 6 h postmortem in our Center; brain tissue of patients 4 to 8 was obtained through the NIH NeuroBioBank (https://neurobiobank.nih.gov). Two age-matched controls (age 4 and 9 years) were affected by midline pontine glioma without supratentorial tumor localization.

Tissue staining

Five-micrometer-thick formalin-fixed paraffin-embedded tissue sections were routinely stained with Hematoxylin & Eosin and Toluidine blue. Immunohistochemistry was performed as described10 using affinity-purified antibodies targeting human ASA (1:100). For affinity purification recombinant human ASA (1 mg) was coupled to agarose beads using the Micro Link Protein Coupling Kit (Thermo Fisher Scientific) following the manufacturers recommendations. Antibodies from a polyclonal rabbit anti-human ASA antiserum were then allowed to bind to the column and were eluted as described.11 Specificity of the affinity-purified rabbit anti-human ASA antibodies was validated in murine endothelial (bEnd.3) cells, Chinese hamster ovary (CHO-K1) cells, and human (HuH7) hepatoma cells (Fig. 1). Antibodies against the microglia/macrophages markers Iba-1 (1:10.000, Wako CTJ0605) and CD45 (1:100, Dako 0701), the myelin marker proteolipid protein (PLP, 1:3000, Biorad MCA839G), the astrocyte marker glial fibrillary acidic protein (GFAP, 1:500, Millipore AB4451), and the anti-inflammatory marker CD163 (1:300, Cell Marque MRQ-26) were also employed. Double fluorescent stain against ASA and OLIG2 was performed using the TSA Plus Fluorescence Kit (Perkin Elmer NEL763E001KT) according to the manufacturer’s specifications.

Five-micrometer-thick frozen tissue sections were stained with Oil red O and with antibodies against the pro-inflammatory markers CD40 (1:500, Dako ab13545) and CD64 (1:250, Abcam ab104273), anti-inflammatory marker mannose receptor (MR, CD206, 1:500, Dako ab125028), and the oligodendrocyte lineage-specific marker Olig2 (1:100, Millipore AB9610) as described.12

Figure 1. Validation of the affinity-purified rabbit anti-human ASA antibody. Each of the murine cell cultures (bEND3) shown in A, B, and C were stained with this antibody (red) and with an antibody against LAMP2 to visualize lysosomes (green). Cells in panel A did not receive any ASA for endocytosis and thus show no red staining, since these cells lack human ASA. Cells in panels B and C were incubated with recombinant human ASA for endocytosis. Cells were either incubated with mannose-6-phosphate (M6P) blocking the endocytosis of recombinant ASA (B) or glucose-6-phosphate (G6P) allowing endocytosis. There is no red staining in cells which were either not incubated with ASA (A) or in which the endocytosis was blocked with M6P (B). These two samples show that when no human ASA is present in these cells no red staining occurs, excluding unspecific cross-reactivity of the antiserum. Only in the presence of G6P, which allows endocytosis of ASA, red staining and lysosomal colocalization with LAMPT2 is seen. (D) and (E) show CHO-K1 cells, which were either mock transfected (D) or transfected with a plasmid expressing human ASA cDNA (E). Cells were stained with the ASA antibody (red) and LAMP2 antibody (green). Only the transfected cells show a red signal. (F) and (G) display the findings in human hepatoma cells HuH7, which were stained with the ASA antibody (red) and with a Ki67 antibody (green) visualizing nuclei. Cells in A had not received recombinant human ASA for endocytosis whereas cells in B had been exposed to ASA.
Frozen tissue was also used for double staining of markers CD40 and MR, respectively. CD40 immunoreactivity was visualized with an Envision + system HRP-labeled antibody and 3,3'-Diaminobenzidine (Dako, K4002, K3467). MR was visualized with liquid permanent red (1:100, Dako, K00640) after secondary incubation.
with biotinylated secondary antibody (1:100, Dako, E0432) followed by streptavidin with an alkaline phosphatase conjugate (1:100, Sigma-Aldrich, 11089161001). Sections were counterstained with hematoxylin. Fluorescence in situ hybridization (FISH) against chromosomes X and Y was performed using a XY CEP probe (Abbott, 05J10-051) and a FISH Accessory Kit (Dako, K5799). Electron microscopy was performed on the white matter of the second frontal gyrus as described. Briefly, tissue was fixed in 2% glutaraldehyde, 4% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4), postfixed in 1% osmium tetroxide, 1% potassium ferrocyanide, dehydrated, and embedded in epoxy resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined.

**Image acquisition and analysis**

Light microscopy pictures were taken with a Leica DM6000B microscope (Leica microsystems). The number of positive pixels was quantified using the color deconvolution plugin for imaging software ImageJ. Threshold settings in ImageJ were done blinded. Total cell numbers were counted with the ImageJ cell counter, also blinded, on 5 images per patient per area at 10× magnification (ASA, PLP), 10 pictures per patient per area at 20× magnification (Olig2, CD40, MR), or 15 pictures per patient per area at 40× magnification (IBA1, CD45). Electron microscopy pictures were taken with a FEI Tecnai 12 electron microscope, at 9300x or 11000x magnification. The G ratio (diameter axon/diameter myelinated fiber) was measured using ImageJ software and compared between transplanted MLD patients (633 axons in total) and the two non-MLD controls (713 axons in total).

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism v7.0a and data displayed as mean ± standard error of the mean. An unpaired t-test, linear regression, or the non-parametric Kruskal–Wallis test with multiple comparisons were performed to evaluate the differences between controls, nontransplanted, and HSCT-treated patients (considered significant when P < 0.05).

**Results**

**Patients**

Patient 1 (male) presented at age 2 years with peripheral demyelinating polyneuropathy and mildly delayed myelination on brain MRI, without signs of demyelination (Fig. 2). Undetectable ASA activity and mutations c.251C>T p.(Pro84Leu) and c.1174C>T p.(Arg392Trp) in ARSA confirmed the diagnosis of late-infantile MLD. Cognitive function was age adequate. Because there was no clear clinical CNS involvement, HSCT from mismatched (five of six matched) unrelated female cord blood donor was performed. Unfortunately, disease progressed albeit rapid successful engraftment and full donor chimerism with ASA activity in the normal range 2 months after HSCT (Fig. 2). He died 1 year after HSCT. Patient 2 (female) was diagnosed at age 7 years with gait abnormalities due to mild spasticity, ataxia, and demyelinating polyneuropathy. MRI showed extensive white matter abnormalities (Fig. 2). Diagnosis of juvenile MLD was confirmed by low ASA activity; ARSA mutation analysis revealed c.836_837delTCinsAA p.(Ile279Lys) and c.1283C>T p.(Pro428Leu). Total IQ was 74. HSCT was performed with a fully matched unrelated male cord blood donor. Despite fast successful engraftment and full donor chimerism with normal ASA activity 2 months after HSCT, the disease rapidly progressed. She died 1 year after diagnosis. HSCT in patients 1 and 2 was performed as earlier described. Patient 3 (female) was 26 months old when diagnosed with late-infantile MLD because of motor regression, confirmed by low ASA activity and mutations c.251C>T p.(Pro84Leu) and
c.293C > T p.(Ser98Phe) in ARSA. MRI showed extensive white matter abnormalities. She continued to deteriorate and died 3 years after diagnosis from disease progression. Patient 4 (NBB5505) died at age 3 years from late-infantile MLD, after having been diagnosed at age 22 months with delayed acquisition of independent walking, low

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ASA activity, and mutations c.847G > T p.(Asp283Tyr) and c.1010A > T p.(Asp337Val) in ARSA. Disease progression characterized by severe feeding difficulties and a respiratory infection led to his death. Patient 5 (NBB3308) died at age 21 years, patient 6 (NBB1144) at age 12 years, patient 7 (NBB5381) at age 7 years, and patient 8 (NBB5509) at age 6 years. No further information was available for these patients.

**Histopathology**

We first questioned whether donor cells were able to reach the brain upon HSCT. In the two transplanted patients, donor macrophages were found throughout the brain white matter, confirmed by FISH studies for X and Y chromosomes, informative because of donor sex mismatch. Donor macrophages seemed less intensely PAS-positive than macrophages in untreated patients and were able to degrade sulfatides, as demonstrated by their being orthochromatic and Oil red O positive (Fig. 2). Still, many macrophages were loaded with sulfatides (metachromatic cells). Immunostaining against ASA confirmed the absence of positive cells in the white matter of untreated MLD patients. By contrast, ASA-positive cells were present in treated subjects with a granular perinuclear pattern, suggesting intralysosomal localization of the enzyme. These cells costained against LN3, confirming they were macrophages. We could not detect ASA immunoreactivity in resident oligodendrocytes or astrocytes. Quantification showed that HSCT only resulted in immunoreactive areas shows that HSCT slightly increases the amounts of enzyme in transplanted patients, but that such increase does not reach the levels found in control tissue. *P < 0.05.

The same double stains in HSCT-treated MLD patients show that there is no cross-correction to astrocytes (G) nor to oligodendrocytes (I), whereas the presence of ASA-positive cells is compatible with macrophages, while in age-matched control tissue (C) staining is present in all cell types. (D) Double stain of control tissue against ASA (green) and GFAP (red) shows the presence of ASA with a granular cytoplasmic distribution in GFAP-positive white matter astrocytes. (E) Double stain of control tissue against ASA (green) and LN3 (red) shows perinuclear presence of ASA in LN3-positive microglia. (F) Double stain of control tissue against ASA (green) and the pan-oligodendrocyte marker olig2 shows perinuclear immunoreactivity for ASA in oligodendrocytes. The same double stains in HSCT-treated MLD patients show that there is no cross-correction to astrocytes (G) nor to oligodendrocytes (I), whereas ASA can be seen with a granular distribution inside the cytoplasm of LN3-positive microglia-macrophages (H). (J) Quantification of ASA-immunoreactive areas shows that HSCT slightly increases the amounts of enzyme in transplanted patients, but that such increase does not reach the levels found in control tissue. *P < 0.05.

**Discussion**

The concept of HSCT in lysosomal storage disease is based on the assumption that the lacking enzyme – in this case ASA – is partially secreted by donor cells, and that this secreted enzyme is efficiently endocytosed by the mannose-6-phosphate receptor of the cells of the recipient. Since mannose-6-phosphate receptor delivers enzyme to lysosomes, endocytosed ASA is localized to the correct cellular compartment. In our immunofluorescent studies in...
Figure 4. Transplantation prevents loss of white matter oligodendrocytes and is associated with remyelination. Stain with Klüver-PAS of whole mount coronal brain slices of an untreated (A, patient 3) and a transplanted patient (B, patient 1) shows a variable degree of myelin loss in the periventricular and deep white matter, with relative sparing of the subcortical white matter and U-fibers. This pattern of distribution is typical of MLD. (C, patient 4) Klüver-PAS stain of an untreated MLD patient shows the centrifugal progression of the demyelinating process, with the periventricular white matter (below the dotted line) containing less myelin than the subcortical white matter (between dotted and solid line). LV, lateral ventricle; Cx, cortex. (D) Quantification of immunoreactivity against the myelin protein proteolipid protein (PLP) confirms that myelin loss is more marked in the cerebellar white matter of untreated compared to transplanted MLD patients, whereas myelin amounts in the subcortical frontal and parietal regions are comparable. The overall degree of PLP immunoreactivity is nonetheless less than in control tissue. (E–G) Stain against the oligodendrocyte lineage-specific marker olig2 shows marked loss of oligodendrocytes in untreated MLD patients (E, patient 6), but preservation of cells in this transplanted patient (F, patient 2). Quantification (G) confirms that oligodendrocyte numbers are higher in the white matter of treated compared to untreated MLD patients. In the parietal lobe of treated patients, oligodendrocyte numbers closely approach what is found in non-MLD controls. (H–J) Electron microscopy shows that compared to non-MLD controls (H) myelin sheaths are thinner in treated MLD patients (I). Measurements of the G ratio confirm that the difference in myelin sheath thickness is significant ($P < 0.0001$). Scale bars: C, E, and F 10 µm; H and I 200 nm. *$P < 0.05$. 

Figure 5. Abundant monocyte-derived cells in the white matter of transplanted MLD patients and predominance of an alternatively activated anti-inflammatory phenotype. (A) Double staining against the markers Iba-1 (brown) and CD45 (red) reveals higher abundance of double-positive monocyte-derived cells in the white matter of transplanted (gray bar) compared to untreated MLD patients (white bar) and non-MLD controls (dark gray bar). (B–E) Stain against CD40, a marker for classically activated pro-inflammatory macrophages, shows more abundance of these cells in the brains of untreated compared to transplanted MLD patients (B), whereas stain against CD163, a marker of alternatively activated anti-inflammatory macrophages, shows the opposite (C). This is also confirmed in double stains (D). Quantification of differentially activated cells demonstrates lower numbers of classically activated cells and greater numbers of alternatively activated and intermediately activated cells in treated compared to untreated MLD patients. ****$P < 0.0001$. Scale bars: A and D 20 µm; B and C 10 µm.
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Transplanted MLD patients, we could clearly detect ASA in a granular lysosomal pattern in monocytes/microglia. However, we were not able to detect ASA in oligodendrocytes neither in astrocytes, suggesting that true cross-correction to these cell types has not occurred. Here it is important to recall that ASA secreted by macrophages and microglia does, in contrast to other cells, not bear mannose-6-phosphate.19 Thus, enzyme secreted by macrophages is not available for mannose-6-phosphate receptor-dependent endocytosis of neighboring macroglial cells. Alternatively, the antibody may not be suitable to detect very low amounts of ASA present in cells after cross-correction. Since the signal in normal individuals was, however, strong, this would at least allow the conclusion that only little amounts of enzyme are present in the cells of the recipient.

Even so, oligodendrocyte precursors and mature myelin-forming oligodendrocytes were present in higher numbers in brains of transplanted than untreated MLD patients. These findings, in combination with ultrastructural evidence of remyelination, suggest that HSCT does support survival, proliferation, and differentiation of oligodendrocytes, which in turn are capable of restoring myelin and provide an explanation for the improvement of MRI white matter changes in successfully treated patients.5,6 This supportive effect on oligodendrocytes could derive indirectly from influences of donor cells on the environment of resident cells. Ongoing myelin loss with local accumulation of myelin debris is a potent inhibitor of oligodendrocyte progenitor maturation,20 and this debris is digested by (metabolically competent) donor macrophages21 after transplantation. In addition, sulfatides in the extracellular matrix are thought to play a role in oligodendrocyte maturation22, and this balance might be restored or at least improved by HSCT.

Another indirect support mechanism for oligodendrocytes may be the higher number of alternatively activated macrophages. In brain white matter, classically activated macrophages are considered to be pro-inflammatory and detrimental to white matter regeneration, whereas alternatively activated cells are thought to be anti-inflammatory cells that support oligodendrocyte survival and remyelination.23 Following demyelination, alternatively activated microglia signal to OPCs, inducing them to proliferate and mature into myelin-forming cells.24 Classically activated macrophages dominate early after myelin loss and promote OPC proliferation. Appearance of alternatively activated cells is then required to induce timely OPC differentiation.25 Dominance of anti-inflammatory macrophages is also observed in active multiple sclerosis lesions undergoing remyelination.23

In both treated and untreated patients, we found that many macrophages expressed an intermediate activation status. Since efficient remyelination necessitates repopulation of pro-inflammatory microglia by pro-regenerative microglia,21 this finding supports that the activation status of microglia/macrophages is dynamic in vivo and occurs as a continuum, with the classically activated pro-inflammatory and alternatively activated anti-inflammatory phenotypes on either end of this spectrum.17,18,25 We finally found that the presence of these intermediately activated cells overexpressing markers of alternative activation was associated with evidence of remyelination in HSCT-treated patients' white matter.

The obvious limitation of our study is that transplantation in our two patients was not sufficient to halt disease progression. Besides, we were not able to prove that the abundant alternatively activated anti-inflammatory ASA-positive cell population present in white matter is exclusively donor derived in our treated MLD patients. Nonetheless, the fact that we did observe donor cells and remyelination even in these unsuccessfully transplanted patients points to the robustness of our data. Another limitation is the age difference in treated and untreated MLD patients in this study; it was not possible to obtain exactly age-matched autopsy material.

Our study provides important insights in the effects and therapeutic mechanism of HSCT in MLD, indicating that, besides myelin restoration, immunomodulation may be necessary to promote white matter recovery. Cross-correction does not play a role for remyelination. These results are valuable especially since therapeutic strategies for MLD (and other lysosomal and peroxisomal disorders) are changing: noncellular options as intrathecal enzyme replacement therapy and substrate inhibitors are being explored,26,27 and gene therapy by autologous genetically manipulated HSCT has been shown to be effective,8,9 although long-term data for the latter are still lacking. In order to improve outcome, future MLD treatment approaches will probably be multimodal.28 They should take proper posttranslational modification of ASA into account to achieve cross-correction of macroglial cells as well. Besides, the additional beneficial effects of immunomodulation by HSCT need to be further exploited, probably not only for MLD, but also for other leukodystrophies treated with HSCT (e.g., Krabbe disease, adrenoleukodystrophy) and acquired white matter disorders such as multiple sclerosis.

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**Conflict of Interest**

The authors declare that they have no competing interests regarding this submission.

**Author Contributions**

NIW and MB conceived and supervised the study, collected and analyzed all data and wrote the manuscript. ASRW, MB, BP, SB, SlDV, and MHPK performed stains, electron microscopy, cell counts, and statistical analysis. VG and UM validated and provided the ASA antibody. DFvR, SB, AV, PvH, MSvDK, CL, and JJB interpreted and collected patient data. All authors critically reviewed the manuscript.

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