Secretion of phosphomannosyl-deficient arylsulphatase A and cathepsin D from isolated human macrophages

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The transfer of macrophage-secreted arylsulphatase A (ASA) to enzyme-deficient brain cells is part of the therapeutic concept of bone marrow transplantation in lysosomal storage diseases. Here we have investigated this transfer *in vitro*. The uptake of ^{125}I-labelled recombinant human ASA purified from ASA-overexpressing mouse embryonic fibroblasts deficient for mannose 6-phosphate (M6P) receptors in a mouse ASA-deficient astroglial cell line was completely inhibited by M6P. In contrast, when ASA-deficient astroglial cells were incubated with secretions of [^{35}S]methionine-labelled human macrophages or mouse microglia, containing various lysosomal enzymes, neither ASA nor cathepsin D (CTSD) were detected in acceptor cells. Co-culturing of metabolically labelled macrophages with ASA-deficient glial cells did not result in an M6P-dependent transfer of ASA or CTSD between these two cell types. In secretions of [^{33}P]phosphate-labelled macrophages no or weakly phosphorylated ASA and CTSD precursor polypeptides were found, whereas both intracellular and secreted ASA from ASA-overexpressing baby hamster kidney cells displayed ^{33}P-labelled M6P residues. Finally, the uptake of CTSD from secretions of [^{35}S]methionine-labelled macrophages in rat hepatocytes was M6P-independent. These data indicated that lysosomal enzymes secreted by human macrophages or a mouse microglial cell line cannot be endocytosed by brain cells due to the failure to equip newly synthesized lysosomal enzymes with the M6P recognition marker efficiently. The data suggest that other mechanisms than the proposed M6P-dependent secretion/recapture of lysosomal enzymes might be responsible for therapeutic effects of bone marrow transplantation in the brain.

Key words: endocytosis, mannose 6-phosphate, metachromatic leucodystrophy, microglia.

INTRODUCTION

Lysosomal enzymes are synthesized in the endoplasmic reticulum as N-glycosylated precursor forms. During the passage to the Golgi the N-linked oligosaccharides of lysosomal enzymes are processed, which includes the formation of mannose 6-phosphate (M6P) residues. In a two step enzymic reaction catalysed by UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (phosphotransferase) and an α-*N*-acetylglucosaminidase (phosphodiesterase), high-mannose-type oligosaccharides on lysosomal enzymes are equipped with M6P residues. Following the binding to M6P-specific receptors (MPRs), the enzyme–receptor complexes are segregated from the secretory pathway and exit the *trans*-Golgi network in clathrin-coated vesicles. Upon fusion with the acidic endosomal/prelysosomal compartment, the dissociation of ligands occurs. The delivery of several enzymes to lysosomes is accompanied by a series of proteolytic cleavages into the mature enzyme form [1]. A variable fraction, depending on the enzyme and the cell type studied, of newly synthesized lysosomal enzyme precursors escapes the binding to MPRs in the Golgi and instead is secreted. These enzymes can, however, be endocytosed by neighbouring cells and be delivered to lysosomes via MPRs localized at the cell surface [2].

Arylsulphatase A (ASA; EC 3.1.6.8) is a lysosomal enzyme involved in the degradation of sulphated glycolipids. It is synthesized as a 62 kDa polypeptide with three high-mannose-type oligosaccharide chains of which two are phosphorylated [3,4].The deficiency of ASA activity in humans leads to metachromatic leucodystrophy (MLD), a progressive demyelinating lysosomal storage disorder. Mutations in the ASA gene result in the intralysosomal accumulation of 3-*O*-sulphogalactosylceramide (sulphatide) preferentially in glial cells. Lipid storage causes cellular dysfunction particularly in oligodendrocytes and results in progressive demyelination. In the most severe forms the disease begins at the age of about 2 years with various neurological symptoms, including seizures, ataxia, blindness and loss of speech, leading to a widespread neurological regression during early infancy [5].

Bone marrow transplantation (BMT) combined with gene therapy is considered to be a potentially useful therapeutic approach for lysosomal storage disorders with central nervous system (CNS) manifestations. It has been shown that bone-marrow-derived monocytes/macrophages can cross the blood–brain barrier and become perivascular macrophages and microglia [6,7]. The secretion of lysosomal enzymes from haematopoietic donor cells and the recapture by surrounding diseased brain cells are proposed to be the corrective mechanism. Thus BMT has yielded improvement of neurological functions and somatic manifestations as well as increased life span, with some variations depending on the disease, the onset of clinical symptoms and the animal model [8–12]. With the exception of cats suffering from α-mannosidosis and of fucosidase-deficient dogs transplanted before the onset of clinical signs, neither reduction of the stored material in the brain nor morphological changes in neurons have been detected [13,14]. BMT has also been performed in patients suffering from various lysosomal storage diseases. Whereas improvements in symptoms of visceral organs are well documented the effects on the CNS, e.g. in children with MLD, are still controversial [15,16].

Abbreviations used: ASA, arylsulphatase A; ASOR, asialo orosomucoid; BHK, baby hamster kidney; BMT, bone marrow transplantation; CNS, central nervous system; CTSD, cathepsin D; DMEM, Dulbecco's modified essential medium; M6P, mannose 6-phosphate; MLD, metachromatic leucodystrophy; MPR, M6P-specific receptor; PMP-BSA, pentamannose 6-phosphate coupled to BSA; RT-PCR, reverse transcriptase PCR.

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In this study, we examined the capability of macrophages and microglial cells to transfer ASA and the lysosomal protease cathepsin D (CTSD) to brain cells in an *in vitro* co-culture system. We show that both cell types secrete lysosomal enzymes incompetent for MPR-dependent uptake by brain cells due to the lack of M6P residues.

EXPERIMENTAL

[^{35}S]Methionine (> 1000 Ci/mmol), Na^{125}I (> 15 Ci/mg of iodide), [^{32}P]P_i and [^{33}P]P_i (3000 and 10 Ci/ml respectively), and Rainbow-coloured protein molecular mass markers were purchased from Amersham Biosciences (Freiburg, Germany). Recombinant human ASA purified from ASA-overexpressing mouse embryonic fibroblasts deficient for MPRs [17], a gift kindly provided by Dr T. Dierks (University of Göttingen, Göttingen, Germany), was iodinated with IODO-GEN (Pierce Chemical Co., Rockford, IL, U.S.A.), yielding a specific activity of 30–70 μCi/μg. Pentamannose 6-phosphate was prepared from *Hansenula holstii* phosphomannan, which was kindly provided by Dr M. Slodki (United States Department of Agriculture, Northern Regional Research Center, Peoria, IL, U.S.A.). Pentamannose 6-phosphate coupled to BSA (PMP-BSA) was prepared and iodinated to a specific activity of 125 μCi/μg as described previously [18]. M6P, mannan fraction II, Protein A–agarose and protease inhibitor cocktail (P-2714) were purchased from Sigma. Orosomucoid was a gift from the American Red Cross Research Laboratory (Bethesda, MD, U.S.A.) and was desialylated as described [19].

Isolation of monocytes and hepatocytes

Monocytes were isolated from human buffy coats by means of diatrizoate (Histopaque-1077; Sigma) followed by centrifugal elutriation (JE6-B rotor; Beckman, Munich, Germany) at a flow rate of 21.5 ml/min [20]. Monocytes were eluted at 2500–1500 rev./min. The cell preparation was characterized by FACScan analysis (Becton Dickinson, Heidelberg, Germany) for the expression of CD14 and CD45. About 60–70 % of the cells were CD14 and CD45 antigen-positive.

The cells were plated at a density of 2×10^6 cells/ml on 35 mm dishes (Primaria Falcon; Becton Dickinson) and cultured in RPMI plating medium containing 10 % fetal calf serum (Gibco BRL, Life Technologies, Karlsruhe, Germany) and penicillin/streptomycin. After 24 h the medium was replaced with Dulbecco's modified essential medium (DMEM) containing 10 % fetal calf serum and penicillin/streptomycin.

Hepatocytes were isolated from rat liver with collagenase by a recirculating *in situ* perfusion technique [21] and cultured as reported previously [22].

Cell lines and medium

Baby hamster kidney (BHK)-ASA cells overexpressing human ASA were described previously [23]. ASA-deficient astroglia cell line 17 −/− A1 was cultured in DMEM containing 10 % fetal calf serum and 4 mM L-glutamine [24]. The murine microglial cell line BV-2 [25] was kindly provided by Dr E. Blasi (University of Perugia, Perugia, Italy). Mixed rat cortex cells were prepared and provided by Dr M. Schweitzer (University of Hamburg, Hamburg, Germany). The cells were used 4–6 days after plating [26]. C6 glial cells (CCL-107) were purchased from the ATCC, and SH-SY5Y human neuroblastoma cells were kindly provided by Dr D. Isbrand (University of Hamburg). Cells were cultured in DMEM containing 10 % fetal calf serum and penicillin/streptomycin in a humidified atmosphere containing 5 % CO_2, at 37 °C.

Antibodies

Polyclonal antibodies against human placenta ASA [27] and human and murine CTSD [28,29] were described previously. Antibodies against CD14 and CD45 were purchased from Becton Dickinson.

Metabolic labelling of cells

For preparation of media used as a source of lysosomal enzymes for endocytosis experiments, cells were labelled with [^{35}S]methionine (50–200 μCi/ml) for 20–24 h at 37 °C. The media were precipitated by $(NH_4)_2SO_4$ (50 %, w/v) and dialysed against DMEM containing 0.1 % BSA (DMEM/BSA). For labelling with [^{32}P]P_i and [^{33}P]P_i, cells were preincubated in phosphate-free minimal essential medium (ICN Biomedicals) containing 0.1 % BSA for 1 h at 37 °C followed by another 4–5 h in the same medium supplemented with ^{32}P (0.3–0.5 mCi/ml) or ^{33}P (0.15 mCi/ml) respectively.

Endocytosis and immunoprecipitation of radiolabelled lysosomal enzymes and M6P-containing ligands

Cells were incubated for various periods with ^{125}I-labelled recombinant ASA, ^{125}I-labelled PMP-BSA (200000–750000 c.p.m.) or secretions of [^{35}S]methionine-labelled cells ($7–14 \times 10^6$ trichloroacetic acid-precipitated c.p.m./plate) in the presence or absence of 10 mM M6P for 1–6 h ([^{125}I]ASA, [^{125}I]PMP-BSA) and 20–24 h ([^{35}S]methionine), respectively. Thereafter the cells were washed at 4 °C and the cell-surface-bound material displaced by M6P. The cells were either solubilized in Laemmli sample buffer and directly analysed by SDS/PAGE and autoradiography ([^{125}I]ASA, [^{125}I]PMP-BSA) or lysed in lysis buffer (PBS containing 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.2 % SDS, 1 % BSA and protease inhibitor cocktail) followed by immunoprecipitation of ASA and cathepsin D as described previously [30,31]. The radiolabelled polypeptides were separated by SDS/PAGE (10 % acrylamide) under reducing conditions, visualized by autoradiography and quantified by densitometric scanning (Hewlett Packard Scan Jet 4c/T). The activity of ASA and β-hexosaminidase was measured photometrically [32]. The cellular protein content was determined by the procedure of Bradford [33].

RNA isolation and reverse transcriptase PCR (RT-PCR)

Total RNA was isolated using the NucleoSpin RNA II purification system (Macherey-Nagel, Düren, Germany). The reverse transcription reaction was performed with an input of 1 μg of total RNA using the GeneAmp first-strand cDNA synthesis kit for RT-PCR (Roche, Mannheim, Germany). Primers used for PCR were synthesized by MWG-Biotech AG (Ebersberg, Germany). The sequences for the forward and reverse primers were 5′-GCAGCGGCAGTGGGACCAGGT-3′ and 5′-TCCACCTCTGCTCTCCCACCA-3′ for the phosphotransferase gene [34] and 5′-ACATCAACGAGAGCCAAGCCA-3′ and 5′-CCACAGTCCAGCTCATCACAG-3′ for the phosphodiesterase gene [35]. PCR involved heating for 10 min at 94 °C and then cycling 30 times through a 40 s denaturation step at 94 °C, a 40 s annealing step at 63 °C and a 40 s extension step at 72 °C in a Mastercycler gradient apparatus (Eppendorf, Hamburg, Germany). PCR products were separated on a 1.5 % agarose gel and visualized by ethidium bromide staining.

Phosphodiesterase activity

The phosphodiesterase-catalysed uncovering of M6P residues was determined as described previously [36] with CTSD secreted from [^{35}S]methionine- and [^{32}P]P_i-labelled microglial cells.

RESULTS

Uptake of [^{125}I]ASA by ASA-deficient astroglial cells

To investigate the capability of the immortalized ASA-deficient murine astroglial cell line 17 −/− to endocytose ASA, cultures were incubated in the presence or absence of 10 mM M6P with the ^{125}I-labelled recombinant human ASA. Densitometric evaluation of autoradiographs revealed that the astroglial cell line 17 −/− endocytosed [^{125}I]ASA in a time-dependent manner which was completely inhibited by M6P (Figure 1). M6P-dependent uptake of [^{125}I]ASA was also observed in the rat glial cell line C6, human neuroblastoma cell line SH-SY5Y and in mixed embryonic rat cortex cells cultured for 6 days (results not shown).

Uptake of ASA and cathepsin D secreted from macrophages by ASA-deficient astroglial cells

Macrophages are believed to serve as bone-marrow-derived donor cells for deficient lysosomal enzymes. To study the amounts and properties of macrophage-secreted lysosomal enzymes, human macrophages, cultured for 20 days, were labelled with [^{35}S]methionine for 24 h followed by immunoprecipitation of ASA and CTSD from cells and media. About 54 % of newly synthesized ASA was immunoprecipitated from the macrophage medium as the 62 kDa form, whereas a doublet of 58 and 54 kDa ASA polypeptides was detectable in the cell extract (Figure 2). As control, ASA was immunoprecipitated from cell extracts and media of [^{35}S]methionine-labelled BHK cells overexpressing human ASA (BHK-ASA). Here about 46 % of newly synthesized ASA was secreted into the medium and no size difference of ASA between cells and media was observed. Sequential immunoprecipitation of the lysosomal protease CTSD from the same cell extracts of macrophages revealed the 53 kDa precursor, the 47 kDa intermediate and the 31 kDa mature form (Figure 2). About 60 % of newly synthesized CTSD was secreted as precursor and mature forms. Next, the murine immortalized ASA-deficient

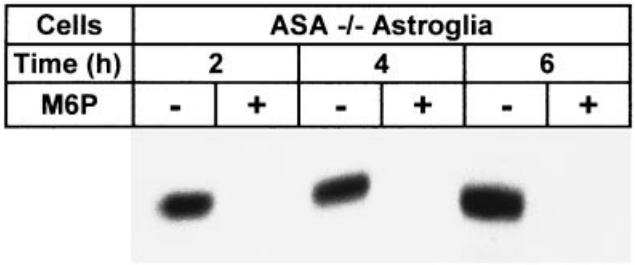


Figure 1 M6P-dependent uptake of ^{125}I-labelled recombinant ASA

Mouse ASA-deficient (ASA −/−) astroglia cell line 17−/− was incubated with [^{125}I]ASA (250000 c.p.m./plate) for 2–6 h in the presence or absence of 10 mM M6P. After removal of the medium and displacement of cell surface bound material by M6P at 4 °C, cells were solubilized. The samples were analysed by SDS/PAGE and the internalized ASA was visualized by autoradiography. Following autoradiography the ^{125}I-labelled bands were excised and counted in a gamma counter. After 2, 4 and 6 h endocytosis, the internalized [^{125}I]ASA corresponded to 401, 733 and 975 c.p.m., respectively. A representative experiment out of five is shown.

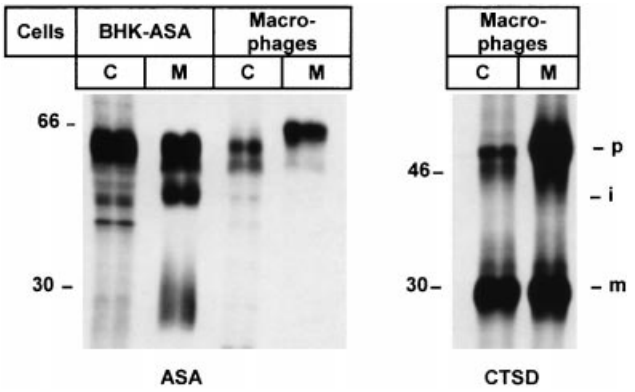


Figure 2 Macrophages secrete lysosomal enzymes

Human macrophages cultured for 20 days were labelled with [^{35}S]methionine for 24 h. ASA and CTSD were analysed in extracts of cells (C) and medium (M) by sequential immunoprecipitation. The precipitates were separated by SDS/PAGE and visualized by fluorography. The position of the mature (m), intermediate (i) and precursor (p) forms of CTSD and of molecular-mass marker proteins (in kDa) are indicated. As a control, ASA was precipitated from [^{35}S]methionine-labelled BHK cells transfected with the human ASA cDNA (BHK-ASA).

astroglial cell line 17 −/− was incubated with media collected from [^{35}S]methionine-labelled macrophages, and BHK-ASA cells in the presence or absence of 10 mM M6P. Subsequently ASA and CTSD were immunoprecipitated from the cell extracts. Endocytosis of ASA by the ASA-deficient astroglia could only be observed when media of BHK-ASA cells were used (Figure 3). This uptake was completely inhibited by M6P. In contrast, when astroglial cell line 17 −/− was exposed to media derived from macrophages, no ASA could be immunoprecipitated from cell extracts (Figure 3). The small quantities of CTSD endocytosed from media of macrophages (about 1.5 % of the CTSD offered

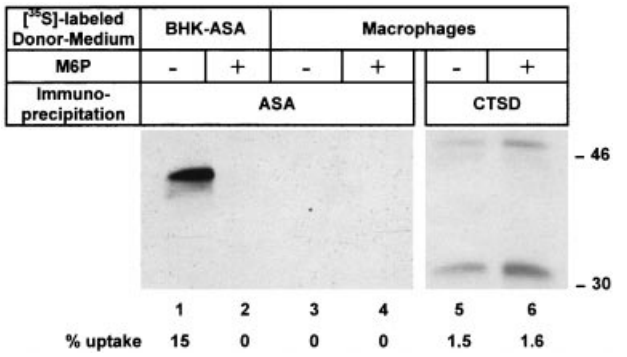
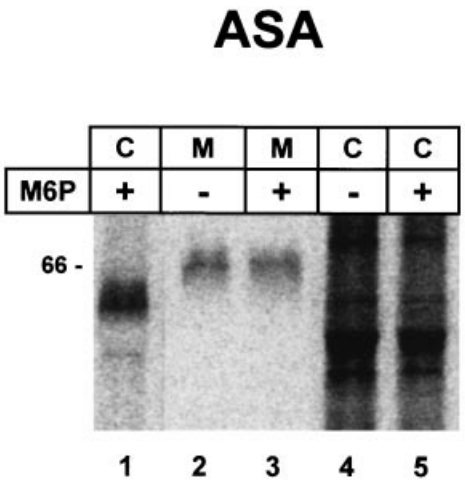


Figure 3 ASA-deficient astroglia cells fail to endocytose macrophage-derived ASA and CTSD

Secretions of [^{35}S]methionine-labelled BHK-ASA cells or macrophages cultured for 20 days were collected, dialysed, adjusted for adequate radioactivity and/or ASA activity and used for endocytosis. The medium used for endocytosis contained (12–24) × 10^6 c.p.m./plate and the ASA activity in the macrophage-derived medium was about one-third of that in [^{35}S]methionine-labelled media of BHK-ASA cells. Prior endocytosis aliquots (1/10) of the ^{35}S-labelled donor medium were processed for immunoprecipitation of ASA and CTSD. ASA-deficient astroglial cells were incubated with ^{35}S-labelled secretions from BHK-ASA cells (lanes 1 and 2) or macrophages (lanes 3–6) in the presence (+) or absence (−) of 10 mM M6P for 24 h. ASA (lanes 1–4) and CTSD (lanes 5 and 6) were immunoprecipitated from cell extracts and visualized by SDS/PAGE and phosphorimaging. The autoradiograph was quantified by densitometric scanning and expressed as a percentage of the total radiolabelled ASA or CTSD in the donor medium precipitated prior to endocytosis. The experiment was repeated four times with similar results.



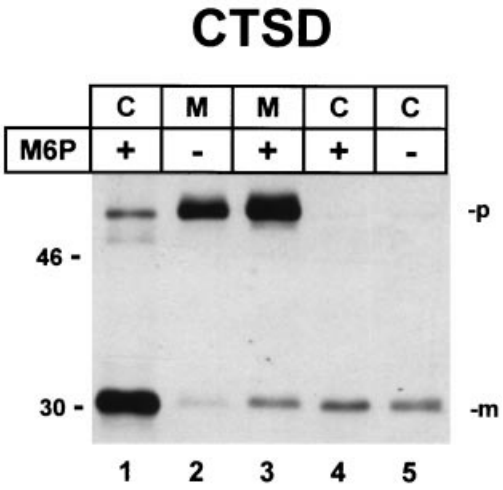


Figure 4 Failure of ASA-deficient astroglial cell line 17 −/− to endocytose macrophage-derived lysosomal enzymes in co-culture

ASA-deficient astroglial cell line 17 −/− grown on polycarbonate filters for 2 days were transferred to a Transwell chamber containing macrophages cultured for 14 days. The co-cultures were further incubated in [^{35}S]methionine-containing medium (0.1 mCi/well) for 24 h in the presence or absence of 10 mM M6P. ASA and CTSD were immunoprecipitated from cell extracts (C) of macrophages (lanes 1), ASA −/− astroglial cell line on filters (lanes 4 and 5), and aliquots (1/3) of the common media (M; lanes 2 and 3). The positions of the precursor (p) and mature (m) CTSD forms are indicated.

for endocytosis) or microglia were presumably due to unspecific fluid pinocytosis, because no inhibition by M6P was observed. When the ASA-deficient astroglial cell line 17 −/− was incubated with increasing amounts of secretions from macrophages the uptake of [^{125}I]ASA was hardly affected (results not shown), indicating the absence of uptake-inhibitory lysosomal enzymes in secretions of macrophages competing with the endocytosis of [^{125}I]ASA.

To examine whether the efficacy of uptake of lysosomal enzymes is affected by the differentiation status of macrophages used as donor cells for the collection of media, human macrophages were cultured for 8, 21 and 28 days, and labelled in parallel with [^{35}S]methionine for 24 h. Subsequently, the dialysed media were adjusted for adequate radioactivity and used for

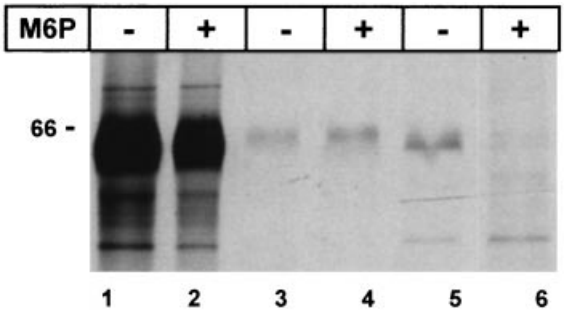


Figure 5 Radiolabelled ASA diffuses efficiently through polycarbonate filters

BHK-ASA cells grown on filters were co-cultured with ASA-deficient astroglial cells grown at the bottom of the wells in [^{35}S]methionine-containing medium in the absence or presence of M6P for 24 h. ASA was immunoprecipitated from BHK-ASA cells (lanes 1 and 2), aliquots (1/10) of the medium (lanes 3 and 4) and ASA-deficient astroglial cell extracts (lanes 5 and 6), separated by SDS/PAGE and visualized by fluorography.

endocytosis in ASA-deficient astroglia cells in the presence or absence of 10 mM M6P. Again, small quantities of CTSD were immunoprecipitated from cell extracts (1–2% of the CTSD offered), which were not affected by the presence of M6P (results not shown).

Co-culture of macrophages and astroglial cell line 17 −/−

In another approach the ASA-deficient astroglial cell line 17 −/− was grown on polycarbonate filters for 2 days prior to co-culture with macrophages. Co-culture was carried out for 24 h in the presence of [^{35}S]methionine followed by immunoprecipitation of ASA or CTSD from the medium and cell extracts of macrophages and the astroglial cell line (Figure 4). Whereas the 58 and 62 kDa radiolabelled ASAs were immunoprecipitated from cellular extracts from macrophages (Figure 4, top panel, lane 1) and from the media of co-cultures (Figure 4, top panel, lanes 2 and 3) respectively, no ^{35}S-labelled human ASA was found in the mouse astroglial cell line 17 −/− (Figure 4, top panel, lanes 4 and 5). The 46 kDa labelled protein band precipitated with the antibody against ASA was unspecific because it was also detectable in astroglial cell-line extracts cultured and labelled in the absence of macrophages (results not shown). Immunoprecipitation of CTSD from ASA-deficient mouse astroglial cells co-cultured with human macrophages revealed small amounts of the 31 kDa mature CTSD form (Figure 4, bottom panel, lane 4). The intensity of the polypeptide band was not affected by M6P present during the incubation (Figure 4, bottom panel, lane 5) suggesting uptake by fluid endocytosis. To exclude the possibility that the failure of enzyme transfer was due to a slow diffusion rate of ASA and/or to a filter barrier, [^{125}I]ASA was added to the upper medium and the appearance of radioactivity in the lower medium was measured over time. The equilibrium of radioactivity between both compartments was reached after 6 h (results not shown). When BHK-ASA cells grown on filters were co-cultured with the ASA-deficient astroglial cell line in [^{35}S]methionine-containing medium for 24 h, ^{35}S-labelled ASA was immunoprecipitated from the medium (Figure 5, lanes 3 and 4) as well as from ASA-deficient cells (Figure 5, lane 5). This uptake of ASA in ASA-deficient cells was completely inhibited by M6P (Figure 5, lane 6). These data confirm that human macrophages secrete ASA and CTSD, which could not be endocytosed by ASA-deficient acceptor cells efficiently.

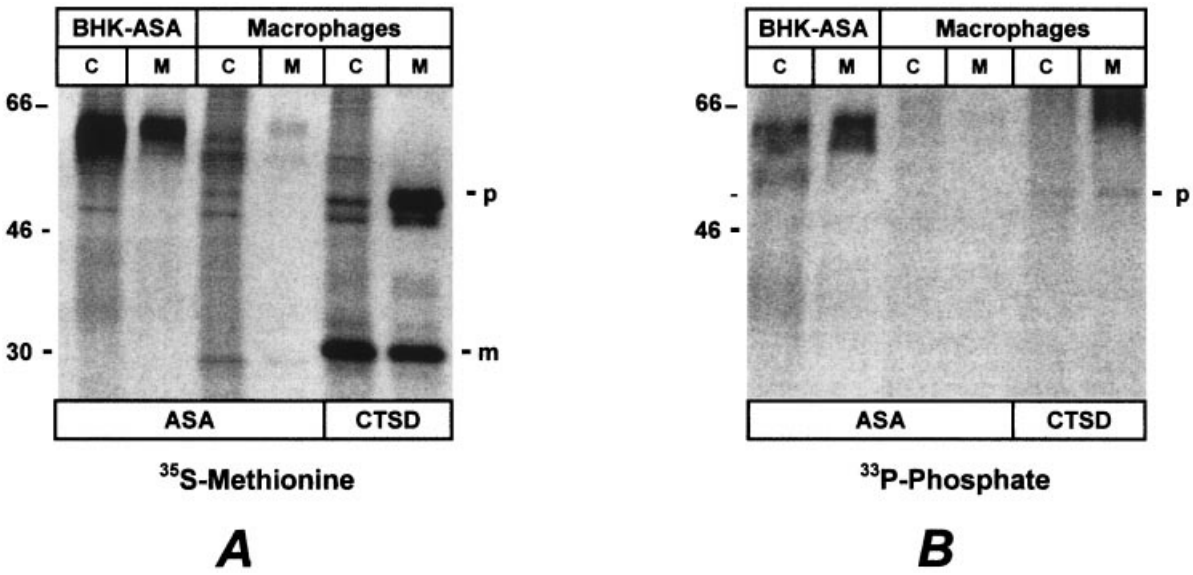


Figure 6 Secretion of non-phosphorylated ASA and CTSD from macrophages

Macrophages cultured for 20 days, and as control BHK-ASA cells were labelled in parallel either with (**A**) [^{35}S]methionine (0.1 mCi/plate) or with (**B**) [^{33}P]P_i (0.25 mCi/plate) for 20 h in the presence of 10 mM M6P followed by immunoprecipitation of ASA and CTSD from cell (C) and media (M) extracts. The precipitates were separated by SDS/PAGE and visualized by fluorography and Phosphorimaging. The experiment was repeated three times with either [^{32}P]P_i or [^{33}P]P_i with identical results. The positions of the CTSD precursor (p) and mature (m) forms are indicated.

Phosphorylation of lysosomal enzymes

To examine whether the failure to take up lysosomal enzymes secreted from human macrophages by the astroglial cell line 17 −/− was due to the lack of the M6P recognition marker, cells were metabolically labelled with [^{32}P]P_i or [^{33}P]P_i (results not shown). No ^{32}P incorporation could be observed in ASA immunoprecipitated from cell and media extracts of macrophages (Figure 6), whereas ASA secreted from BHK-ASA cells was phosphorylated in both the secreted and cellular forms. Weak signals of phosphorylated precursor forms of CTSD were observed in immunoprecipitates from macrophage cell extracts and media. In parallel, ASA and CTSD were immunoprecipitated under identical conditions from cell extracts and media of macrophages labelled with [^{35}S]methionine, indicating that ASA and almost all CTSD are secreted in a non-phosphorylated form. RT-PCR analysis revealed the expression of both phosphotransferase and phosphodiesterase in macrophages (results not shown).

To determine whether the lysosomal enzymes secreted by macrophages can be endocytosed specifically by receptor mechanisms other than via MPR, cultured primary rat hepatocytes were used as acceptor cells expressing various carbohydrate-recognizing receptors [37]. Rat hepatocytes were incubated with ^{35}S-labelled secretions from macrophages cultured for 8 or 21 days, in the presence or absence of various effectors of receptors recognizing M6P residues, mannose (0.1 mg/ml mannan II), galactose [0.1 mg/ml asialo orosomucoid (ASOR)] and fucose (5 mM fucose). The total amount and the processing of internalized CTSD were different when media deriving from ^{35}S-labelled macrophages cultured for 8 or 21 days were used (Figure 7A). None of the effectors alone inhibited the uptake of CTSD in hepatocytes, whereas a combination of mannan and ASOR inhibited the uptake by 38 % (Figure 7A, lane 12). In contrast, the uptake of ^{125}I-labelled recombinant ASA in hepatocytes was strongly inhibited by M6P (75 % inhibition), but not by mannan or fucose, and only marginally (23 %) by ASOR alone. The combination of mannan and ASOR, M6P and ASOR, or mannan, ASOR and fucose inhibited the uptake of ^{125}I-ASA by 23, 82 and 31 %, respectively (Figure 7B). Furthermore, the uptake of the ^{125}I-PMP-BSA was completely inhibited by M6P but not by other effectors of carbohydrate receptors (Figure 7C). These data show that M6P-containing ligands are endocytosed preferentially (ASA) or exclusively (PMP-BSA) via MPR. The failure of M6P to inhibit specifically the uptake of CTSD into hepatocytes confirmed the ^{32}P-incorporation data, demonstrating that lysosomal enzymes secreted by macrophages at different differentiation status lack the M6P recognition marker.

Microglial cells secrete weakly phosphorylated cathepsin D

To examine whether other bone-marrow-derived cells, such as microglia can secrete uptake-competent lysosomal enzymes, the mouse microglial cell line BV-2 was investigated. Immunoprecipitation of CTSD from BV-2 cells and media labelled with [^{35}S]methionine for 24 h revealed the presence of the 53 kDa precursor, the 47 kDa intermediate and the 31 kDa mature form in cell extracts, and the secretion of the 53 kDa precursor form into the medium (Figure 8A). Because no antibodies are available against murine ASA, this enzyme could not be precipitated from the microglial cell line BV-2. When ASA-deficient astroglial cell line 17 −/− was incubated with media collected from [^{35}S]methionine-labelled microglial cell line BV-2 followed by immunoprecipitation of CTSD, small quantities of non-processed CTSD could be detected in extracts of astroglial cell line 17 −/−. No inhibition by M6P was observed, suggesting non-specific uptake (Figure 8B). Metabolic labelling with [^{35}S]methionine and [^{32}P]P_i for 4 h followed by immunoprecipitation revealed that small quantities of secreted CTSD from the microglial cell line incorporated [^{32}P]P_i (Figure 8C) compared with the secreted CTSD from [^{35}S]methionine-labelled cells. The

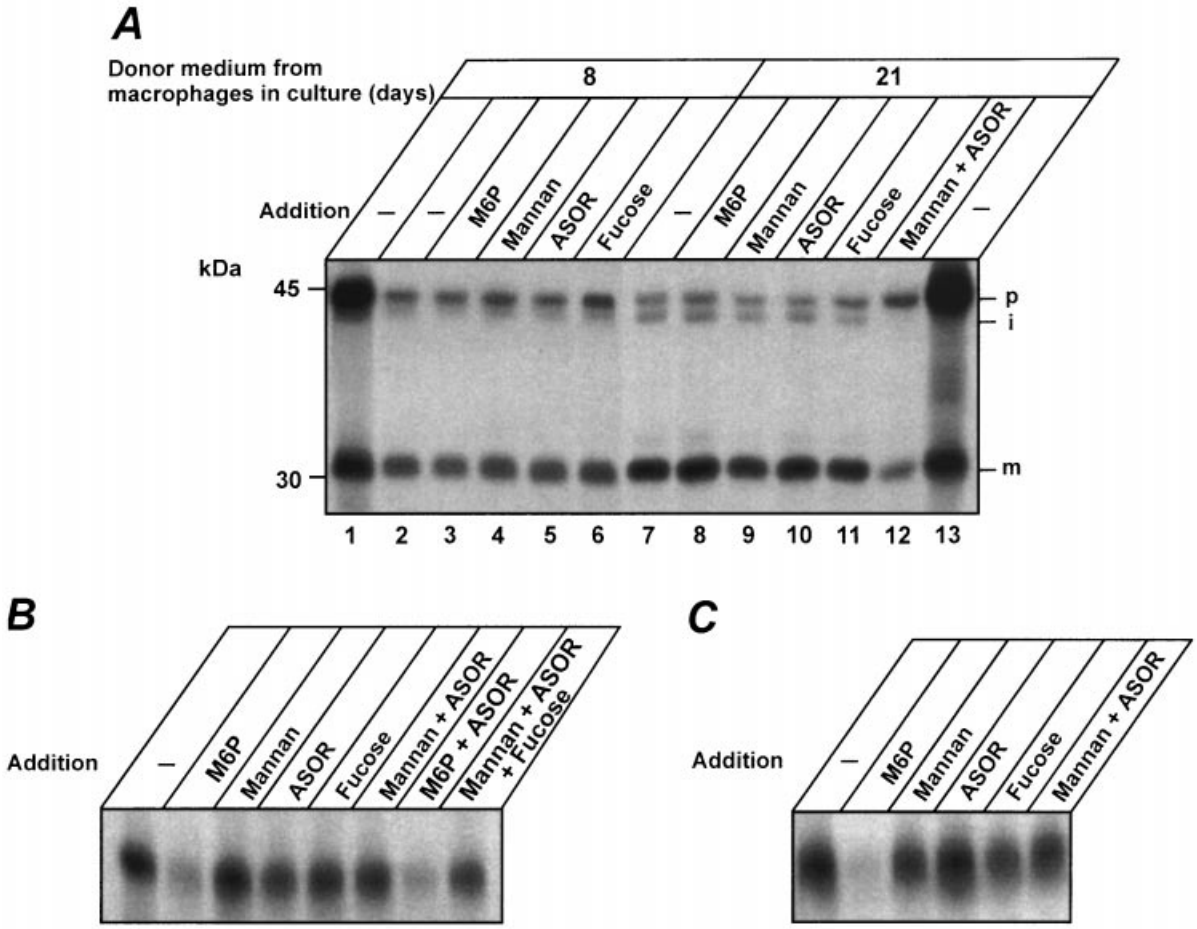


Figure 7 M6P-independent uptake of macrophages-derived CTSD in rat hepatocytes

(**A**) Secretions from [^{35}S]methionine-labelled macrophages cultured for 8 and 21 days were collected, dialysed, adjusted for adequate radioactivity, and used for endocytosis (1.6×10^7 c.p.m./plate). Prior endocytosis aliquots (30%) of the ^{35}S-labelled donor medium were processed for immunoprecipitation of CTSD (lanes 1 and 13). Rat hepatocytes were incubated with ^{35}S-labelled secretions from macrophages cultured for 8 (lanes 2–6) or 21 days (lanes 7–12), in the absence (lanes 2 and 7) or the presence of 10 mM M6P (lanes 3 and 8), 0.1 mg/ml mannan (lanes 4 and 9), 0.1 mg/ml ASOR (lanes 5 and 10), 5 mM fucose (lanes 6 and 11) or a combination of mannan and ASOR (lane 12) for 24 h. CTSD was immunoprecipitated from cell extracts, followed by SDS/PAGE and fluorography. The position of the precursor (p), intermediate (i) and mature (m) forms of CTSD are indicated. The experiment was carried out three times with three different preparations of ^{35}S-labelled secretions from macrophages and rat hepatocytes with similar results. (**B**, **C**) Rat hepatocytes were incubated in parallel with ^{125}I-ASA (500 000 c.p.m./plate) for 2 h (**B**) or with ^{125}I-PMP-BSA (750 000 c.p.m./plate) for 1 h (**C**) in the absence or presence of the indicated effectors at concentrations described above. After removal of the medium and displacement of cell-surface-bound material by M6P at 4 °C, cells were solubilized, analysed by SDS/PAGE, and the internalized ^{125}I-labelled ligands were visualized by autoradiography.

^{32}P-labelled CTSD in the medium is completely sensitive to treatment with alkaline phosphatase, indicating the presence of 'uncovered' phosphomonoester residues (Figure 8C).

DISCUSSION

The rationale of BMT for the treatment of MLD is the secretion of ASA by donor-derived macrophages repopulating various tissues of the recipient, in particular the brain. The recapture of ASA by deficient glial and neuronal cells is believed to correct the metabolic defect and reduce the stored material [6,38].

In this study we have demonstrated, by two independent experimental approaches, that ASA secreted from isolated, cultured human macrophages cannot be endocytosed by immortalized ASA-deficient astroglial cells *in vitro*. First, incubation of ASA-deficient acceptor cells with secretions of [^{35}S]methionine-labelled human macrophages, and second, co-culturing of ASA-deficient cells with macrophages separated by permeable filters failed to demonstrate the uptake of ASA into the glial acceptor cells. Furthermore, secretions of macrophages revealed a low capability for inhibiting the M6P-dependent uptake of recombinant [^{125}I]ASA into astroglial cells (N. Muschol, unpublished work), indicating the lack of uptake-competent lysosomal enzymes in secretions of macrophages. In contrast, both recombinant [^{125}I]ASA and ASA from secretions of ASA-overexpressing BHK cells were internalized by primary brain cells or brain cell lines in an M6P-dependent manner. The lack of endocytosis of another lysosomal enzyme, CTSD, in acceptor cells, indicates a more general rather than an ASA-specific inability of macrophages to secrete uptake-competent M6P-containing lysosomal enzymes. The lack of ^{32}P incorporation into ASA secreted by macrophages demonstrates the absence of the M6P-recognition marker which is essential for efficient uptake into brain cells. Finally, the present data on the M6P-independent uptake of CTSD secreted by macrophages at a different differentiation status support the conclusion that macrophage-derived lysosomal enzymes lack the M6P recognition marker. Hepatocytes endocytose M6P-containing ligands, such as recombinant

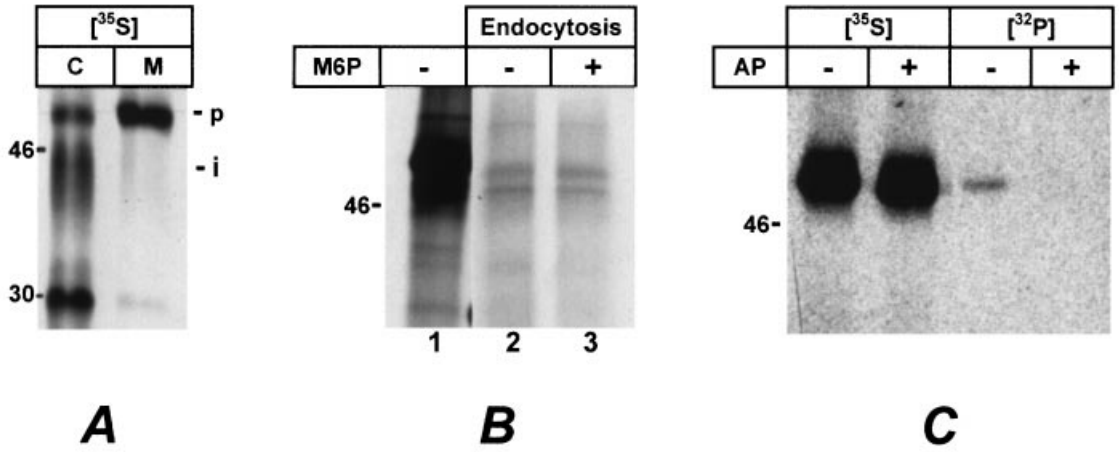


Figure 8 Secretion and phosphorylation of CTSD in the murine microglial cell line BV-2

(**A**) Mouse microglial cell line BV-2 was labelled with [^{35}S]methionine for 24 h. CTSD was immunoprecipitated from extracts of cells (C) and medium (M) followed by SDS/PAGE and fluorography. The precursor (p) and intermediate (i) forms of CTSD are indicated. (**B**) Secretions from [^{35}S]methionine-labelled BV-2 cells were incubated with cell line 17 −/− in the presence (+) or absence (−) of 10 mM M6P for 24 h followed by immunoprecipitation of CTSD from cell extracts, SDS/PAGE and fluorography. Lane 1, aliquots (1/5) of the ^{35}S-labelled microglial donor medium; lanes 2 and 3, immunoprecipitates from astroglial cell extracts after endocytosis. (**C**) Microglial cell line BV-2 was labelled in parallel either with [^{35}S]methionine (0.1 mCi/plate) or with [^{32}P]P_i (0.4 mCi/plate) for 4 h followed by immunoprecipitation of CTSD from the medium. Half of the immunoprecipitates were incubated with (+) or without (−) 6.2 units of alkaline phosphatase (AP) under standard conditions for 24 h at 37 °C. After solubilization, the precipitates were separated by SDS/PAGE and visualized by fluorography.

ASA or the neoglycoprotein PMP-BSA, preferentially via MPR whereas receptors recognizing galactose or mannose residues appear to be marginally involved in lysosomal enzyme uptake [39]. Whereas the RT-PCR data showed that in human macrophages both the mRNA of the γ-subunit of the *N*-acetylglucosamine-1-phosphotransferase and the M6P-uncovering enzyme are expressed, the amounts of functional active enzyme protein is unknown. The significance of the small quantities of weakly phosphorylated 53 kDa CTSD precursor form in the medium of macrophages is unclear and might present enzyme-specific variations in phosphorylation due to differences in the structure of the protein, oligosaccharide chains, in the kinetics of intracellular transport or the expression level. Indeed, the higher molecular mass of ASA secreted by macrophages compared with the uptake-competent ASA found in secretions of BHK-ASA cells suggest that an altered glycosylation may impair the proper phosphorylation of mannose residues, at least of ASA. Furthermore, the data led to the conclusion that in these cells the majority of newly synthesized lysosomal enzymes is transported in an MPR-independent manner.

In the brain, bone-marrow-derived macrophages differentiate predominantly into perivascular microglia and to a lesser extent into resident microglia [38]. The analysis of the murine microglial cell line BV-2 showing that the secreted 53 kDa CTSD precursor fails to be endocytosed by astroglial cells confirms the data obtained with human macrophages. Little information is available on the transport and phosphorylation of lysosomal enzymes in human macrophages and microglial cells. Thus only a small portion of CTSD and β-hexosaminidase released by the human monocytic cell line U937 has been reported to contain M6P residues [40]. Additionally, there is evidence that in U937 cells, rat bone marrow macrophages and rabbit alveolar macrophages CTSD is transported in an MPR-independent, membrane-associated form to the endosomes [41–43]. The MPR-dependent pathway can be disrupted by the addition of NH_4Cl, which interferes with the acidification of intracellular compartments involved in receptor-dependent intracellular sorting of lysosomal enzymes. Addition of NH_4Cl to macrophages used in this study had no effect on lysosomal enzyme sorting (N. Muschol, unpublished work), which provides additional evidence that M6P-dependent sorting does not play a major role in these cells. Our results clearly demonstrate that ASA and CTSD synthesized by macrophages and microglial cells are barely phosphorylated or equipped with the proper M6P-recognition marker. This explains the failure of brain cells to recapture the enzymes from secretions efficiently, whereas intracellular enzyme forms are transported to the lysosome in an MPR-independent manner. Additionally, it has been shown that a mutant ASA lacking M6P-bearing oligosaccharides can be internalized and delivered to the lysosomes of astoglial cells by fluid pinocytosis in a concentration-independent manner [44].

Transplantation of ASA-deficient mice which have a mild phenotype with bone marrow cells overexpressing ASA from a retroviral vector [14] resulted in partial correction of sulphatide storage in visceral organs [38]. In the CNS only minor effects on pathology were observed. Similarly, the BMT of β-hexosaminidase-deficient mice did not increase the β-hexosaminidase level in neurons and consequently did not affect the glycolipid storage in the brain [10,13]. The slight improvement of neuronal symptoms and the prolongation of the life span in β-hexosaminidase-deficient mice after BMT has been ascribed to the extensive suppression of microglial activation and neuronal death in treated animals, rather than to the enzyme transfer into the brain [45].

These data are in accordance with the *in vitro* data presented here, which indicate that lysosomal enzymes secreted by macrophages/microglial cells are not equipped with M6P residues. If this also occurs *in vivo*, macrophages that have entered the brain in the course of BMT would not be able to supply deficient brain cells with enzyme. Thus our data suggest, at least, a molecular mechanism other than the secretion/recapture of lysosomal enzymes underlying the therapeutic effects of BMT in the brain. Whereas in the brain the M6P-dependent, MPR-mediated uptake of lysosomal enzymes appears to be insufficient, the direct transfer of the missing enzyme mediated by cell-to-cell

contact [46,47], the suppression of activated microglia and the microglia-mediated removal of damaged storage neurons [12], or other still unknown mechanisms, might be responsible for the amelioration of CNS pathology.

We are grateful to Dr J.-G. Scharf and Dr I. Probst (University of Göttingen, Göttingen, Germany) for their help with the rat hepatocyte analyses. This work was supported by the Deutsche Forschungsgemeinschaft grant UL 133/2-1 Graduiertenkolleg 336 and the SHS-Company, Heilbronn, Germany.

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Received 11 February 2002/17 September 2002; accepted 24 September 2002
Published as BJ Immediate Publication 24 September 2002, DOI 10.1042/BJ20020249