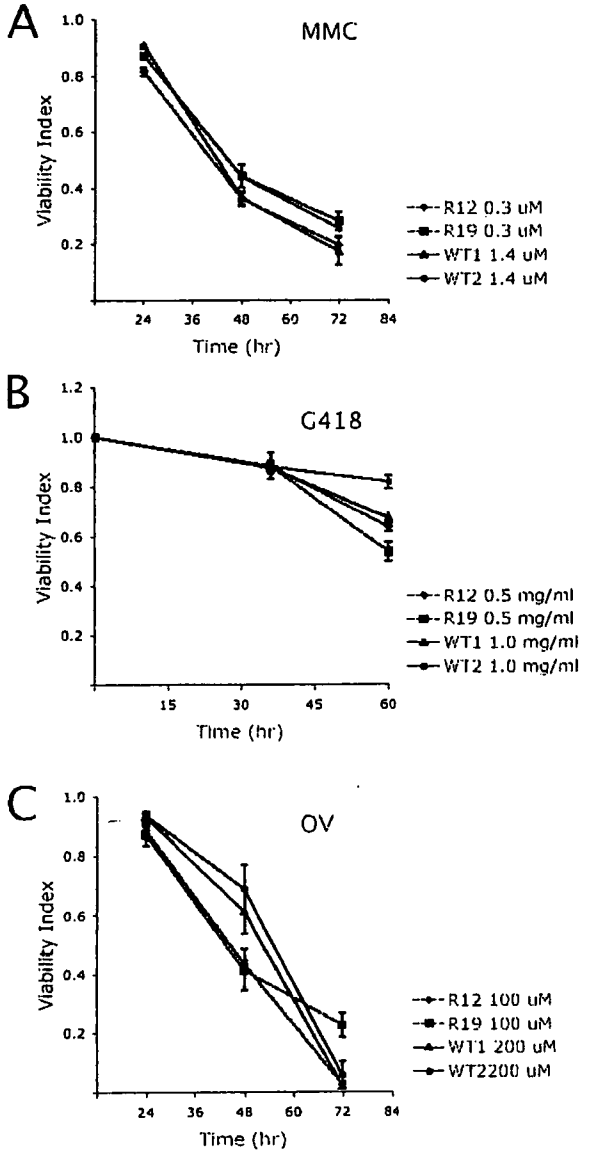

Figure 2. Equivalence dose viability curves. Viability curves obtained for each dose applied to control and RBS cell lines were analyzed and replotted such that curves generated for both RBS and LCLs were similar. (A) MMC doses were 0.3 μM for the RBS cell lines and 1.4 μM for the control. (B) G418 doses were 0.5 mg/ml for RBS cells and 1.0 mg/ml for control cell lines. (C) Orthovanadate doses were 100 μM and 200 μM for RBS and control cell lines respectively.

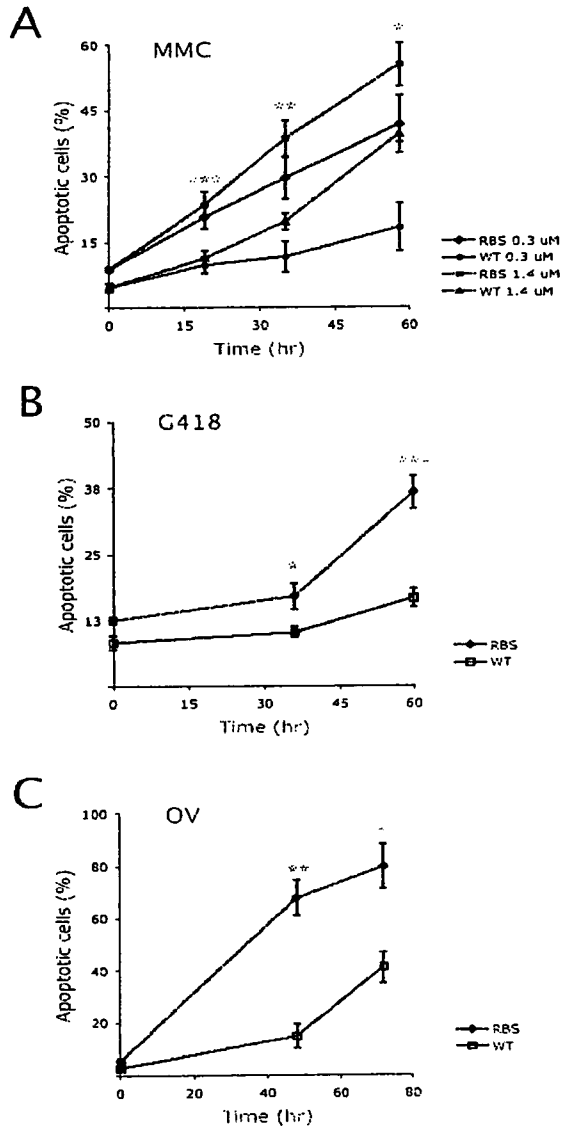

Figure 3. Apoptosis induction by MMC, G418 and orthovanadate in RBS and control LCLs. Cells were exposed to the indicated concentrations of each drug and assayed at different time points. (A) Comparison of apoptosis induction in RBS and control lymphoblasts treated with 0.3 and 1.4 μM for up to 58 hr. (B) Cell lines were exposed to 0.75 mg/ml G418 and apoptosis measured at 36 and 60 hours. (C) Apoptotic response to 100 μM sodium orthovanadate after 48 and 72 hours treatment. *p<0.05, **p<0.03, ***p<0.005

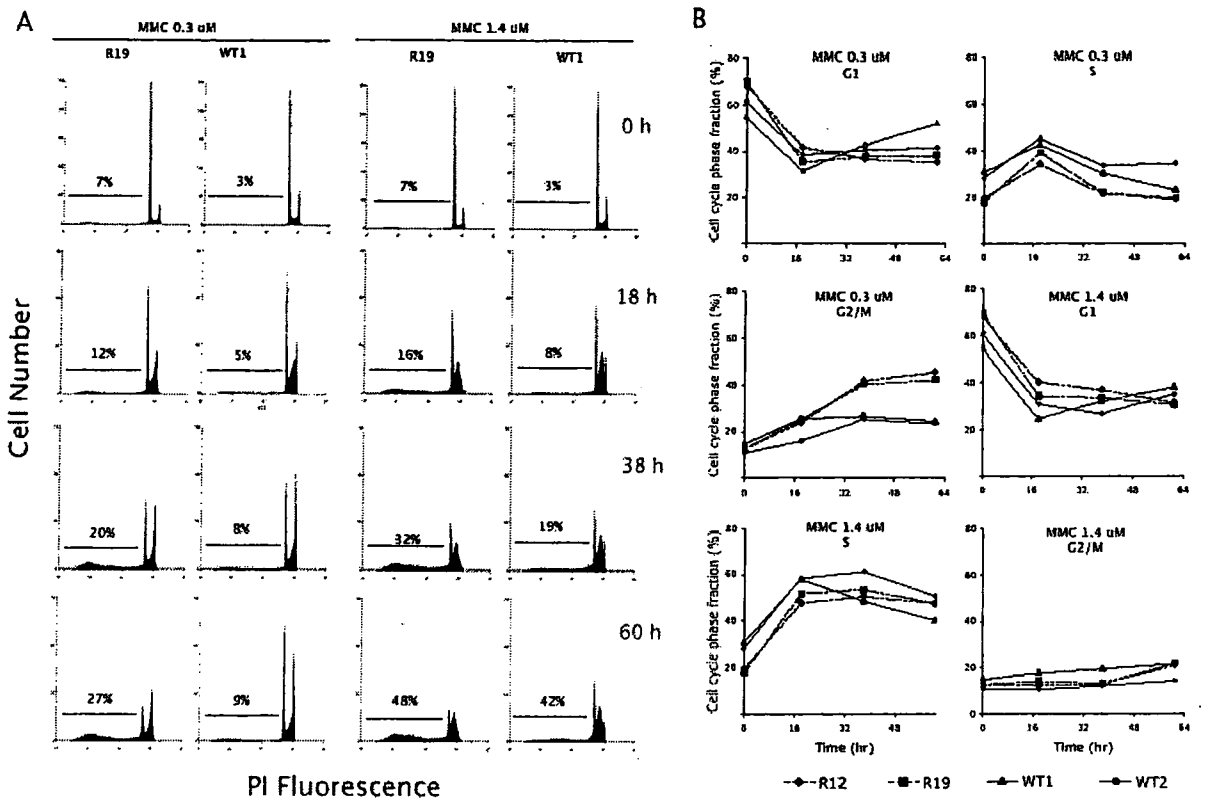

Figure 4. MMC-induced cell cycle changes and apoptosis in RBS and control cell lines. Cell lines were exposed to the indicated concentrations of MMC and assayed at 18, 38 and 60 hours of treatment. (A) PI-stained cells were analyzed by flow cytometry, allowing detection of apoptotic cells as sub-G0/G1 material, indicated by horizontal bars. Percentages of apoptotic cells for each sample are indicated. (B) Time course for the effect of MMC on cell cycle distribution. Cells treated with 0.3 and 1.4 μM MMC were fixed after the periods indicated. PI-stained cells were analyzed by flow cytometry, and percentages of cells present in the different phases of the cell cycle were quantified.

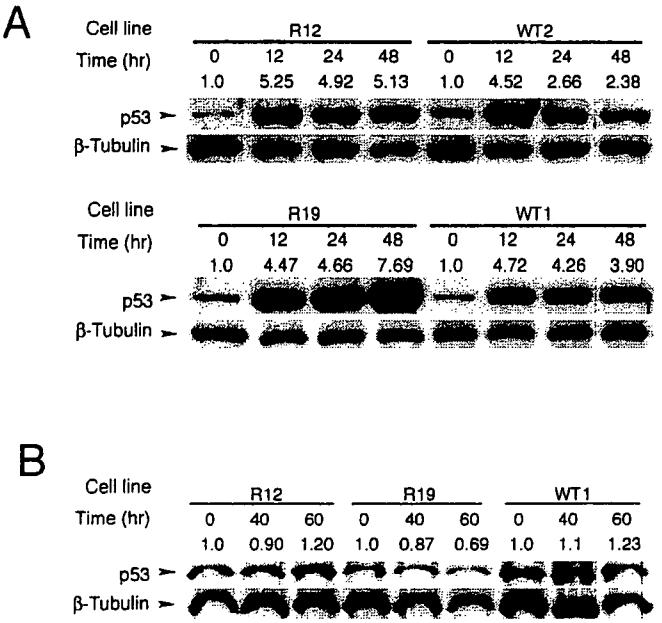

Figure 5. Expression level of p53 in RBS and control cell lines treated with orthovanadate and G418 was evaluated by Western blotting. Cell lines were exposed to (A) 100 μM orthovanadate or (B) 0.75 mg/ml G418 for the indicated times and cell lysates were prepared. α-tubulin levels were used as loading controls. The level of p53 was compared with the level of α-tubulin. The ratio of the level of p53 to the level of α-tubulin at 0 hour was defined as 1.0.

apoptotic cells, reduces the PI fluorescence intensity to a level less than that of diploid cells. For orthovanadate nuclear condensation and fragmentation was assessed by morphologic analysis of May-Grünwald/Giemsa stained cells. Comparison of the numbers of apoptotic cells after exposure to 0.3 μM and 1.4 μM MMC, 0.75 mg/ml G418 and 100 μM OV clearly distinguished RBS from control cells (Fig. 3A, B. C). MMC induced apoptosis increased with the time in a dose dependent manner (Fig. 3A). For MMC, the difference between RBS and normal cells was greater at 0.3 μM MMC yet remained significant after 58 hr of cell culture in the presence of 1.4 μM MMC($p<0.05$). When treated with 0.75mg/ml G418, the difference between RBS and normal cells in apoptosis was detected after 60 hr treatment (Fig. 3B). RBS cells exposed to 100 μM OV exhibited massive apoptosis after 48 and 72 hr treatment (Fig. 3C). These results suggest that the mechanism of MMC-, G418- and OV-induced cell death in RBS LCLs appears to be through apoptosis.

To understand the cellular basis for the hypersensitive phenotype, we examined the cell cycle response upon MMC, G418 and OV treatment by flow cytometric analysis of propidium iodide-stained nuclei. Treatment with 0.3 μM MMC during 18 hr resulted in a sharp decrease in the number of G1 cells in both RBS and control LCLs with a simultaneous increase in late S and G2/M fractions (Fig. 4A, B). At later time points, the fraction of cells in S phase returned to near normal levels for both RBS and control cells while the number of cells in G2/M increased steadily in RBS LCLs until cells in G1 and G2/M became equal. However the absolute number of cells in each fraction was reduced as a result of many cells having undergone apoptosis (Fig. 2A). From this experiment, the cell cycle phase from which cells undergo apoptosis cannot be inferred. Thus, upon treatment with 0.3uM MMC, RBS cells progress from G1 to G2/M, and following accumulation in G2/M, either partially transit to G1 or exit the cell cycle to undergo apoptosis. At 1.4 μM MMC, there were no differences in the effect over cell cycle distribution between RBS and control cells as both became persistently blocked in S phase. As depicted in Fig.4A and B, the fraction of G1 cells was largely reduced with a consequent increase in the number of cells in S phase while the G2/M fraction remained constant . After treatment with G418 or OV, no obvious changes in cell cycle distribution were found except for a slight increase in G1 accompanied by reductions in S and G2/M detected for G418 treatment. Furthermore, no clear differences were noted between RBS and control LCLs (data not shown)

To gain insight into the molecular mechanism leading to cell death in RBS and control LCLs, we investigated the expression of p53, one of the predominant modulators of cell death in response to diverse forms of cellular stress (29). We examined p53 expression by immunoblotting in RBS and normal cell lines. As shown by representative experiments in Fig, 5A, treatment with 100 μM OV resulted in p53 induction at 12 hr in both RBS and control cell lines. However, in control cells p53 levels decreased over time whereas in RBS cells P53 levels increased or at least remained constant. These results are in agreement with data from other cellular types that indicates that OV treatment cause p53 activation, which is required for apoptosis induction by vanadate (30). For G418 treatment, no induction of p53 over basal level was detected in either RBS or control cells (Fig. 5B). To our knowledge, p53 induction has not been studied in G418 induced cell death. However studies of cell death induced by hygromycin B, another aminoglycoside antibiotic, indicate that apoptosis in response to these drugs occur independently of p53 (31). In agreement with previous reports (32) MMC treatment induced accumulation of p53 but not differences were observed between normal and RBS cells (data not shown).

DISCUSSION

Roberts syndrome is a developmental disorder with a variety of cell phenotypes including defects in sister chromatid cohesion (4). RBS hypersensitivity to MMC, and G418 has been reported but the cellular mechanisms underlying these defects are not known. In the present study we found that apoptosis is the main reason for the growth inhibition induced by these drugs. We also found that, after culture in

the presence of MMC, there is a relation between the accumulation of cells in G2/M and apoptosis. Further, we report on the hypersensitivity to sodium orthovanadate, which was related to high and sustained levels of p53.

Studies on drugs sensitivity in RBS cells have been performed by either colony forming assays or automated cell counting (9, 10, 11). Colony forming assays require cells with high plating efficiency, are time-consuming and laborious. Furthermore, colony forming assays and automated cell counting do not discriminate between drug induced cell death or growth inhibition. The requirement of cells with high proliferation efficiency makes more difficult the analysis of drug sensitivity by these methodologies in cells with genetic defects that compromise their proliferation as is it has been reported for Roberts Syndrome (7). To overcome these drawbacks, we used flow cytometry and FDA to measure viable cells after drug treatment in RBS and control LCLs (23). In contrast to colony-forming assays that can require weeks of incubation for colony formation, the flow cytometric assay was able to detect differential effects on cell viability between RBS and control LCLs in a maximum of two days after drug treatment. Despite the intrinsic differences between colony forming assays and the flow cytometry and FDA techniques, we found equivalent dosages for MMC and G418 that are in agreement with previous reports, demonstrating that the flow cytometric assay is a valid surrogate for colony-forming assays when investigating the response to stress-inducing agents on RBS and other cells having limited proliferative capacity, such as those derived from individuals affected with DNA repair disorders akin to Fanconi anemia, Werner Syndrome and ataxia telangiectasia (33, 34, 35).

The hypersensitivity of RBS cells to crosslinking agents such as MMC has been reported previously (9, 10). In the present report, we analyzed the induction of apoptosis and the cell cycle response to MMC in RBS LCLs. We observed that RBS cells experience higher levels of apoptosis and display prolonged accumulation in G2/M following lower dose MMC treatment. The effect of 0.3 μM MMC treatment on cell cycle distribution suggests that the onset of apoptosis was preceded by a sharp decrease of the G1 cell fraction with subsequent accumulation in G2 (Fig. 3A and 4). At higher doses, such as 1.4 μM, both RBS and control cells accumulated in S phase in a similar manner (Fig. 4) and differences in the amounts of cell death and apoptosis become less evident or disappear (Fig. 1A, B and 3). These results indicate that apoptosis is the major reason for the hypersensitivity of RBS cells to MMC and that apoptosis is related to dose dependent specific delays in the cell cycle.

Mitomycin C (MMC) is a cytotoxic antitumor antibiotic that induces chromosome aberrations, micronuclei, and sister chromatid exchanges (36). The molecular mechanism of MMC action is due primarily to the formation of both DNA intrastrand and, to a greater extent, interstrand crosslinks (37, 38). The repair of interstrand crosslinks proceeds via the formation of a double-strand break (DSB) in S phase followed by DSB repair via homologous recombination (39). A substantial amount of evidence indicates that sister chromatid cohesion plays a critical role in DSB repair (19, 20, 21). We recently reported that RBS is caused by mutations in *ESCO2* (12). ESCO2 protein is a homolog of *S. cerevisiae* Eco1, a protein essential for the establishment of sister chromatid cohesion as well as for efficient DNA double-strand break repair in G2/M (13, 14, 21). Hypersensitivity of RBS cells to different DNA damaging agents suggests that sister chromatid cohesion is involved in DNA repair in humans.

The role of sister chromatid cohesion in the postreplicative repair of double-strand breaks is not yet understood. Under normal physiological conditions mammalian cells respond to DNA damage with inhibition of cell cycle progression followed by either the repair of the damage or the induction of programmed cell death (40). It has been proposed that chromatid cohesion enables broken DNA ends to find and invade their sister sequences, thus facilitating DNA repair. However, the possibility that cohesin containing structures are required for detecting DNA damage and for activating the necessary proteins for double-strand break repair remains (21). Should the latter hypothesis be correct, one may predict that defects in cohesion will lead to defective signaling of cell

cycle arrest in response to damage. Within such a context, no inhibition of cell cycle progression would be observed (21). Our finding that RBS cells are arrested in G2/M in response to low doses of MMC suggests that sister chromatid cohesion and, more specifically, ESCO2, is not required for checkpoint signaling. We support the hypothesis that, after low dose MMC treatment, the cohesion defect in RBS cells precludes efficient DSB repair, resulting in the persistent accumulation of cells in G2/M as a consequence of the activation of the G2/M DNA damage checkpoint. Finding that the hypersensitivity of Roberts syndrome cells to cisplatin does not result from a deficiency in the nucleotide excision repair (NER) pathway (11) is not inconsistent with this hypothesis as NER is not the main pathway involved in the repair of DSB (39).

The pattern of MMC-induced apoptosis and cell cycle delay over RBS lymphoblastoid cell lines that we report is similar to that found in cells from patients with Fanconi anemia (FA), whose best known cellular phenotype is sensitivity to DNA cross-linking agents (41, 42). FA is an autosomal recessive disorder characterized by congenital malformations (such as radial aplasia), progressive pancytopenia, and, eventually, fatal malignant disease. Some of the congenital malformations in FA and RBS overlap and reclassification of RBS patients, first described as FA, has been reported (43). We report here another similar cellular phenotype between RBS and FA cells: no alteration in sensitivity towards the DNA topoisomerase inhibitor camptothecin in RBS cells as it has also been described for FA cells (44). This result also implies selectivity of the hypersensitive response of RBS cells to different clastogenic agents. Other agents known to selectively elicit a hypersensitive response in RBS include cisplatin, hygromycin B and G418. Here, we show that the decrease in viable cell populations after G-418 treatment appears to be the result of apoptosis induction but appears not to be mediated by p53 and to be unrelated to cell cycle abnormalities.

We found that RBS cells are hypersensitive to the inhibitor of tyrosine phosphatases, sodium orthovanadate, but not to the serine/threonine phosphatases inhibitor, Okadaic acid, or the protein kinase inhibitor, staurosporine. Sodium orthovanadate induced massive cell death via apoptosis both in RBS and control cells but the extent and the time of induction was higher in RBS LCLs. In addition, persistent p53 expression in RBS cells was induced by OV treatment. There was no association between these effects and cell cycle phase distribution. Protein tyrosine dephosphorylation by protein tyrosine phosphatases have been shown to play prominent roles in a variety of cell functions, particularly in the control of cellular proliferation and differentiation (45). Interestingly, phosphorylation and dephosphorylation of proteins related to cohesion are crucial to sister chromatid cohesion (25, 46, 27, 28). One possible explanation for RBS cells hypersensitivity to OV could be that ortovanadate treatment results in the perturbation of processes related to sister chromatid cohesion and, consequently, to cell death. If this explanation is accurate, then one may predict the accumulation of cells in M phase due to activation of the mitotic spindle checkpoint, as it has been described for cells with defects in phosphorylation of cohesion subunits (46). Our results do not support this possibility, as no cell cycle perturbations were found after OV treatment.

An alternative explanation of RBS hypersensitivity comes from studies showing that vanadium salts, including sodium orthovanadate, are able to induce micronuclei, aneuploidy, sister chromatid exchanges, DNA strand breaks and DNA–protein cross-links (47, 48, 49, 50). In addition, it is known that reactive oxygen species generated by vanadate-mediated reactions cause DNA damage. One reasonable hypothesis, therefore, is that the hypersensitivity to OV of RBS cells might result from unrepaired DNA damage produced during treatment. In the response to cellular stress, including DNA damage response, the p53 protein plays a key role (29). Normal mammalian cells exposed to DNA-damaging agents, exhibit increases in the cellular level of p53 which, in turn, can cause growth arrest, permit the induction of the DNA repair process, or direct cells to undergo apoptosis (29). Unrepaired orthovanadate-induced DNA damage in RBS cells might activate signal transduction pathways leading to the sustained increase in p53 observed in RBS cells and to subsequent apoptosis. Indeed, induction of p53-dependent

apoptosis by vanadate has been described in other cellular models (30). Further experimentation could confirm our hypotheses and ultimately provide more insight into the role of sister chromatid cohesion in DNA repair.

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ライソゾーム病の酵素補充療法

8

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酵素補充療法は，正常な酵素が発現しない遺伝病の中で，ライソゾーム病を直接のターゲットとして開発された．異物として投与された正常の酵素分子が細胞に取り込まれ，ライソゾームに運ばれるからである．これまでにゴーシェ病，ファブリー病，ムコ多糖症(3 病型)，ポンペ病に対する試みがなされ，わが国では最初の 2 つの病気についてすでに臨床の場での治療が行われている．しかし，小児の重度脳障害の原因としてのこのグループの病気に対して，中枢神経系へのアプローチは達成されていない．今後の問題として残される．

Key words
1 酵素補充療法
2 ゴーシェ病
3 ファブリー病
4 ライソゾーム病

はじめに

現在，遺伝代謝病と定義されている疾患群は，単一遺伝子の構造変化によって生じた全身病である．そして，この変異遺伝子の異常な情報をもとに，細胞内で合成された変異蛋白質が正常に働かないことが複雑な病態のすべての始まりである．つまり，本来働くべき蛋白質が存在しないということである．そこで代わりに，正常な働きを持つ蛋白質を生体外から補給しようという発想が生まれ，いくつかの病気に試みられてきた．ただし，そのためには一定の条件がある．本来体内に存在すべき蛋白質は，細胞が自分で作り上げ，目的に応じて必要なコンパートメントに運び，機能を発現させている．ところが，正常の蛋白質といっても，細胞外から投与する場合，いわば異物として取り込まれ，必ずしも機能の発現を期待することができるとは限らない．

酵素補充療法が成立するための必要条件

酵素補充療法が成立するためには，以下の条件を満たす必要がある．すべての酵素欠損症に漫然と蛋白質を投与しても効果は期待できない．

①正常蛋白質の大量合成：ヒト個体の治療に用いるためには，大量の蛋白質精製・供給が必要である．しかも，特に生物製剤としてウイルスや毒性化合物など不純物が微量でも存在することは許されない．わが国でも一部の製剤に混在したウイルスや微量金属と特定の疾患発生との関係が大きく取り上げられたこともあり，供給者はこの点に特に厳重な管理体制を整えている．

②蛋白質の投与：蛋白質を経口投与すれば，腸管で分解され本来の機能を失うので，原則として静脈内注入が必要である．投与された蛋白質はそ

のまま血液中に長時間とどまっているわけではない．速やかに処理される．

③標的臓器へのターゲティング：投与した蛋白質は，できるだけ多く特定の標的臓器に到達するような工夫がなされねばならない．疾患によりその標的が異なるため，製剤も目的に合わせた分子修飾が必要である．つまり，細胞の取り込み効率を可能な限り上げることである．

④細胞内での安定な活性発現：異物として取り込まれた蛋白質は，細胞内で安定に存在し，しかも本来活性を発現するコンパートメントに運ばれ，活性を発現しなければならない．酵素の場合，基質との出会いの場が必要である．

このような条件を満たすのがライソゾーム病である．ライソゾーム酵素を細胞外から投与すると，異物として認識され，そのままライソゾーム，つまりこの酵素が本来働く場所に運ばれ，酸性の条件で安定に活性を発現することが期待されるからである[1]．したがって，酵素補充療法はライソゾーム病を対象とする治療法として理解される．ただし，血友病など血漿蛋白質の欠損症に対する蛋白質補充療法は，臨床医学で広範に実用化されていることは周知のとおりであるが，酵素補充療法とは別のコンセプトのアプローチであることを付け加えておく．

ライソゾーム病に対する酵素補充療法

1970年代から，血漿や白血球の注入，臓器移植などの方法により，正常酵素を患者体内に供給しようとする試みが行われたが，広く実用化されるには至らなかった．その後Bradyらは，酵素をヒト胎盤から精製して患者に供給する試みを始めた．まずゴーシェ(Gaucher)病(β-グルコシダーゼ欠損症)が対象となり，すでに世界各国で広く治療に使われるようになった．わが国では現在，オーファンドラッグとして実用化されている．

以後いくつかの病気について順次開発が進められ，臨床治験に進んでいる製剤が多い．わが国では，ファブリー(Fabry)病(α-ガラクトシダーゼA欠損症)の治験も終了し，治療薬として採用された．このほかに，国外ではいくつかのムコ多糖症(ハーラー(Hurler)病：α-イズロニダーゼ欠損症，ハンター(Hunter)病：イズロン酸スルファターゼ欠損症，マロトー・ラミー(Maroteaux-Lamy)病：アリルスルファターゼB欠損症)，ポンペ(Pompe)病(α-グルコシダーゼ欠損症)などの治験が行われているが，わが国ではこの段階に至っていない．

ゴーシェ病の酵素補充療法

1．酵素製剤の開発

1974年Bradyらは，ヒト胎盤から精製したグルコセレブロシダーゼ(β-グルコシダーゼ)を成人型ゴーシェ病症例に投与し，肝臓のグルコセレブロシド蓄積の減少，血液中の脂質の消失が長時間続くことを報告した[2]．以後多数の患者に対する治療実験を行い，ある程度の効果を認めたが，実際に大量に蓄積しているマクロファージには到達しないことがわかった[3]．そこで，マクロファージへのターゲティングのためにこの酵素の糖鎖を改変し，末端がマクロファージに親和性のあるマンノースを露出した分子として成人型ゴーシェ病患者に投与したところ，著しい臨床効果のあることが確認された[4]．当初はヒト胎盤から精製した酵素製剤(セレデース®；Genzyme)が用いられたが，その後遺伝子組み換え製品が開発され，広く用いられている(セレザイム®；Genzyme)．第1の酵素製剤は病原微生物の混入の可能性を完全に否定できないため，現在は市販されておらず，実際には遺伝子組み換え製剤が治療薬として用いられている．

2．臨床効果

胎盤製剤と遺伝子組み換え製剤に効果の差はない[6][7]．臨床効果は貧血，血小板減少などの血液学的所見，臓器腫大(肝脾腫)，骨症状などの改善であ

る．

ゴーシェ病は中枢神経障害を伴わない非神経型と，中枢神経障害を伴う急性神経型，亜急性神経型の3病型に大きく分類される．酵素蛋白質は血液脳関門を通過せず，酵素補充療法により中枢神経系の症状を改善することは期待できないので，当初は非神経型症例に適応が限られていたが，2つの神経型症例でも全身状態が改善されるので，現在使用されているセレザイムについては，すべての病型のゴーシェ病に適応が拡大された．

3．治療方法・成績

①初期治療：体重kgあたり60単位セレザイムを隔週1回，6～12ヵ月間静脈内投与．肝脾腫・血液所見の改善，血清アンジオテンシン変換酵素(ACE)・酸ホスファターゼ(ACP)活性の低下を指標とする．

②維持療法：体重kgあたり15～30単位セレザイムを各週1回，維持投与．最小有効量は15単位．

上記の臨床所見を指標として，投与量を増減する．

この治療基準をもとに，わが国では6例の非神経型症例の治験を行った[8]．すべて肝脾腫があったが，2例は酵素治療前に脾摘手術を受けていた．すべての症例について上記の初期治療により著しい効果がみられた．身長体重増加が正常化し，骨密度の改善もあり，生活の質の著しい改善のみられた症例があった一方，著しい肝脾腫の存在のため食欲不振，骨病変が進行し，成長が改善されない症例もあった．早期治療が重要であるとの結論が得られた．

4．副作用

皮膚掻痒感，蕁麻疹，嘔吐・下痢，脱力・発熱・疲労感などの全身症状などもあったが，重篤な身体症状はなかった．特に抗体出現には十分な注意が必要である．

4 ファブリー病の酵素補充療法

1．酵素製剤の開発

ゴーシェ病の場合と同様，ヒト胎盤その他の組織体液から精製したα-ガラクトシダーゼAの投与が試みられた．胎盤由来の酵素治療により，血中セラミドトリヘキソシドの現象が確認された[9]．しかし，血漿と脾臓由来の酵素製剤のクリアランスが異なることがわかった[10]．そして，その理由が酵素製剤の糖鎖構造の違いにあることが明らかになった．以後，組み換え遺伝子による酵素大量発現系を用いた大量生産法が確立され，2つの企業が並行して製剤開発を進め(Brady RO：TKT，Desnick RJ：Genzyme)，それぞれ独自に効果を報告した．

2．臨床効果

Schiffmannら[11]は，糖鎖末端にマンノースリン酸を持つ酵素製剤リプラガル®(agalsidase alpha；TKT)をファブリー病患者10例に1回投与し，肝臓と尿沈渣の蓄積脂質がそれぞれ平均31％，38％減少したことを報告した．さらに，26例の患者に二重盲検試験を行った[12]．酵素製剤0.2 mg/kgを隔週12回投与したところ，投与群では対照群と比較して腎メサンギウム増生が減少し，正常な糸球体数が増加した．臨床的にはクレアチニンクリアランスの低下が起こらず，血液・尿・腎組織の脂質蓄積が減少した．治療経過中，疼痛スコアが減少し，臨床的改善がみられた．対照群にはこれらの変化がみられなかった．過半数の症例に注射後悪寒が発生したが，抗ヒスタミン薬とステロイド薬の投与により軽快した．少数例に酵素に対するIgG抗体が出現したが，臨床効果や副反応との相関はなかった．このグループはヒト上皮細胞に酵素蛋白質を発現させ，精製した製剤を使った．

他方Engら[13]は，CHO細胞に発現させた酵素製剤ファブラザイム®(agalsidase beta；Genzyme)を29例のファブリー病患者に投与し，非投与群と比較した．1 mg/kgの酵素製剤隔週投与20週後，20例(69％)で腎毛細血管内皮細胞への脂質蓄積が消失していた．皮膚や心臓への脂質蓄積も減少していた．非投与群には脂質消失例はなかった．血漿中酵素レベルと脂質蓄積とは明確な相関があった．この対照実験終了後，対象群には同量を隔週投与し，6ヵ月間，臨床効果を観察した．同様に，腎・皮膚の毛細血管脂質蓄積が消失した．この投与量でファブリー病の腎不全や心不全が予防できるという結論が得られた．疼痛については投与群と非投与群との間に差がみられなかったが，これは治療実験中も鎮

表1　わが国における臨床治験成績

検査評価法	組織・体液	変化率
組織病変スコア	腎	1.0→0
	皮膚	2.0→0
蓄積脂質免疫測定(ELISA)	腎	51.9％
	尿	55.4％
	血漿	100％
クレアチニンクリアランス		有意差なし
QOL	疼痛スコア	有意差なし
	全体的健康感	有意差なし
	精神的健康度	有意差なし

ファブリー病男性患者13例．平均年齢26.6歳．ファブラザイム1 mg/kgを隔週静注，20週間(合計11回投与)．投与前と投与後の検査データを比較した．
(文献14より改変して引用)

痛剤の使用を継続したためと考えられた．副反応として悪寒，発熱，頭痛のみられた症例があった．また，酵素製剤に対するIgG抗体が検出された症例があったが，臨床効果に影響はなかった．

わが国における臨床治験成績[14]を表1にまとめた．この治験の対象は腎不全，心不全などのある重症例は除いてあり，全般的に検査所見に大きな異常のない症例であるため，著しい変化はみられなかったが，蓄積脂質および病理組織学的変化の改善は確認された．

3．投与方法・副反応

上述のように，2つの製剤の投与量は異なる．リプラガルは1回0.2 mg/kg，ファブラザイムは1回1 mg/kgの投与であり，隔週，継続投与する．わが国ではファブラザイムが薬価収載され，診療に用いられている．悪寒，発熱，頭痛などの副反応があるが，静注速度の調整や抗ヒスタミン薬などの投薬により，これまでのところ重大な問題は起こっていない．

ムコ多糖症の酵素補充療法

1．ムコ多糖症1型(ハーラー病)

デルマタン硫酸とヘパラン硫酸の蓄積による全身組織の変形・異常増殖のために，骨関節系，皮膚粘膜，心臓などの形態機能異常，中枢神経障害の起こる病気である．欧米ではCHO細胞に発現させたα-L-イズロニダーゼ酵素製剤(ラロニダーゼ；Laronidase)が認可されているが，わが国ではまだ使用許可が得られていない．治療により関節可動域，心不全の改善がみられた[15]．発熱，頭痛，血管運動反応(顔面紅潮，欠陥性浮腫)などがみられたが，投薬を中止するほどの重篤な例はなかった．

2．ムコ多糖症2型(ハンター病)

やはりデルマタン硫酸とヘパラン硫酸の蓄積症であるが，ハーラー病に比べて臨床徴候は軽症である．X染色体に責任遺伝子があり，原則として男児に発症する．ヒト線維芽細胞に発現させたイズロン酸スルファターゼ[16]を用いた治験が欧米で開始されている．効果や副反応についての結果は公表されていない．

3．ムコ多糖症6型(マロトー・ラミー病)

デルマタン硫酸の蓄積症である．骨関節系の症状が中心で，中枢神経障害は起こらない．CHO細胞に発現させたN-アセチルガラクトサミン4-スルファターゼ(アリルスルファターゼB)の酵素製剤が試みられている[17]．尿中ムコ多糖排泄が減少し，関節拘縮が改善されたとのことであるが，詳細は公表されていない．

ポンペ病に対する酵素補充療法

ポンペ病は，グリコーゲン蓄積症の中で唯一，ライソゾームの加水分解酵素であるα-グルコシダーゼの欠損により，骨格筋，心筋にグリコーゲンが蓄積する疾患である．大部分は乳児期に発症する重症例であるが，学童期以後に進行性筋疾患として発症することがある．中枢神経障害はない．製剤は酵素を発現するCHO細胞[18]またはト

ランスジェニックウサギ乳汁[19)20)]から精製された．治験の詳細は公表されていないが，有効例では骨格筋グリコーゲンの減少，心肥大の改善，運動機能の改善などが認められた．わが国では治験は行われていない．

今後の課題

1．投与方法・投与量

これまでに数種の疾患についての基礎的臨床的な治療実験が行われているが，製剤により投与量が異なること，そして病型・症例により効果に差があることを考慮せねばならない．一般にライソゾーム病は乳児期の重症型の症例数が多いが，同じ遺伝子変異の異なった表現型に対して，個別的な治療計画の設定が必要になるであろう．これまでのところ論理的な治療基準はなく，経験的な結果をもとに一応の投与量，投与方法が示されているのみである．

酵素製剤により，標的組織・臓器への親和性に違いがあることは当然であり，最初の2つの疾患，ゴーシェ病とファブリー病のように，酵素蛋白質の糖側鎖の修飾によって細胞への取り込み効率が著しく増大することがわかったが，他の因子の可能性も考慮しながら酵素製剤の改善を図ることが必要である．

それぞれの酵素製剤を供給するには莫大な費用がかかり，したがって治療費がきわめて大きいのが現状である．この点も近い将来，何か技術的な改革を迫られるであろう．

2．副反応

蛋白質製剤の静注という限られた投与法に頼るこの治療法は，当然，即時の副反応が発生する可能性を常に考慮し，対応しなければならない．幸い，これまでのところ，きわめて重篤な反応は避けられてきたようであるが，生物製剤である以上，夾雑物を完全に除去することはきわめて困難である．この点は酵素補充療法のひとつの限界であるが，それでも多くの患者症例への治療経験をもとに，さらに十分な対応法を確立する努力をすべきである．

3．標的組織への酵素取り込み

標的組織の異なるいくつかの酵素製剤は分子修飾がなされ，特定の臓器に対しては治療効果がみられるようになったが，中枢神経系のほか，骨組織，肺組織への導入効率は高くない．ライソゾーム病の大部分は乳児期の脳の病気である．高分子の蛋白質製剤を，血液脳関門を通過して神経細胞に運ぶためには，今後特別の輸送システムの開発が必要になるであろう．現在，比較的低分子の物質については，リポゾームを使ったターゲティングが実験的に試みられているが，実際に臨床レベルで使われるには多くの問題があるし，まして高分子化合物をこのシステムでうまく神経細胞まで運ぶことができるかどうか，予測は困難である．

この大きな課題を克服した段階で，酵素補充療法は大きく飛躍し，真にライソゾーム病の臨床治療薬として一般化されるようになるであろう．

おわりに

本特集のメインテーマは「神経疾患の先端的治療」である．酵素補充療法が真にこの目的のために有効に使われているかどうかは，現在の段階では疑問である．かろうじてライソゾーム病における筋疾患に対する治療の可能性は示されているが，本来のターゲットである中枢神経系についてはこれからの課題として残されることを強調しておきたい．われわれは最近，この方向とは全く違った，中枢神経系を直接の標識臓器とした新しい治療の可能性を探っている(ケミカルシャペロン療法)[21)22)]．このような異なる立場からの治療法の開発が望まれる．

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Enzyme replacement therapy in Japanese Fabry disease patients: The results of a phase 2 bridging study

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Summary: Fabry Disease (α-galactosidase A deficiency) is an X-linked hereditary disorder leading to the pathological accumulation of globotriaosylceramide (GL-3) in lysosomes, particularly in the vascular endothelium of the kidney, heart and brain. We report the results of an open-label phase 2 study that was undertaken to evaluate whether ethnic differences exist that would affect agalsidase beta (Fabrazyme) treatment of Fabry patients in the Japanese population, relative to safety and efficacy. The study design mirrored the design of the completed phase 3 clinical trial that led to approval of the product agalsidase beta. The 13 Japanese, male Fabry patients enrolled in the study received the enzyme replacement therapy over a period of 20 weeks as biweekly infusions. All selected efficacy end points showed improvements that were comparable with findings from the phase 3 study. These improvements included reductions of GL-3 accumulation in both kidney and skin capillary endothelial cells to (near) normal levels (92% of patients). Kidney and plasma GL-3 levels decreased by 51.9% and 100%, respectively, by ELISA. Renal function remained normal. Fabry-associated pain, and quality of life, showed improvement over baseline in multiple categories. Related adverse events were mild or moderate in intensity and mostly infusion-associated (fever and rigors). As expected, IgG antibody formation was observed in 85% of the patients, but had no effect on treatment response. These results suggest that treatment with agalsidase beta is safe and effective in Japanese patients with Fabry disease. With regard to safety and efficacy, no differences were observed as compared to the caucasian population.

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Fabry disease (McKusick 301500) is an X-linked inborn error of metabolism characterized by deficient activity of the lysosomal hydrolase α-galactosidase A (α-GalA; EC 3.2.1.22). The prevalence in males is estimated as 1:40 000 to 1:60 000 (Desnick et al 2001; Meikle et al 1999). In classically affected male individuals, residual activity of α-galactosidase A is (nearly) absent, which results in the pathological accumulation of α-galactosyl-terminated neutral glycosphingolipids, predominantly globotriaosylceramide (GL-3), in cellular lysosomes. Accumulation of GL-3 occurs in virtually all tissues of the body, but particularly in the endothelial, perithelial and smooth-muscle cells of blood vessels, ganglion cells of the autonomic nervous system, glomeruli and tubules of the kidney and the cardiomyocytes of the heart (Desnick et al 2001). In the classical phenotype, deterioration of renal function will ultimately progress to end-stage renal disease in the third to fifth decades of life (Desnick et al 2001). Cardiac manifestations causing significant morbidity may include cardiomyopathy, angina pectoris, congestive heart failure, myocardial ischaemia and arrythmias (Linhart et al 2002). Cerebrovascular involvement can lead to transient ischaemic attacks, stroke and other neurological disorders (Kolodny and Pastores 2002). The clinical spectrum of Fabry disease also includes a 'renal variant' and a 'cardiac variant' phenotype in patients without classic symptoms who predominantly develop end-stage renal disease (Nakao et al 2003) and cardiac manifestations (Elleder et al 1990; von Scheidt et al 1991), respectively. Heterozygotes are also prone to manifest disease symptoms owing to the phenomenon of random X-chromosome inactivation (lyonization) (Lyon 2002). The disease spectrum in female carriers may range from asymptomatic disease to the classic phenotype.

Treatment of Fabry disease used to be limited to supportive care such as management of pain and hypertension (Desnick and Wasserstein 2001). Clinical trials with two enzyme replacement therapies, agalsidase alfa (Replagal; Transkaryotic Therapies, Inc., Cambridge, MA, USA) (Schiffmann et al 2000, 2001) and agalsidase beta (Fabrazyme; Genzyme Corporation, Cambridge, MA, USA) (Eng et al 2001a,b) have led to the commercial availability of these two products: both agalsidase beta and agalsidase alfa in the European Union and only agalsidase beta in the United States.

A phase 2 open-label trial was undertaken in patients of Japanese descent to evaluate whether ethnic differences exist that would affect treatment of Fabry disease with agalsidase beta in this population, in relation to safety and efficacy. The study design, including the safety and efficacy end points, mirrored the design of the completed phase 3 double-blind study with agalsidase beta (Eng et al 2001b). This choice of design was based on the concern that the relatively small number of patients with Fabry disease in Japan would not adequately power a primary end point in an open-label, single treatment group study design. The results of this phase 2 bridging study are reported here.

PATIENTS AND METHODS

Study design, patients, treatment regimen: This multicentre, phase 2, open-label trial was designed to evaluate the efficacy and safety of agalsidase beta treatment

and enrolled 13 male patients with confirmed Fabry disease. The patients received 1 mg/kg of agalsidase beta administered as 11 biweekly infusions over 20 weeks. Participating study sites were The Jikei University School of Medicine, Chubu National Hospital, Osaka University School of Medicine, Nagoya University School of Medicine, and Kyusyu University School of Medicine. The protocol was approved by the relevant institutional review boards and the trial was conducted in accordance with the Good Clinical Practice guidelines.

Patient eligibility criteria, as well as the clinical, biochemical, biopsy tissue (kidney, heart, skin) and safety assessments, were identical to those applicable for the phase 3 double-blind study. Refer to the publication by Eng et al (2001b) for details. For the management of potential infusion-associated reactions, the patients were pretreated with acetaminophen ($\leqslant$500 mg) or ibuprofen ($\leqslant$200 mg) and hydroxyzine (up to 30 mg) administered 1 h prior to each infusion. The infusion rate was less than 0.25 mg/min.

Evaluation of efficacy: The renal efficacy end point was the proportion of patients with a zero score for GL-3 deposits at week 20 (11 infusions). Additional tissue efficacy end points included microvascular endothelial deposits of GL-3 in the heart, skin and other kidney cell types. Biopsy tissues were scored (0–1–2–3 scoring system) by three blinded, independent pathologists. Specimens with no microvascular endothelial deposits or only trace amounts (normal or nearly normal) were given a score of 0; specimens in which the majority of vessels had evidence of a single endothelial inclusion (mild GL-3 accumulation) were given a score of 1; those with multiple aggregates of granules in the majority of capillaries were given a score of 2 (moderate GL-3 accumulation); and those with numerous aggregates of granules within the endothelium (often bulging into the lumen) in the majority of vessels were given a score of 3 (severe GL-3 accumulation). Majority scores were calculated per organ as well as summed for all organs. Change from baseline to week 20 was also assessed for GL-3 concentrations (ELISA assay) in kidney, urine sediment and plasma. Quality of life measurements included the Short Form McGill Pain Questionnaire and SF-36 Health Status Survey.

Statistical analysis: An exact binomial matched pair procedure was used primarily for the analysis of GL-3 accumulation in the capillary endothelium of the kidney to analyse the proportions of patients with a score of 0 at baseline compared to week 20. This test was also used to analyse GL-3 accumulation in the other cell types in the kidney and skin. A one-sample Wilcoxon signed-rank test was used to determine whether there was a significant difference from zero in the median change score from baseline to week 20. Descriptive statistics (n, mean, standard deviation, minimum, median, maximum) were displayed at baseline, at week 20, and for changes from baseline to week 20 for multiple parameters, such as the kidney, urinary and plasma GL-3 (ELISA), GL-3 accumulation in additional cell types, and the quality of life results as measured by the Short Form McGill Pain Questionnaire and SF-36 Health Status Survey. Change in SF-36 parameters was analysed by the Wilcoxon signed-rank test.

RESULTS

Patients: Baseline characteristics and demographic data for the 13 patients enrolled in the study are presented in Table 1.

Kidney GL-3 clearance

Kidney capillary endothelial cells. Reduction in kidney capillary endothelial cell GL-3 accumulation from non-0 scores to a 0 score (clearance) by week 20 was achieved by 12/13 (92%) patients ($p < 0.001$). At baseline, GL-3 accumulation was mild (score = 1) for 10 of 13 (77%) patients and moderate (score = 2) for 3/13 (23%) patients. All patients (10/10) with mild accumulation showed clearance at week 20. The same observation was made for 2 of 3 (67%) patients who had moderate accumulation at baseline. The third patient exhibited a reduction from moderate to mild. The overall change in median histology score for all 13 patients was −1.0 ($p < 0.001$).

Other kidney cell types. All 11 patients with kidney glomerular endothelial cell GL-3 accumulation (non-0 score) at baseline achieved clearance (0 score) at week 20. Of these patients, 7/11 (64%) had severe GL-3 accumulation (score = 3) at baseline, and 4/11 (36%) had moderate accumulation (score = 2).

All 12 patients with kidney noncapillary (arteriolar) interstitial endothelial cell GL-3 accumulation (non-0 score) at baseline achieved a 0 score at week 20. Of these 12 patients, 10 (83%) had severe GL-3 accumulation and 2 (17%) had moderate GL-3 accumulation in noncapillary (arteriolar) interstitial endothelial cells at baseline.

Skin GL-3 clearance

Skin capillary endothelial cells. Twelve of 13 (92%) patients achieved a reduction of skin capillary endothelial cell GL-3 from non-0 scores to a 0 score (clearance) at week 20 ($p < 0.001$). For these 12 patients, at baseline GL-3 accumulation was severe

Table 1 Patient details

Age (years)	
Mean ± SD	26.6 ± 5.5
Range	16–34
Weight (kg) (mean ± SD)	59.1 ± 8
Sex (*n*)	
Male	13
Female	0
Plasma α-GAL activity	BDL[a]
Leukocyte α-GAL activity	BDL[a]
Serum creatinine (mg/dl) (mean ± SD)	1.1 ± 0.28
Completed study *n* (%)	13 (100)

[a] BDL, below detectable level (<0.78 nmol/h per ml for plasma α-GAL and <0.78 nmol/h per mg for leukocyte α-GAL)

(score = 3) for 5 patients, moderate (score = 2) for 6, and mild (score = 1) for 1 patient. One patient with moderate GL-3 accumulation at baseline achieved a reduction to mild at week 20. The overall change in median histology score for all patients was −2.0 ($p < 0.001$).

Other skin cell types. Of patients with deep-vessel endothelial cell GL-3 accumulation in the skin (non-0 score) at baseline, 10/12 (83%) patients achieved reduction to a 0 score at week 20, and 2/12 (17%) patients achieved a decrease in GL-3 accumulation from moderate (score = 2) to mild (score = 1) at week 20.

Heart GL-3 clearance—heart capillary endothelial cells: Only one patient met the criteria for baseline cardiac biopsy as defined in the protocol. The cardiac capillary endothelial cells from this patient showed reduction of GL-3 from 'mild' (score = 1) GL-3 accumulation in cardiac capillary endothelial cells at baseline, to complete clearance at week 20.

GL-3 clearance—kidney, urine, plasma (by ELISA): The results of GL-3 clearance in the kidney, urinary sediment and plasma are shown in Table 2. In the 13 patients, the median kidney, urinary, and plasma GL-3 levels decreased by 51.9% ($p = 0.003$), 0.003), 55.4% ($p = 0.244$), and 100% ($p < 0.001$), respectively, between baseline and week 20.

Creatinine clearance: Creatinine clearance was used as a measure of the change in renal filtration function from baseline to week 20. The median creatinine clearance was 125.9 ml/min (mean ± SD = 126.6 ± 41.8) at baseline and 120.2 ml/min (mean ± SD = 115.3 ± 30.4) at week 20. This difference was not statistically significant ($p = 0.216$; Wilcoxon signed rank test). Median serum creatinine levels also

Q1 Table 2 Mean change from baseline to week 20 (11 infusions) for GL-3 levels in kidney, urine, and plasma (ELISA)

Organ		*Baseline*	*Week 20*	*% Change from baseline (week 20)*	*p-Value*[a]
Kidney (ng/mg)	Mean ± SD	2972 ± 1529	1667 ± 1760	−46.2	0.003
	Median	3149	1182	−51.9	
	Range	341–5098	171–6122		
Urine (nmol/filter)	Mean ± SD	4085 ± 2077	2687 ± 2514	65.1[b]	0.244
	Median	3680	1278	−55.4[b]	
	Range	62–7340	313–8080		
Plasma (ng/μl)	Mean ± SD	3.9 ± 2.7	0.2 ± 0.8	−89.4	<0.001
	Median	3.6	0	−100	
	Range	0–9	0–3		

[a] *p*-Value derived from a Wilcoxon signed rank test on change from baseline to week 20
[b] Values are correct. The upper end of the range (min–max) consisted of only a few patients; therefore, the median fell on a negative value

remained relatively stable at week 20 (not shown), suggesting maintenance of renal function.

Clinical assessments: Results from laboratory tests indicate that treatment with r-hαGAL appears to have no toxic effect. Ophthalmic, ECG, and echocardiogram findings further support this observation.

Quality of life assessments: Overall, median pain scores were at the low end at baseline and showed slight improvement at week 20 in all parameters. The median change score for present pain intensity (PPI) approached statistical significance ($p = 0.063$). Likewise, there was improvement in all categories for the SF-36 Health Status Survey. Statistically significant improvement was observed in median values for the General Health ($p = 0.023$) and the Mental Component Scale (MCS) scores ($p = 0.048$). The median values from baseline to week 20 for the category Role – Emotional approached statistical significance ($p = 0.063$). p-Values presented in this section refer to the Wilcoxon signed rank test.

Safety: All patients completed the study and each received all 11 infusions of agalsidase beta. All patients reported at least one adverse event (AE). Relation of AEs to the study drug were defined as possible, probable, definite or unknown. The most frequently reported related AEs were rigors (chills) and fever. These were infusion-associated reactions (related events that occurred on the same day as the infusion) and were mild or moderate in intensity (Table 3). These events were often managed with antihistamines and antipyretics or with a reduction in the infusion rate.

One patient experienced a serious adverse event considered to be related to the infusion. The patient was hospitalized overnight for observation owing to persistent malaise (related to fever) and limb pain after the infusion (probably related). The patient recovered without sequelae.

IgG seroconversion occurred in 11/13 (85%) patients. The mean time to seroconversion was 63.3 days. Seroconversion did not affect the patient's response to treatment. No IgE antibody formation was detected in any of the patients.

Table 3 Related adverse experiences occurring in >10% of patients

	Severity of adverse experience (% of patients)	
WHOART preferred term	*Mild*	*Moderate*
Rigors	3 (23)	2 (15)
Fever	2 (15)	2 (15)
Malaise	2 (15)	0
Dyspnoea	2 (15)	0
Rhinitis	2 (15)	0
Hypertension	1 (8)	1 (8)

AE counted once (most severe occurrence) if reported more frequently. There were no adverse experiences of severe intensity