electron donor that can transfer electrons to cytochrome *c*. TMPD reduced by ascorbate renders electrons to cytochrome *c*, which transfers electrons to complex IV. The fact that ATP production in the presence of succinate plus rotenone and in the presence of TMPD plus ascorbate is similar in the two groups of mitochondria indicates that the respiratory chain downstream of complex II is not affected by chronic exposure to rapamycin. Moreover, immunoblotting revealed that expressions of respiratory chain proteins including complex I, III, IV, and V were not affected by rapamycin treatment. Antibodies used in the present study were raised against 39 kDa subunit in complex I, core II in complex III, subunit I in complex IV, and subunit α in complex V. In these subunits, subunit I in complex IV is derived from an mtDNA-encoded gene; the others are from nuclear genes (Hunte 2001, Richter & Ludwig 2003, Scheffler 2008, Zickermann *et al.* 2009), indicating that rapamycin does not affect the expression of respiratory proteins derived from mtDNA or nuclear genes in islets. Considered together, these results do not support the notion that rapamycin reduces ATP production by reducing activity of the respiratory chain. Because the decrease in ATP production was found in the presence of substrates that are metabolizable in the Krebs cycle by rapamycin treatment, the reduction in ATP production may be attributable to reduced carbohydrate metabolism in the Krebs cycle.

Glucose oxidation reflects the velocity of carbohydrate metabolism in the Krebs cycle in which CO_2 is released in the reaction mediated by dehydrogenases. To clarify the link between reduced mitochondrial ATP production in the presence of substrates metabolizable in the Krebs cycle and reduced glucose oxidation by rapamycin, recovery of insulin release and ATP content in the presence of high glucose by ROS scavengers and activity of enzymes in the Krebs cycle were examined. Ouabain-induced endogenous ROS suppresses mitochondrial metabolism in the Krebs cycle, subsequently reducing ATP production, and reduces glucose-induced insulin release and ATP levels in the presence of high glucose, which is recovered by the suppression of endogenous ROS production and by ROS scavenge (Kajikawa *et al.* 2002, Kominato *et al.* 2008). High glucose raises ROS level in β-cells (Bindokas *et al.* 2003, Sakai *et al.* 2003), which is also found in our previous study (Kominato *et al.* 2008). However, our previous study shows that ROS scavenging does not affect glucose-induced insulin secretion from control islets, but increases that from GK-diabetic islets. A more profound increase in high glucose-induced ROS was observed in diabetic islets compared with control islets. These results suggest that a physiological level of ROS increase by glucose does not impair stimulus-secretion coupling, while a pathophysiological increase in ROS impairs stimulus-secretion coupling. Administration of H_2O_2, the most abundant ROS, to mitochondria reduced the activity of Krebs cycle enzymes including aconitase, KGDH, and succinate dehydrogenase (Tretter & Adam-Vizi 2000, Nulton-Persson & Szweda 2001). Because α-tocopherol is a lipid-soluble antioxidant, it is often used as membrane-permeable ROS scavenger. α-tocopherol reduces ROS production in various kinds of cells (Saito *et al.* 2003, Brookheart *et al.* 2009, Yang *et al.* 2009) including β-cells (Kajikawa *et al.* 2002). As ascorbate is water soluble, it is not necessarily membrane permeable. However, it is useful to prevent oxidization of α-tocopherol in the medium and to maintain the ROS-scavenging effect of α-tocopherol. Because insulin release and ATP content in the presence of high glucose in rapamycin-treated islets were not increased by the addition of α-tocopherol plus ascorbate, overproduction of endogenous ROS seems not to participate in reduced mitochondrial carbohydrate metabolism due to rapamycin treatment.

Impaired metabolism-secretion coupling in β-cells due to reduced activity of enzymes in the Krebs cycle has been reported. Exposure to fatty acids for 48 h inhibits glucose-induced insulin secretion from islets with decreased activity in PDH (Zhou & Grill 1995). Interleukin-1β-induced nitric oxide production leads to inhibition of glucose-induced insulin secretion together with reduced aconitase activity (Welsh *et al.* 1991). In the present study, activity of KGDH was decreased by rapamycin treatment. The reaction catalyzed by KGDH is one of the slowest steps in the Krebs cycle, and thus can be the rate-limiting step in islets (Ashcroft 1981) and in other tissues (Tretter & Adam-Vizi 2000, Nulton-Persson & Szweda 2001). Inhibition of KGDH alters mitochondrial function in N2a neuroblastoma cells (Huang *et al.* 2003). These findings suggest that decreased activity of KGDH might reduce mitochondrial ATP production and result in decreased glucose-induced insulin secretion from rapamycin-treated islets. To investigate this, suppression of mitochondrial ATP production by inhibition of KGDH was examined using KMV, a specific competitive inhibitor of KGDH (Huang *et al.* 2003). KMV dose dependently suppressed mitochondrial ATP production in the presence of malate and pyruvate. This dose dependency of KMV on mitochondrial ATP production is consistent with the dose-dependent effect of KMV on KGDH activities previously described (Huang *et al.* 2003). These results indicate that reaction at KGDH may limit the velocity of carbohydrate metabolism in the Krebs cycle and thus mitochondrial ATP production, which is consistent with the result that KGDH limits the amount of NADH available for the respiratory chain (Tretter & Adam-Vizi 2000). These results support the notion that a slight alteration in KGDH activity may affect mitochondrial ATP production.

While rapamycin shares with tacrolimus a similar molecular structure and binding ability to FK-binding protein 12 (FKBP12), the FKBP12–rapamycin complex has no effect on calcineurin, a phosphatase that is known to be inhibited by the FKBP12–tacrolimus complex (Saunders *et al.* 2001). This is consistent with our finding in the present study that rapamycin had no effect on glucokinase activity, but tacrolimus suppresses glucokinase activity in islets (Radu *et al.* 2005). MTOR has an FKBP12–rapamycin binding domain to which phosphatidic acid (PA) can also bind.

MTOR expresses biological effects by forming two types of complexes, MTORC1 and MTORC2, which includes MTOR and PA commonly and Raptor and Rictor respectively. FKBP12–rapamycin is believed to inhibit MTOR signaling by preventing the interaction between MTOR and PA and thus forming MTOR complexes (Foster & Toschi 2009). Since low concentrations of rapamycin (0·5–100 nM) target MTORC1 and higher concentrations of rapamycin (0·2–20 μM) target MTORC2 (Foster & Toschi 2009), our result may be derived from inhibition of signaling mediated by MTORC1.

Recently, it has been revealed that MTOR is a nutrient sensor that balances energy metabolism by transcriptional control of mitochondrial oxidative function using peroxisome proliferator-activated receptor γ coactivator-1α in skeletal muscle cells (Cunningham *et al.* 2007). Further investigation of suppression of KGDH activity by rapamycin is required to clarify adaptation of mitochondrial oxidative function and insulin secretion according to nutrient supply.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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ABC transporter A3 facilitates lysosomal sequestration of imatinib and modulates susceptibility of chronic myeloid leukemia cell lines to this drug

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ABSTRACT

Background
Inhibition of BCR-ABL tyrosine kinase activity has evolved as a mainstay of therapy for patients with chronic myeloid leukemia. However, a fraction of leukemic cells persists under targeted therapy and can lead to disease progression on cessation of treatment.

Design and Methods
We analyzed bone marrow progenitor cells with the side population phenotype, and characterized the role of the intracellular ABC transporter A3 in imatinib detoxification.

Results
BCR-ABL-positive leukemic cells contribute to the side population cell compartment in untreated patients. Such leukemic side population cells, as well as CD34-positive progenitors from chronic myeloid leukemia samples, strongly express the intracellular ABCA3. Functionally, ABCA3 levels are critical for the susceptibility of chronic myeloid leukemia blast cell lines to specific BCR-ABL inhibition by imatinib. The transporter is localized in the limiting membrane of lysosomes and multivesicular bodies, and intracellular [^{14}C]-labeled imatinib accumulates in such organelles. The lysosomal storage capacity increases with ABCA3 expression, thus regulating imatinib sequestration.

Conclusions
The intracellular ABC transporter A3 is expressed in chronic myeloid leukemia progenitor cells and may contribute to intrinsic imatinib resistance by facilitating lysosomal sequestration in chronic myeloid leukemia cells.

Key words: imatinib resistance, ABC transporter, lysosome, chronic myeloid leukemia, side population.

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The online version of this article contains a supplementary appendix.

Introduction

Targeting the disease-specific BCR-ABL translocation by tyrosine kinase inhibition with imatinib represented a major therapeutic advance for patients with chronic myeloid leukemia (CML).[1-5] Cessation of imatinib treatment is, however, is followed by disease recurrence, and the subgroup of patients who relapse under imatinib therapy often display mutations in the ATP-binding site of BCR-ABL, a mechanism of acquired imatinib resistance.[6-9] These observations suggest that imatinib effectively controls the disease, but may not eradicate the leukemic clone at the level of rare persistent cells with clonogenic capacity.

Elucidation of the characteristic propensities of acute myeloid leukemia (AML) cells with side population (SP) phenotype showed that the intracellular ATP-binding cassette (ABC) transporter A3 (ABCA3) is strongly expressed in leukemic SP cells.[10-13] ABCA3 has a crucial function in surfactant production and secretion from type 2 pneumocytes in the mammalian lung, leading to fatal respiratory distress syndrome in neonates carrying dysfunctional gene mutations.[14] In AML cells, however, we found ABCA3 expression to be associated with drug resistance against a broad spectrum of cytostatic substances, mediated by sequestration of drugs into lysosomal organelles.[15] In this study we analyzed the bone marrow of patients with CML for leukemic SP cells, measured the expression of ABCA3 in such cells, recognized a critical role for ABCA3 in imatinib susceptibility, and discovered subcellular sequestration as an efficient mechanism of cellular imatinib detoxification.

Design and Methods

Patients, Hoechst 33342 staining, cell sorting, and fluorescence in situ hybridization

The bone marrow specimens examined (n=35) were archived material collected from a cohort of 30 adult CML patients before therapy and under surveillance during imatinib treatment at the University Hospital in Goettingen, Germany. This retrospective study was submitted to the Ethics Committee of the University of Goettingen, and no objections were raised. As control samples, adult human bone marrow progenitor cells were isolated from routine diagnostic posterior iliac crest aspirates of individuals without marrow disease involvement. Mononuclear cells were separated from whole bone marrow aspirates using density centrifugation with Ficoll (Pharmacia). For SP cell analysis, the mononuclear cell fractions were stained with the fluorescent dye Hoechst 33342 (Sigma) as previously described.[8] Fluorescence *in situ* hybridization (FISH) analysis was performed following standard protocols with BCR-ABL dual color/dual fusion probes (Vysis, Downers Grove, IL, USA).

Cell culture

K562, LAMA84 and BV17.3 cells (DSMZ, Braunschweig, Germany) were propagated in RPMI 1640 medium, and HEK293 cells in Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES, GlutaMAX I (Gibco-BRL), and penicillin/streptomycin (Sigma, Biochrom). K562, LAMA84 and HEK293 cells were supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL), while 20% serum was used for BV173. The stable ABCA3-eGFP-expressing transgenic cell line[16] was additionally supplemented with 300 μg/mL G418. For all assays, the transfected cell lines were propagated without G418 for four passages, without losing transgene expression as evaluated routinely by fluorescence microscopy.

Enforced expression and small interfering RNA of ABCA3

pEGFP-N1-ABCA3 wild-type or pEGFP-N1-ABCA3 N568D mutant plasmids[16] as well as the pre-designed short interfering RNA (siRNA) against ABCA3 and scrambled siRNA (Qiagen) were delivered by electroporation as previously described.[15] Using siRNA knockdown, the nadir of ABCA3 mRNA and *ABCA3* protein expression was documented 72 h after electroporation, as tested by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and western blotting (*Online Supplementary Appendix Figures S2*).

Cytotoxicity assays

Cell viability was determined using the MTT assay as previously described.[15] The specific viability is expressed as the ratio of the absorbance with drug versus the absorbance of vehicle control. The IC_{50} was defined as the concentration of drug causing a 50% inhibition of cell growth, compared with the vehicle control. To analyze the clonogenicity, 500 (K562 or LAMA84) to 1000 (BV173) cells were seeded in triplicate into 1 mL methylcellulose medium supplemented with increasing amounts of imatinib ranging from 0 to 1000 nM. Cells were allowed to form colonies by incubating at 37°C, and the number of colonies was counted on day 10 of culture. Specific clonogenicity was expressed as a fraction of vehicle control.

Accumulation of intracellular [^{14}C]-imatinib, nuclear fractions, subcellular fractionation and in vitro [^{14}C]-imatinib exposure to isolated lysosomal fractions

Intracellular accumulation of [^{14}C]-imatinib (a gift from Novartis, Basel, Switzerland) was measured as previously described.[17] For subcellular fractionation, cells were incubated with 2.14 μM imatinib (0.1 μCi [3.7 kBq]) for 2 h. After washing with ice-cold phosphate-buffered saline, cells from each cell line were harvested from three 15 cm dishes and homogenized in 0.25 M sucrose buffered with 3 mM imidazole/HCl pH 7.3, 1 mM EDTA and a 1:500 dilution of a protease inhibitor cocktail (Sigma) using five strokes of a G27 syringe. A post-nuclear supernatant was prepared (5 min /500 g/4°C) and 6 mL of this protein normalized post-nuclear supernatant was adjusted to a final concentration of 20% percoll solution (12 mL) in 0.25 M sucrose, 3 mM imidazole/HCl, 1 mM EDTA pH 7.3 and centrifuged for 30 min at 35,000g without brakes in a vertical tube rotor Vti 65.1 (Beckmann Instruments). After removal of percoll, the

supernatant and membranes were prepared by centrifuging each of the 23 fractions for 1 h at 100,000g. Membranes were resuspended in 0.5% Triton X-100/phosphate-buffered saline and used for further analysis. For *in vitro* exposure of [^{14}C]-imatinib to isolated lysosomal fractions, subcellular fractionation was performed as described above. The lysosomal fractions, harboring at least 85% of total β-hexosaminidase activity, were pooled, divided into five samples and each sample adjusted to 1x *in vitro* reaction buffer (0.25 M sucrose, 20 mM KCL, 2.5 mM Mg/acetate, 125 μM DTT and 25 mM HEPES pH7.2). Subsequently 3.8 μM [^{14}C]-imatinib was applied to isolated lysosomes for 2 h under different conditions, i.e. 4°C, 37°C, and supplementation of an ATP-regenerating system. Unlabeled imatinib was added as a competitor to [^{14}C]-imatinib in 650x excess, and chloroquine was added at a concentration of 10 μM. After 2 h of incubation with imatinib, lysosomal membranes were pelleted by 1 h of 100,000g centrifugation at 4°C. Membranes and supernatant were collected for quantification of [^{14}C] and β-hexosaminidase activity.

Measurements of lysosomal matrix enzymes and western blotting

The enzyme activity of β-hexosaminidase, β-glucuronidase and α-mannosidase was measured using either 10 mM p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, 20 mM p-nitrophenyl-β-D-clucuronide or 10 mM p-nitrophenyl-α-D-mannopyranoside (all from Sigma) as substrates, prepared at 10 mM in 0.1 M sodium citrate (pH 4.6). Aliquots of the sample (5-10 μL) were mixed at 4°C with 100 μL of the substrate, then incubated for 0.5–4 h at 37°C. Reactions were stopped by adding 0.5 mL of 0.4 M glycine/NaOH (pH 10.4), and the specific activities calculated by measuring the absorbance at 405 nm.

Western blotting was performed as previously described.[15] The monoclonal mouse antibody to EEA1 was obtained commercially (Transduction Laboratories), the antibodies to LAMP-1 (code H4A3) and Na^+/K^+-ATPase (code 6Fα) were provided by the Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA). P-Crkl(Tyr207) was studied with a commercially available monoclonal antibody (clone 32H4, Cell Signaling Technologies, Danvery, MA, USA). The murine monoclonal antibody against green fluorescent protein was a generous gift from A. A. Noegel (Biochemistry I, University of Cologne, Germany), the polyclonal rabbit antibody against human ABCA3 was provided by N. Inagaki and N. Ban.[18] Cross-reactivity to other ABC transporters of this antibody has not been observed (*data not shown*). Actin controls were run in parallel throughout, except in the p-Crkl blot, in which Ponceau-red staining of protein was applied. The phosphorylation status of the BCR-ABL adaptor protein, Crkl, was determined by western blotting conducted according to the manufacter's recommendation.

Statistical evaluations

Differences between samples treated with different dose levels were analyzed using the two-way ANOVA with Bonferroni's post-test, differences between cohorts of samples were tested using Kruskal-Wallis test with Dunn's multiple comparison post test using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego California USA, *www.graphpad.com*), Wilcoxon's signed-rank test, or a two-tailed Student's t-test as indicated.

Miscellaneous

Total RNA extraction, quantitative RT-PCR, immunohistochemistry and electron microscopy were conducted as previously described.[15]

Results

Detection of the malignant clone in bone marrow side population cells of patients with chronic meyloid leukemia

In previous studies on AML, we had discovered that bone marrow SP cells harbor cells of the leukemic clone.[11] As CML is a stem cell disease, in this study we analyzed such early hematopoietic stem cells by flow cytometric cell sorting and consecutive FISH probing of the BCR-ABL translocation. SP cells were readily detected in the bone marrow samples of patients with CML, albeit at low frequencies (0.6%, range 0.1% to 1.8% of all measured cells, n=11). However, the proportion of BCR-ABL-positive cells in the SP compartment closely mirrored the leukemic involvement in the main population (*Online Supplementary Table S1*). We found BCR-ABL-positive cells in the SP cells in all of seven cases of active disease, while the SP cells in three samples from patients in complete cytogenetic remission under imatinib treatment did not contain BCR-ABL-positive cells (*Online Supplementary Table S1*). Thus hematopoietic stem cells with the SP phenotype are involved by the leukemic clone, and are devoid of gross leukemia in imatinib-induced cytogenetic remission.

Expression of ABCA3 in chronic myeloid leukemia progenitor cells

By examining the dye efflux mechanisms contributing to the SP phenotype, we had previously determined the expression of intracellular ABCA3 in leukemic progenitor cells from patients with AML.[11-13] Here, we analyzed the expression of ABCA3 in cells from patients with CML, and found significantly higher levels of ABCA3 mRNA in the bone marrow of CML patients, compared to in the marrow of normal volunteers (Figure 1A, box plot). ABCA3 expression mainly originated from leukemic stem cells with the SP phenotype, as shown by RT-PCR (Figure 1A), as well as from CD34-positive leukemic progenitors, as shown and enumerated by immunocytology (Figure 1D, quantification in C). In normal bone marrow, strong ABCA3 expression was only observed in a fraction of physiological CD34-positive hematopoietic progenitor cells, this heterogeneity was also found in the fraction of leukemic CD34-positive progenitors, although the proportions were generally higher (Figure 1D). Significant ABCA3 levels were also detected in the CD34-negative fractions of patients with CML (Figure 1D, lower right panel), but not in CD34-negative cells from the bone marrow of healthy volunteers. Furthermore, to search for *in vitro* model systems, we screened the well-established

CML cell lines K562, LAMA84 and BV173 and detected high ABCA3 expression levels, comparable to those in primary CML progenitor cells (*Online Supplementary Figure S6*). In addition, we investigated the expression of the plasma membrane transporter ABCG2 in SP versus non-SP cells, and detected no differences in expression levels (*Online Supplementary Figure S1*). Thus, intracellular ABC A3 is expressed in the bone marrow of patients with CML, mainly in SP and CD34-positive progenitor cells.

ABCA3 expression levels are critical for cellular susceptibility to imatinib

Using the K562, LAMA84 and BV173 cell lines as *in vitro* models, we modulated ABCA3 levels by either down-regulation with specific siRNA (*Online Supplementary Figure S2*), or up-regulation by ectopic expression, examining the effect of such manipulations on imatinib susceptibility. With regards to clonogenicity measured by colony formation in methylcellulose, down-regulation of ABCA3

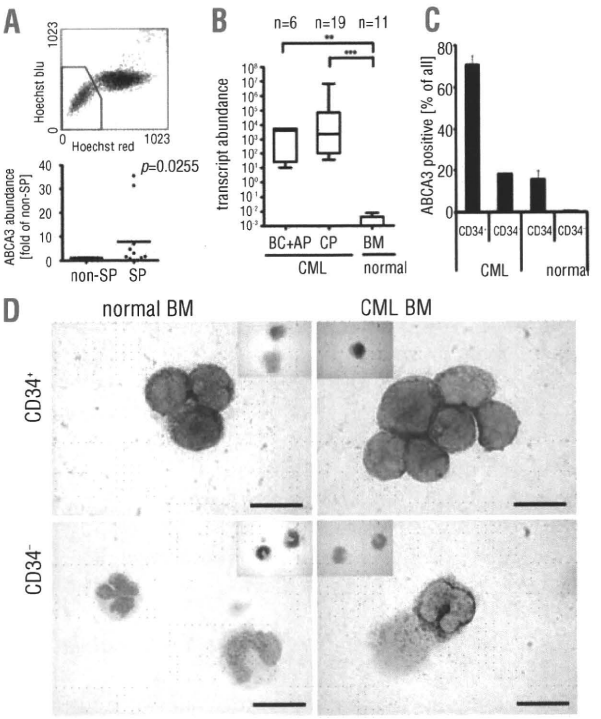


Figure 1. **Detection of the leukemic clone in bone marrow SP cells of patients with CML, and expression of ABCA3 in the stem cell compartment of patients with CML. Bone marrow samples of CML patients were stained with Hoechst 33342 and subsequently sorted into cells with the SP phenotype (A upper panel, as boxed) and the remaining non-SP cells. In the samples from patients with untreated CML (n=11, blast crisis excluded), ABCA3 transcript levels, as detected by quantitative RT-PCR were higher in the SP cell fraction than in the non-SP cell fraction (A, lower panel, Wilcoxon's signed rank test). As for whole marrow, ABCA3 transcripts were detected by quantitative RT-PCR in 25 bone marrow samples from patients with CML (19 cases in chronic phase, 4 cases in acceleration, and 2 cases in blast crisis), with significantly higher levels of expression in leukemic samples than in bone marrow cells of healthy volunteers (B, middle panel, Kruskal-Wallis test with Dunn's multiple comparison post-test, ** and *** indicate $p<0.01$ and $p<0.001$, respectively). In bone marrow from healthy volunteers, immunocytological detection of the ABCA3 protein revealed a small fraction of positive cells (17%) in sorted CD34-positive cells, and no ABCA3-positive cells in the CD34-negative fraction (D, left column, quantification in C). The scale bars represent 10 μm, the respective isotype control stains are shown as inserts. In the marrow samples of CML patients, ABCA3-positive cells were found in both the CD34-positive and in the CD34-negative fractions, with a significantly higher proportion of positive cells in the CD34-positive fraction (D, right column, quantification in C). One hundred cells each from three representative slide sectors of three different individuals were counted.**

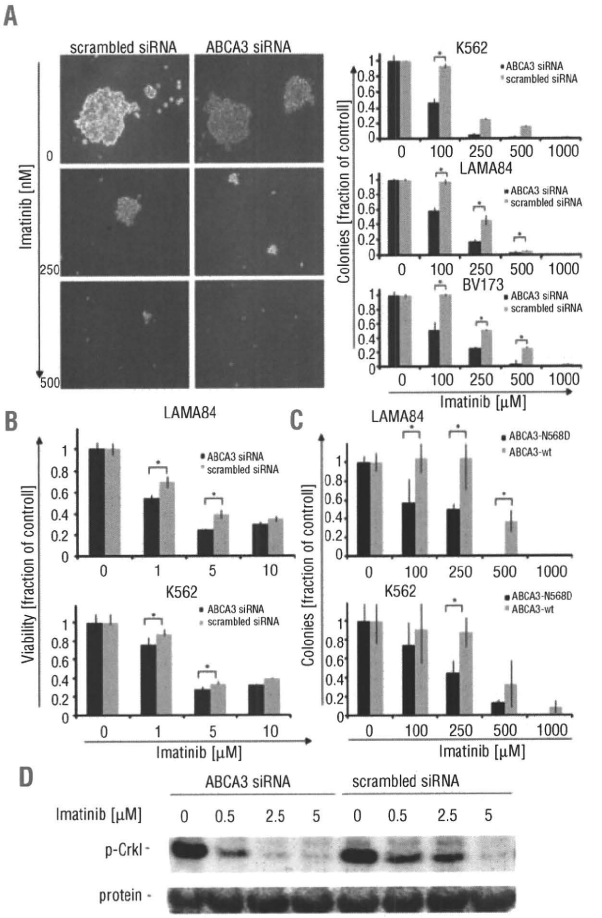
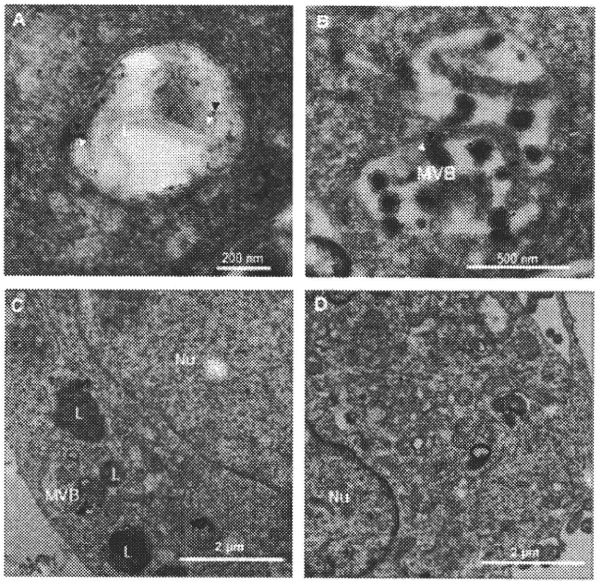


Figure 2. **ABCA3 modulates susceptibility of CML cell lines to imatinib and imatinib-mediated inhibition of Crkl phosphorylation. Clonogenic growth of BCR-ABL-positive cell lines exposed to imatinib was measured in colony-forming-unit (CFU) assays after specific siRNA-mediated suppression of ABCA3 expression levels, or addition of scrambled siRNA. Incubation with increasing doses of imatinib reduced the number and size of colonies, with markedly smaller colonies after specific knock-down of ABCA3 (A, middle and lower panels of the right column). Over an imatinib dose range of 100 to 500 nM, ABCA3 knock-down significantly reduced the number of colonies obtained from the K562, LAMA84 and BV173 cell lines (A, right panel). Correspondingly, ABCA3 knock-down sensitized K562 and LAMA84 to the suppressive effects of imatinib on viability as measured by the MTT test (B). *Vice versa*, increasing ABCA3 levels by ectopic expression protected K562 and LAM84 cells from the suppressive effects of imatinib on clonogenicity (C). Differences were tested for statistical significance by two-way ANOVA with Bonferroni's post-test (*$p<0.05$). Forty-eight hours after transfection with siRNA against ABCA3 or a scrambled siRNA control, K562 cells were exposed to imatinib for 16 h at the concentrations indicated (D). The phosphorylation status of the BCR-ABL adaptor protein Crkl (Tyr207) was visualized by western blot with a phosphorylation-specific antibody. Knock-down of ABCA3 shifted the threshold of suppression in Crkl phosphorylation to lower imatinib concentrations.**



significantly increased the susceptibility of all three cell lines to the suppressive effects of imatinib. Compared to control cells treated with scrambled siRNA, suppression of ABCA3 expression levels yielded smaller cell colonies, and significantly reduced the clonogenic potential under increasing doses of imatinib (Figure 2A). Accordingly, ABCA3 down-regulation increased the efficacy of imatinib in tests for cytotoxicity in the LAMA84 and K562 cell lines (Figure 2B). *Vice versa*, elevating ABCA3 levels significantly reduced the efficacy of imatinib, as measured by colony formation, and shown in the comparison of cells

Figure 3. **Localization of ABCA3 to the limiting membrane of lysosomes and multivesicular bodies. Ultramicroscopy following immunogold staining of ABCA3 (black triangles) and the lysosomal membrane protein LAMP-1 (white triangles) revealed that the transporter was localized in the limiting membrane of lysosomes (L), (A) and multivesicular bodies (MVB), B) of K562 cells. Ectopic expression of ABCA3 significantly increased the number of lysosomes (L) and multivesicular bodies (MVB) in HEK293-ABCA3 cells (C), compared to in the HEK293 wild-type control cells (D).**

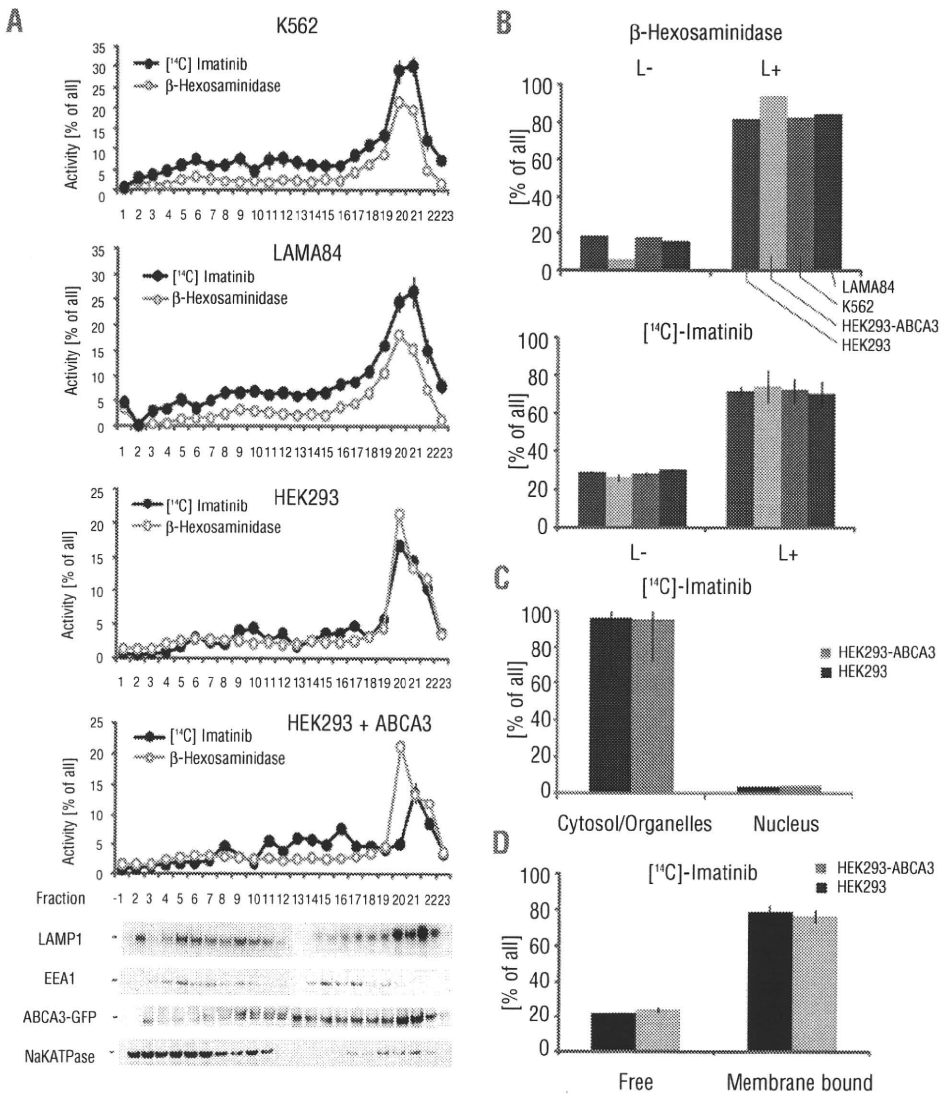


Figure 4. **Biochemical confinement of imatinib to the lysosomal fractions. Following 2 hours of exposure to [^{14}C]-imatinib, cells were homogenized and subcellular fractions of the post-nuclear supernatant separated by density centrifugation. [^{14}C]-imatinib was recovered mainly from the lysosomal fractions (19-22), with an identical pattern of imatinib distribution in the K562, LAMA84, HEK293 and HEK293-ABCA3 cell lines (A). The lysosomal fractions were identified by the lysosomal markers β-hexosaminidase and LAMP-1, and were negative for the early endosome marker EEA1 and the plasma membrane marker Na/K-ATPase (A, lower panel). The ABCA3/GFP fusion protein was also detected mainly in the lysosomal fractions of HEK293-ABCA3 cells (A, lower panel). Comparison of pooled lysosomal (19-23=L+) and non-lysosomal (1-18=L-) fractions confirmed the predominant localization of imatinib to this compartment as a consistent phenomenon in all cell lines (B). Accordingly, most [^{14}C]-imatinib was detected in the post-nuclear supernatant of HEK293 and HEK293-ABCA3 cells (C), and was subsequently co-purified with the intact membranes of organelles, and not in the free cytosol (D).**

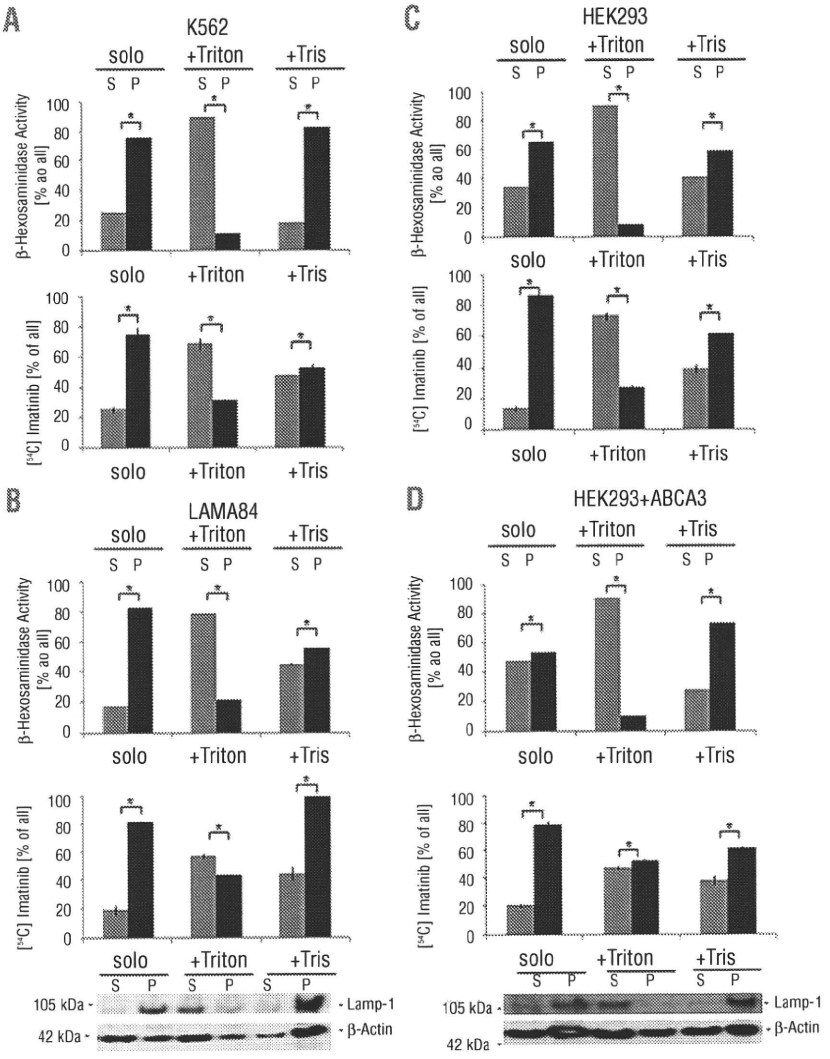


Figure 5. **Release of imatinib from the lysosomal lumen. Following fractionation by density centrifugation, the pooled lysosomal fractions were left untreated (solo), or treated with either Triton-X100 (+Triton) or Tris (+Tris), and then centrifuged again. Supernatant (S) and membrane pellet (P) were then analyzed for imatinib and luminal marker β-hexosaminidase. Disrupting the membrane with Triton-X100, and thereby lysing the lysosomes, released both imatinib and β-hexosaminidase in all cell lines, whereas using Tris as a strong washing buffer, active on the outside of the lysosome, had no such effect (A-D). Importantly, there were no qualitative differences in imatinib release between ABCA3-positive and ABCA3-negative cells (C, D). Western blot of LAMP-1 served as a positive control for the purity of membrane preparations. Differences were analyzed using a two-tailed Student's *t* test (*$p<0.05$).**

with ectopic expression of wild-type ABCA3 and cells expressing the non-functional ABCA3-N568D variant carrying a mutation at the Walker A motif of the transporter (Figure 2C). As a surrogate measure of BCR/ABL function, we analyzed the phosphorylation status of the BCR/ABL adaptor protein, Crkl, and found that knockdown of ABCA3 increased the efficacy of imatinib-mediated inhibition of Crkl phosphorylation in the K562 cell line (Figure 2D). Thus cellular ABCA3 levels are critical for the susceptibility of CML cell lines to imatinib.

Subcellular sequestration of imatinib

The high expression of ABCA3 in the cytoplasm of CML cells, and its protective effects against imatinib, raised our interest in the intracellular mechanisms involved. First, using ultramicroscopy, we detected ABCA3 in the limiting membranes of lysosomes and multivesicular bodies, in proximity to the lysosomal membrane-associated protein 1 (LAMP-1, Figure 3 A,B). Ectopic expression of ABCA3 also induced an abundance of lysosomes and multivesicular bodies in HEK293 cells (Figure 3 C, D). Accordingly, western blot analysis detected the ABCA3/GFP fusion protein from HEK293-ABCA3 cells mainly in the lysosomal fractions after subcellular fractionation, characterized by the concurrent detection of ABCA3 with the lysosomal markers β-hexosaminidase and Lamp1, as well the absence of EEA1 and Na^+K^+-ATPase (Figure 4A, lower panel). These results are consistent with our previous findings in cells from patients with AML and other malignancies.[11,13] We then exposed K562 and LAMA84 cells to [^{14}C]-labeled imatinib, dissected the intracellular content by subcellular fractionation, and found the vast majority of intracellular imatinib in the lysosomal fractions tightly paralleled by the luminal lysosome marker ß-hexosaminidase (Figure 4A, upper panel). An identical pattern of lysosomal imatinib accumulation was also detected in the human embryonic kidney HEK 293 cell line, and in the HEK293-ABCA3 variant with stable transgenic expression of the transporter (Figure 4 A, lower panels). As expected, only small amounts of imatinib were detected in the nuclear fractions (Figure 4 C). We detected a minor proportion (~ 20%) of imatinib as a soluble substance in the cytosol (Figure 4C). For each cell line we pooled the lysosomal fractions of HEK293, HEK293-ABCA3, K562 and LAMA84 cells, and compared these to the non-lysosomal fractions of the same cell

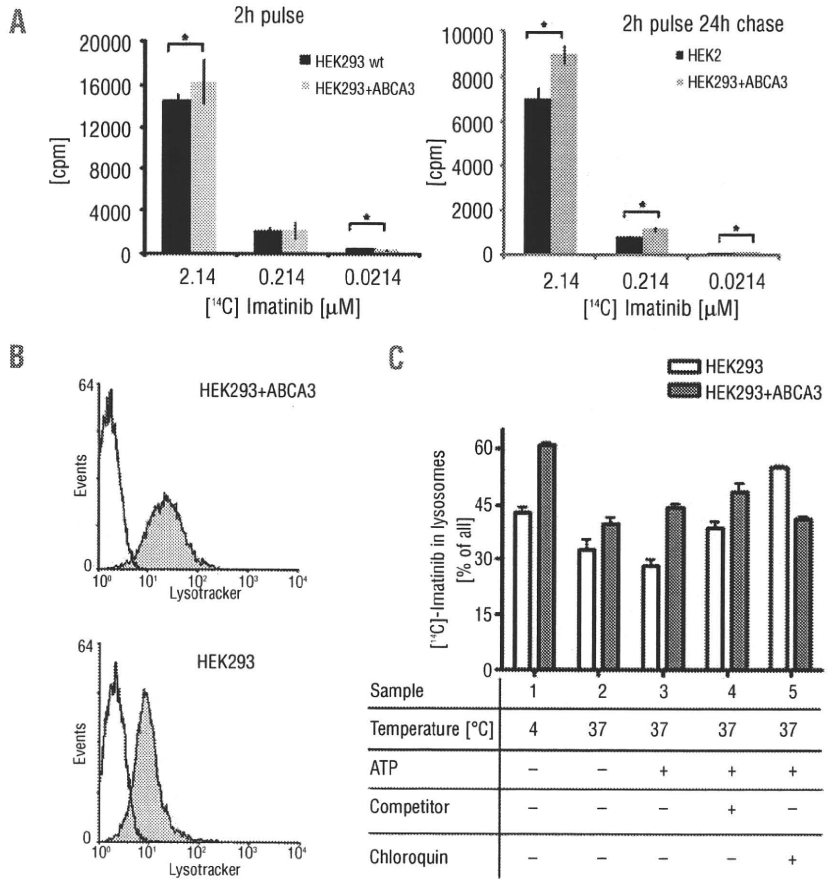


Figure 6. **Impact of ABCA3 on uptake and subcellular sequestration of imatinib. Following exposure to [^{14}C]-imatinib for 2 h at the concentrations indicated, HEK293-ABCA3 cells (gray columns) retained larger amounts of imatinib than did the wild-type HEK293 control cells (black columns) (A). Note the different scale bars between left and right panels, indicating that total cellular [^{14}C]-imatinib is reduced by approximately 50% within 24 h. As shown by using the fluorescent dye Lysotracker red as a marker for lysosomal storage capacity, HEK293-ABCA3 cells retained this substance more efficiently than wild-type HEK293 cells (B). Following incubation of isolated lysosomal fractions with [^{14}C]-imatinib for 2 h (F) at 4°C, uptake was 43% versus 61% of radio-labeled imatinib in the lysosomes of HEK293 and HEK293/ABCA3 cells, respectively (C, sample 1). Incubation at 37°C without ATP (sample 2), with ATP (sample 3), or with ATP and a competitor (sample 4), did not change the ratio of greater imatinib accumulation in ABCA3-positive lysosomes than in ABAC3-negative lysosomes. Differences were tested using a two-tailed Student's *t* test (*$p<0.05$). Representative experiments of four independent replicates are shown.**

lines, confirming the predominant retention of imatinib in the lysosomal fraction as a consistent finding across all four cell lines (Figure 4 B). We further extended this analysis, showing first that imatinib in the lysosomal fractions was indeed confined to membranes (Figure 5 A-D, membrane fractions *solo*). Treatment of the membrane fractions revealed that imatinib could be freed from the lysosomes, together with luminal β-hexosaminidase activity, by membrane distortion with Triton-X100 with quantitative differences between the cell lines, and that it was not dissolved from the outer lysosomal membrane by washing with Tris (Figure 5 A-D). Importantly, such assays revealed no qualitative differences between ABCA3-positive and ABCA3-negative cells.

Whereas the fractionation studies yielded relative results, the HEK293/HEK293-ABCA3 system allowed us to measure the effect of *ABCA3* on imatinib retention in absolute terms. We detected a statistically significant stronger cellular retention of imatinib in the ABCA3 - expressing cells, directly after short-term (2 h) exposure to imatinib (Figure 6A, left panel). Importantly, the amount of radioactive imatinib per cell decreased by approximately 50% within a 24-hour chase period (Figure 6A, right panel), indicating that over a prolonged period of time, subcellular sequestration eventually leads to secretion and cellular depletion of imatinib. Furthermore we found a significantly increased capacity for lysosomal storage in ABCA3-positive cells, as shown by increased accumulation of Lysotracker red in transporter-positive versus transporter-negative HEK293 cells (Figure 6B). In conclusion, intracellular imatinib is sequestered predominantly into the lysosomal lumen in HEK293 and CML cell lines. Finally, we established a cell-free system, in which we exposed the isolated lysosomes to radiolabeled imatinib under different conditions (Figure 6C). In this system, the storage capacity for imatinib of the isolated lysosomes was 2.9 times higher in ABCA3-positive lysosomes than in ABCA3-negative lysosomes. The lysosomes efficiently sequestered imatinib in a process that did not depend on temperature or availability of ATP, but was distorted by chloroquine, which is known to inhibit the ABC-transporter and to disturb lysosomal pH.

Collectively, these findings indicate that the increased imatinib retention in ABCA3-positive cells was due to both an ABCA3-associated increase in lysosomal mass and qualitative changes of ABCA3-positive lysosomal organelles. Imatinib uptake into ABCA3 positive lysosomes occurred by passive mechanisms rather than through active direct transport of imatinib.

Discussion

CML is a disease of the hematopoietic stem cell, and although the specific inhibitor imatinib readily induces complete hematologic and cytogenetic remissions in most patients with chronic-phase disease, evidence of persistent leukemic cells has been observed in the majority of

cases.[19,20] The leukemic cells that are insensitive to imatinib are quiescent in nature, but may give rise to a rapid relapse upon treatment cessation.[20-23] Bone marrow SP cells are very immature hematopoietic progenitors, and here we found that SP cells were involved in all seven cases of active disease. SP cells derived from patients in cytogenetic remission were devoid of BCR-ABL-positive cells, thus indicating that at least a fraction of the SP cell compartment can be cleared of the leukemic clone by imatinib. However, since the average frequency of SP cells in the bone marrow is low (<10%), the absolute number of SP cells accessible for analysis was low, ranging from 6 to 87 cells per patient. As such numbers cannot be considered representative of all hematopoietic SP cells in a given patient, it remains uncertain whether SP cells are truly negative during complete cytogenetic remission, with the leukemia clone hiding in a different hematopoietic compartment, or whether the SP cells in our analysis were negative due to sampling error.

Examining dye and drug extrusion capacity of malignant SP cells as an intrinsic mechanism of drug resistance, we previously found that two ABC transporters are involved, namely ABCG2 and ABCA3.[24,12,13] The plasma membrane ABCG2 protein is a marker of primitive normal hematopoietic stem cells, and was also detected on CML progenitor cells.[25,26] The role of ABCG2 in drug resistance by cellular export of imatinib is controversial because of complex, direct and post-transcriptional interactions between imatinib and this transporter.[26,27,28]

As for ABCA3, this transporter was detected in neoplastic cells of different progeny, with most evidence gathered so far in acute leukemias;[12,13,15,29-31] in AML, ABCA3-expression is positively associated with CD34 positivity.[13] As shown here, ABCA3 is strongly expressed in the progenitor cell compartment of CML, involving both SP cells and CD34-positive cells. However, in contrast to normal hematopoietic cells, in which ABCA3 expression is low and strictly limited to early progenitors such as SP and CD34-positive cells, differentiating CML cells retain notable expression of this transporter beyond the CD34-positive stage (Figure 1). This finding mirrors leukemia-associated disturbances in the expression pattern of other stage-specific markers, and adds to the notion that leukemia stem cells in CML reside in a population with continuous, rather than sharply defined phenotypic boundaries.[32] Importantly, ABCA3 expression occurred in the leukemic cells of untreated patients, documenting an intrinsic, rather than an acquired form of resistance against the drugs sequestered by the ABCA3-mediated mechanism.

In normal physiology, ABCA3 is predominantly expressed in type 2 pneumocytes, and has an essential function in the biogenesis of multilamellar bodies, a specialized type of lysosomal-related organelles in which surfactant is assembled prior to exocytosis.[18,33,34] The precise biochemical role of ABCA3 is unknown, although there is accumulating evidence that ABCA3-mediated transport of lipids, such as phosphatidycholine and phosphatidylglycerol, is crucial for effective biogenesis and composition of such multilamellar bodies.[33,34]

As shown here, ABCA3 function is clearly critical for cellular susceptibility to imatinib, demonstrated by an increase in sensitivity after experimental reduction of ABCA3 levels, as well as by acquired resistance with artificial elevation of protein expression (Figures 2, 3). We traced the intracellular fate of imatinib with a radioactively-labeled substance, and made two surprising, and at first sight, paradoxical findings: first, the net content of imatinib was higher in ABCA3-positive cells than in ABCA3-negative cells, and secondly, the majority of intracellular imatinib was found not in the cytosol, but entrapped in lysosomes (Figure 4). Importantly, the accumulation of imatinib in the lysosomal fraction of cells was similar in all cell types analyzed, i.e. HEK293 reporter cells and the BCR-ABL-positive leukemias, indicating that sequestration of imatinib into lysosomes occurs irrespective of the cell type. The capacity for lysosomal imatinib sequestration did, however, increase with ABCA3 expression, leading to more efficient sequestration and a reduction in cytostatic effects (Figure 2). Efficient subcellular sequestration eventually also leads to efficient export of the drug (Figure 4), presumably by exocytosis. From our experiments on imatinib uptake into isolated lysosomes we conclude that the increased segregation of imatinib in ABCA3-positive lysosomes was not due to an active direct transport of imatinib by ABCA3 as a transmembrane pump, but rather a passive mechanism. So far the ABCA3-dependent qualitative change in the lysosomal organelles, which subsequently leads to the increase in imatinib sequestration capacity, is unknown. As other A-family members of ABC transporters are known lipid translocators, and ABCA3 expression is associated with an increase in lysosomal-related organelles with luminal membrane structures, i.e. multilamellar and multivesicular bodies, we speculate that changes in the lipid composition and structure of lysosomal membranes are responsible for the increased capacity to sequester imatinib. Imatinib is cleared from the circulation through oxidative metabolism catalyzed by hepatic CYP3A, and the resulting metabolites may be pharmacologically active.[35] The role of lysosomal sequestration of such metabolites remains, therefore, to be analyzed.

In conclusion, CML clones involve the stem cell compartment represented by SP cells and CD34-positive progenitors, which express the intracellular ABC transporter A3. In cell line models ABCA3 endows leukemia cells with high lysosomal sequestration capacity, and hence reduces their susceptibility to imatinib. Interference with ABCA3 and the associated subcellular imatinib sequestration mechanism may evolve as a strategy to increase the efficacy of tyrosine kinase inhibition, eventually eliminating CML disease.

Authorship and Disclosures

BC designed and performed research, analyzed data and wrote part of the paper; MP, RK and UR performed research; DW performed research and provided essential reagents; DH performed research; LT contributed support and detailed discussions; GGW designed research, analyzed data and wrote the paper.

We declare that none of the authors have any financial interest related to this work.

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Comparison of Trypsin Inhibitors in Preservation Solution for Islet Isolation

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Islet transplantation has recently emerged as an effective therapy and potential cure for type 1 diabetes mellitus. Recent reports show that the two-layer method (TLM), which employs oxygenated perfluorochemical (PFC) and University of Wisconsin (UW) solution, is superior to simple cold storage in UW for pancreas preservation in islet transplantation. Moreover, we recently reported that islet yield was significantly higher in the ET-Kyoto solution with ulinastatin (MK)/PFC preservation solution compared with the UW/PFC preservation solution in the porcine model and that the advantages of MK solution are trypsin inhibition and less collagenase inhibition. In this study, we compared ulinastatin with another trypsin inhibitor, Pefabloc, in preservation solution for islet isolation. Islet yield before purification was higher in the MK/PFC group compared with the ET-Kyoto with Pefabloc (PK)/PFC group. The stimulation index was higher for the MK/PFC group than for the PK/PFC group. These data suggest that ET-Kyoto with ulinastatin was the better combination for pancreas preservation than ET-Kyoto with Pefabloc. Based on these data, we now use ET-Kyoto solution with ulinastatin for clinical islet transplantation.

Key words: Islet transplantation; Islet isolation; MK solution; Trypsin inhibitor; Preservation solution

INTRODUCTION

Since the report of the Edmonton protocol (37), islet transplantation has advanced significantly and more than 600 type 1 diabetes patients in more than 50 institutions have undergone islet transplantation to cure their disease. The cadaveric pancreas is injured due to brain death, hypotension, and vasopressor therapy, and subsequently from warm ischemia after donor cross-clamping and cold ischemic storage. There is a clear relationship between these injuries and the reduced success of subsequent islet isolation (4,12). In Japan, pancreatic islets are isolated from non-heart-beating donors (NHBDs) for clinical islet transplantation because donations from heart-beating brain-dead donors are only two to five cases per year and most of their pancreata are used for pancreas organ transplantation. We therefore need to develop an efficient isolation technique for NHBD pancreata.

We have recently demonstrated that islet isolation and transplantation with NHBDs using the modified Ricordi method (Kyoto islet isolation method) effectively cures type 1 diabetes (23). The transplantation rate (transplantation number/isolation number) is more than 80%, higher than recently published data using brain-dead heart-beating donors (14,21,31). The isolation method includes an in situ cooling system for pancreas procurement (19), ductal injection (28), the modified two-layer method (MK/PFC) (27,30), and iodixanol-based purification (14). We previously showed that MK/PFC preservation significantly improved islet yields, compared with UW/PFC preservation (30). MK solution includes a trypsin inhibitor (ulinastatin), which is one of the advantages of this solution. Indeed, pancreas preservation using MK solution was superior to preservation with ET-Kyoto solution without the trypsin inhibitor in a rat model (30).

In this study, we compared ulinastatin with another trypsin inhibitor, Pefabloc, in preservation solution for islet isolation.

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MATERIALS AND METHODS

Preservation Solution

We used ET-Kyoto solution (5,32) with ulinastatin (Miraclid®, Mochida Pharmaceutical, Tokyo, Japan; ET-Kyoto + ulinastatin = "MK solution") or Pefabloc (Roche Applied Science, Germany; ET-Kyoto + Pefa bloc = "PK solution"). The components of the solutions are shown in Table 1.

Measurement of Trypsin Inhibition Ability of Solutions

In order to assess the trypsin inhibition of MK solution, PK solution, and ET-Kyoto solution without trypsin inhibitors (control), 3 ml of 0.3 mM *N*-benzoyl-L-arginine ethylester reagent (BAEE; Sigma, Tokyo, Japan) were incubated for 5 min at 25°C and then 5 µl of 1 mg/ml trypsin and 45 µl of each solution were added. Trypsin activity was measured by absorption spectrophotometry (λ253 nm) using BAEE for the trypsin substrate, according to a previous report (17). Absorbance was measured every minute for 6 min. A BAEE unit was defined as a change in optical density of 0.001/min.

Porcine Islet Isolation

Porcine pancreata were obtained at a local slaughterhouse. The operation was started about 10 min after the cessation of heart beating. After removing the pancreas, we immediately inserted a cannula into the main pancreatic duct, infused each preservation solution for ductal protection, and put the pancreas into a two-layer preservation container that had one of the preservation solutions (preservation solution/PFC). Operation time was defined as the time elapsed between the start of operation and removal of the pancreas. Warm ischemic time (WIT) was defined as the time elapsed between cessation of heart beating and placement of the pancreas into the preservation solution. Cold ischemic time (CIT) was defined as the time elapsed between placement of the pancreas into the preservation solution and the start of islet isolation.

Islet isolation was conducted in accordance with the method described in the Edmonton protocol (15,16,33, 34,37). In brief, after decontamination of the pancreas, the ducts were perfused in a controlled fashion with a cold enzyme blend of Liberase HI (1.4 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN). The distended pancreas was then cut into nine pieces, placed in a sterilized Ricordi chamber, and shaken gently. While the pancreas was being digested by recirculating the enzyme solution through the Ricordi chamber at 37°C, we monitored the extent of digestion with dithizone staining by taking small samples from the system. Once digestion was completed, RPMI-1640 medium (Gibco, Carlsbad, CA) was introduced into the system, and the system was cooled to stop further digestive activity. The digested tissue was collected and washed with fresh medium to remove the enzyme. The phase I period was defined as the time between placement of the pancreas in the Ricordi chamber and the start of collecting the digested pancreas. The phase II period was defined as the time between the start and end of collection.

Islets were purified with a continuous density gradient with Iodixanol-Kyoto solution in an apheresis system (COBE 2991 cell processor, Gambro Laboratories, Denver, CO). Because Iodixanol has a lower viscosity than Ficoll, it needs less force during centrifugation, which causes less damage to islets. For the solution, low-density (density: 1.077) and high-density (density: 1.100–1.125) Iodixanol-Kyoto solutions were produced by changing the volumetric ratio of Iodixanol and Kyoto solution.

Islet Evaluation

The crude number of islets in each diameter class was determined by counting islets after dithizone staining (3 mg/ml, final concentration) (Sigma Chemical Co., St. Louis, MO) using an optical graticule. The crude number of islets was then converted to the standard number of islet equivalents (IE; diameter standardizing to 150 µm) (35). Gross morphology was qualitatively assessed by two independent investigators scoring the islets for shape (flat vs. spherical), border (irregular vs. well-rounded), integrity (fragmented vs. solid/compact), uniformity of staining (not uniform vs. perfectly uniform), and diameter (least desirable: all cells <100 µm/most desirable: more than 10% of the cells >200 µm) (16,35). Each parameter was graded from 0 to 2 with 0 equaling the worst and 2 the best score, so that the worst islet preparations were given a cumulative score of 0 and the best a score of 10. Spherical, well-rounded, solid/compact, uniformly stained, and large islets were characterized as the best islets.

Islet viability after purification was assessed using acridine orange (10 µmol/L) and propidium iodide (15 µmol/L) (AO/PI) staining to visualize living and dead

Table 1. Composition of Each Preservation Solution

	MK	PK
Na (mmol/L)	100	100
K (mmol/L)	43.5	43.5
Gluconate (mmol/L)	100	100
Phosphate (mmol/L)	25	25
Trehalose (mmol/L)	120	120
Hydroxyethyl starch (g/L)	30	30
Ulinastatin ($\times 10^3$ U/L)	100	—
Pefabloc (mg/L)	—	1000

islet cells simultaneously (3,16,35). Fifty islets were inspected and their individual viability was determined visually, followed by calculation of their average viability (16).

In Vitro Assessment of Islet Function

Islet function was assessed by monitoring the insulin secretory response of the purified islets during glucose stimulation according to a procedure described by Shapiro and colleagues (37). Briefly, 1200 IE were incubated with either 2.8 or 25 mM glucose in RPMI-1640 for 2 h at 37°C and 5% CO_2. The supernatants were collected and insulin levels were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (ALPCO Insulin ELISA kit; ALPCO Diagnostics, Windham, NH). The stimulation index was calculated by determining the ratio of insulin released from islets in high glucose concentration to the insulin released in a low concentration. The data were normalized to total protein from the cell lysate. All assessments were made in triplicate and the data (mean ± SE) were expressed as a percentage of the control values in each experiment to eliminate variables caused by differences among donor pancreata.

Recently, Goto et al. showed that the measurement of the ADP/ATP ratio correlated with transplantation outcome (8). The ADP/ATP ratio was measured to evaluate the energy status of cultured islets, using the Apo Glow™ kit (Cambrex Bio Science Nottingham Ltd., Nottingham, UK). In brief, 80 IEs were washed in PBS and then mixed with 100 μl of nucleotide-releasing reagent for 10 min at room temperature. Thereafter, 20 μl of nucleotide-monitoring reagent was added to the solution. The ATP levels were measured using a luminometer (FB 12 Luminometer, Berthold Detection Systems GmbH, Pforzheim, Germany) and expressed as the number of relative light units (RLU). After 10 min, the ADP in the solution was converted to ATP by adding 20 μl ADP converting reagent and then measured as the number of RLU. Subsequently, the ADP/ATP ratio of the islets was calculated.

In Vivo Assessment of Islet Function

Mice with severe combined immunodeficiency disease (SCID; CLEA Japan, Inc., Meguro, Tokyo) were used for the experiments. The recipients were rendered diabetic by a single injection of streptozotocin (STZ) at a dose of 220 mg/kg. Hyperglycemia was defined as a glucose level of >350 mg/dl detected twice consecutively after STZ injection. The 2000 IE pig islets obtained from each group were transplanted into the renal subcapsular space of the left kidney of diabetic SCID mice. During the 30-day posttransplantation period, the nonfasting blood glucose levels were monitored three times per week. Normoglycemia was defined when two consecutive blood glucose level measurements showed less than 200 mg/dl. No statistical differences in either pretransplantation blood glucose levels or pretransplantation body weight were observed among the four groups of mice. Mouse studies were approved by the Institutional Animal Research Committees of Kyoto University, Nagoya University, and Fujita Health University.

Statistical Analysis

Values for the data represent the mean ± SE. Two or three groups were compared by Student's *t*-test with Bonferroni correction.

RESULTS

Inhibition of Trypsin Activity

Previous reports show that trypsin inhibition with TLM preservation improves islet yields (17,30). We examined whether MK and PK solutions inhibited trypsin activity. Both solutions inhibited trypsin activity (Fig. 1) compared with ET-Kyoto solution (control) ($p < 0.01$), suggesting that these solutions could be useful in reducing trypsin activity during pancreas preservation.

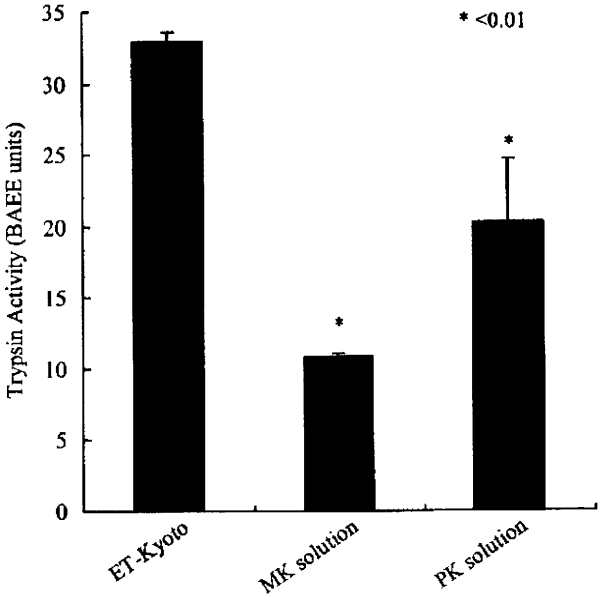

Figure 1. Impact of MK or PK solution on trypsin activity. BAEE was incubated for 5 min at 25°C and then trypsin along with MK solution ($n = 7$), PK solution ($n = 7$), or ET-Kyoto (control; $n = 7$) was added. Trypsin activity was measured by absorption spectrophotometry (λ253 nm) using BAEE reagent. Absorbance was measured every minute for 6 min. A BAEE unit was defined as a change in optical density of 0.001/min. MK and PK solutions inhibited trypsin activity significantly more than ET-Kyoto ($p < 0.01$). Data are expressed as the mean ± SE.

Table 2. Pig Islet Isolation Characteristics

	MK	PK
Pancreas size (g)	105.0 ± 8.9	87.3 ± 3.4
Operation time (min)	8.0 ± 1.0	4.3 ± 0.9
Warm ischemic time (min)	27.2 ± 1.7	25.7 ± 2.8
Cold ischemic time (min)	123.6 ± 1.6	122.3 ± 1.2
Phase I period (min)	10.2 ± 1.8	7.7 ± 1.2
Phase II period (min)	35.0 ± 3.0	29.7 ± 3.2

Data are expressed as mean ± SE.

Table 3. Pig Islet Characteristics

	MK	PK
Viability (%)	97.5 ± 0.5	96.5 ± 1.6
Score	9.2 ± 0.3	8.7 ± 0.2
Purity (%)	66.0 ± 6.8	70.0 ± 5.8
Recovery rate (%)	68.1 ± 8.4	70.5 ± 13.6
Stimulation index	2.50 ± 0.21*	1.37 ± 0.18

Data are expressed as mean ± SE.
*Stimulation index was higher for the MK/PFC group than for the PK/PFC group ($p < 0.05$).

Porcine Islet Isolation Characteristics

The characteristics of porcine islet isolation protocols are shown in Table 2. There were no significant differences in pancreas size, operation time, WIT, or CIT between the two groups. Phase I and phase II periods were also similar for the two groups.

Islet yield before purification was significantly higher in the MK/PFC group ($n = 5$) than the PK/PFC group ($n = 3$) (MK/PFC; 9676 ± 635 IE/g, PK/PFC; 6999 ± 844 IE/g, $p < 0.05$) (Fig. 2A). The islet yield after purification for the MK/PFC group was higher than the PK/PFC group (MK/PFC; 6608 ± 927 IE/g, PK/PFC; 4964 ± 1153 IE/g) but not significantly so (Fig. 2B). Other porcine islet characteristics are shown in Table 3. The stimulation index was higher for the MK/PFC group than for the PK/PFC group ($p < 0.05$). There were no other significant differences in characteristics between the two groups.

Assessment of Islet Function In Vitro and In Vivo

Recently, Goto et al. showed that the measurement of the ADP/ATP ratio correlated with transplantation outcome (8). To assess the islet graft function of each group in vitro, the ADP/ATP ratio was measured. There was no significant difference in ADP/ATP ratio between the groups (data not shown).

To assess the islet graft function of each group in vivo, 2000 IE of each group were then transplanted below the kidney capsule of STZ-induced diabetic SCID mice. There was no significant difference between the groups with respect to the attainability of posttransplantation normoglycemia (data not shown). Morphologic

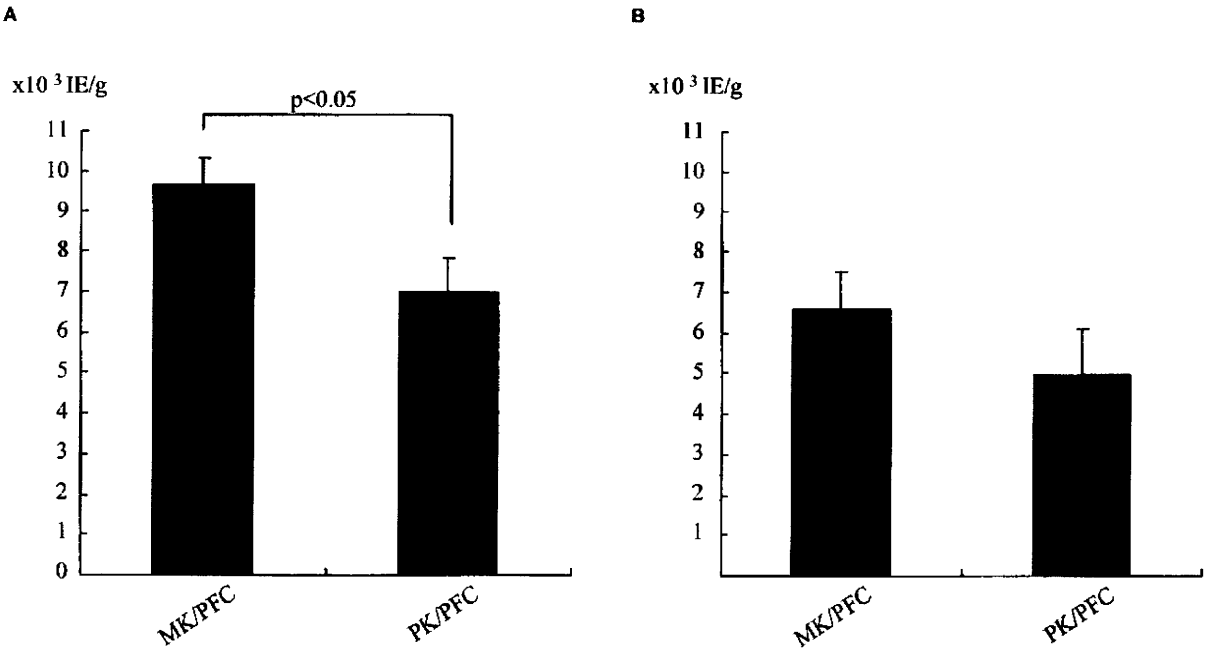

Figure 2. Islet yield before purification (A) and after purification (B). The MK/PFC group had significantly better islet yield before purification than the PK/PFC group ($p < 0.05$). Data are expressed as the mean ± SE.

studies showed the presence of islets under the kidney capsule of all SCID mice 30 days after transplantation. The islet grafts of each group in the normoglycemic mice showed intense insulin staining (data not shown).

Taken together, these data suggest that MK/PFC preservation is superior to PK/PFC preservation.

DISCUSSION

Islet allotransplantation can achieve insulin independence in patients with type 1 diabetes (37). Since the Edmonton protocol was announced, islet transplantations from brain-dead donors (9,10) as well as from non-heart-beating donors (13,14,21) and living donors (15) have been performed. These advances were based on advanced pancreas transport systems (10,16,17,30), revised immunosuppressant protocols (24,25), improved islet isolation methods (17), and enhanced islet engraftment (26). Although experiments of β-cell regeneration from stem cells have proceeded (20,22,29), there is still no reliable method for producing β-cells. Until a new method to generate β-cells is developed, improving the efficacy of islet transplantation seems the most realistic and prudent method to cure diabetes.

Donor pancreata are usually preserved with University of Wisconsin (UW) solution. Recent reports have shown that the two-layer method (TLM), which employs oxygenated perfluorochemical (PFC) and UW solution, is superior to simple cold storage in UW to preserve not only the whole pancreas but also individual islets for transplantation (16,17). However, UW solution has several disadvantages: it is chemically unstable, it must be cold stored until use, and its short shelf life makes it expensive. It is also highly viscous, which may complicate the initial organ flush (39). Recently, our university developed a new preservation solution, ET-Kyoto solution, and its effectiveness in cold lung storage has been demonstrated in clinical lung transplantation (5,32). It also is effective for skin flap storage and its clinical application is beginning in this field (40). Although high potassium in UW solution causes insulin release from pancreatic β-cells (7), ET-Kyoto solution has a high sodium/low potassium composition. Moreover, UW solution inhibits the activity of Liberase, an enzyme blend for pancreatic digestion (6,36), but ET-Kyoto solution with ulinastatin inhibits Liberase less (30).

Trypsin from pancreatic acinar cells destroys islets. Previous studies have shown that trypsin inhibition by Pefabloc during human pancreas digestion improves islet yield and reduces the fraction of embedded islets (11,17), suggesting that trypsin may degrade the ductules and thus reduce the delivery of collagenase solution to the immediate neighborhood of the islets. Previously, we demonstrated that modifying TLM preservation, by including ulinastatin, eliminated trypsin activity during pancreas preservation, and ET-Kyoto/PFC preservation without ulinastatin resulted in lower islet yields (30). In this study, we showed that MK solution was synthetically superior to PK solutions. It may be due to differences in inhibitory effects of cytokines. Ulinastatin has been shown to inhibit not only trypsin activity but also the release of neutrophil elastase. It also downregulates transcription of tumor necrosis factor mRNA, the activation of endothelial cells, and the expression of ICAM-1 induced by endotoxin in vitro (1,2,18). It has been shown that administration of ulinastatin decreased the ischemia-reperfusion injury (38) and attenuated the elevation of inflammatory cytokines and C-reactive protein, a marker of inflammation (41) in transplanted small intestine.

In conclusion, we show that ET-Kyoto with ulinastatin is a better combination for pancreas preservation than ET-Kyoto with Pefabloc. Based on these data, we now use the ET-Kyoto solution with ulinastatin for clinical islet transplantation from NHBD pancreata. MK/PFC preservation makes it feasible to use NHBDs for efficient islet transplantation into type 1 diabetes patients.

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Regular Article

Larger Dosage Required for Everolimus than Sirolimus to Maintain Same Blood Concentration in Two Pancreatic Islet Transplant Patients with Tacrolimus

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Summary: We attempted a switch of mammalian target of rapamycin (mTOR) inhibitors from sirolimus to everolimus, a derivative of sirolimus and now on the market in Japan, in two pancreatic islet transplant patients. Both patients were administered tacrolimus with sirolimus or everolimus. They had been administered 5 or 9 mg sirolimus once a day and had maintained a trough concentration of about 15 ng/mL as measured by high performance liquid chromatography with ultraviolet detection. After the switch from sirolimus to everolimus, they were given 10 or 12 mg/day of everolimus twice a day to maintain a trough concentration of 12-15 ng/mL as measured by a fluorescence polarization immunoassay (FPIA) method. Afterward, the blood concentrations of everolimus and sirolimus after the conversion were measured by high performance liquid chromatography with mass spectrometry and everolimus concentrations were found to be 5-10 ng/mL. These data show that a larger dosage is needed for everolimus than sirolimus to maintain the same trough blood concentration. Data obtained by the FPIA for everolimus should be carefully evaluated after switching from sirolimus to everolimus because of the cross-reactivity of the antibody with sirolimus.

Keywords: everolimus; sirolimus; tacrolimus; pancreatic islet transplantation

Introduction

Pancreatic islet transplantation is a critical treatment for type 1 diabetes when it is difficult to control blood glucose levels despite an optimal insulin regimen and less invasive than pancreatic transplantation. With the Edmonton protocol,[1] results of pancreatic islet transplantation improved markedly. According to the Edmonton protocol, Kyoto University Hospital performed 17 transplantations from non-heart-beating donors for 9 patients as of the end of 2006. The first successful living-donor islet transplantation was carried out on January 19, 2005.[2]

The Edmonton protocol consists of high-dose sirolimus (rapamycin) and low-dose tacrolimus for immunosuppression.[1] Sirolimus suppresses the proliferation of lymphocytes by blocking growth factor-driven signal transduction through the inhibition of mammalian target of rapamycin (mTOR).[3] In Japan, however, sirolimus is not approved by the Japanese government as an immunosuppressant. Everolimus, a derivative of sirolimus, has a shorter elimination half-life than sirolimus,[4-6] and is expected to achieve a steady-state more quickly and adjust blood concentrations more easily. Everolimus has already been approved as an immunosuppressant in Europe and in March 2007, was approved as an immunosuppressant for heart transplant patients in Japan. Hence, we conducted a switch of mTOR inhibitors from sirolimus to everolimus in pancreatic islet transplant patients. Generally, clinical studies on everolimus in organ transplant patients have been performed with the concomitant administration of cyclosporine and steroids. There are a few reports on everolimus using tacrolimus.

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