Phenylalanine sensitive K562-D cells for the analysis of the biochemical impact of excess amino acid

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Although it is recognized that the abnormal accumulation of amino acid is a cause of the symptoms in metabolic disease such as phenylketonuria (PKU), the relationship between disease severity and serum amino acid levels is not well understood due to the lack of experimental model. Here, we present a novel in vitro cellular model using K562-D cells that proliferate slowly in the presence of excessive amount of phenylalanine within the clinically observed range, but not phenylpyruvate. The increased expression of the L-type amino acid transporter (LAT2) and its adapter protein 4F2 heavy chain appeared to be responsible for the higher sensitivity to phenylalanine in K562-D cells. Supplementation with valine over phenylalanine effectively restored cell proliferation, although other amino acids did not improve K562-D cell proliferation over phenylalanine. Biochemical analysis revealed mammalian target of rapamycin complex (mTORC) as a terminal target of phenylalanine in K562-D cell proliferation, and supplementation of valine restored mTORC1 activity. Our results show that K562-D cell can be a potent tool for the investigation of PKU at the molecular level and to explore new therapeutic approaches to the disease.

Metabolic disorders are often characterized by an imbalance of amino acids in plasma. Although it has been recognized that the accumulation of a particular amino acid or associated toxic metabolite(s), or else the deficiency of an essential amino acid, are causes of these diseases, the biochemical linkage between amino acid and pathophysiological changes often remain to be clarified.

Phenylketonuria (PKU) is an autosomal recessive disorder caused by a deficiency in hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1)1,2. Since disease severity correlates with levels of serum phenylalanine, dietary restriction of phenylalanine in combination with the supplemental use of glycomacropeptide or neutral amino acids is the central component of PKU treatment. In a subset of PKU patients, supplementation with the PAH activator sapropterin dihydrochloride (BH4) is sufficient to beneficially reduce plasma phenylalanine levels3.

Amino acids cross the plasma membrane through amino acid transporters and serve as building blocks for protein synthesis, energy-generating metabolites, substrates for enzymes such as nitric oxide synthase (NOS), or carriers for signaling molecule such as nitric oxide4. Recent studies have shown that amino acids regulate cell proliferation and protein synthesis through mammalian target of rapamycin complex (mTORC)5,6. The majority of these studies have focused on amino acid starvation, and a little attention has been paid to the effect of excess accumulation of amino acids7,8.

Hyperalimentation with balanced amino acids has been advocated in metabolic diseases, but this intervention cannot always correct the severe symptoms in congenital metabolic disorders. An elucidation of the mechanisms underlying the pathophysiological effects of amino acid imbalance would contribute to the better understanding of inherited metabolic diseases and to the development of novel therapeutic strategies. Due to the lack of the experimental model in vitro to analyze the biochemical impact of excess phenylalanine, the molecular mechanism(s) of phenylalanine toxicity remain poorly understood. Here, we have developed a cellular model (K562-D cells), which possesses higher sensitivity in cell proliferation to the content of phenylalanine in the culture medium within the clinically observed range in PKU patients. This system enabled us to investigate the molecular mechanism of phenylalanine toxicity.

Results
Differentiated K562-D cells are prone to the excess phenylalanine. It has been reported that oxidative stress status in the blood from PKU patient is closely linked to serum phenylalanine levels9, and nutritional anemias are
prevalent in patients with inborn errors of metabolism. We have found that K562 cells acquire phenylalanine sensitivity in cell proliferation once they differentiated and the phenotype could be used as an in vitro model to assess the effect of excess phenylalanine. In the case of severe PKU patients without dietary restriction of phenylalanine, the serum phenylalanine level may increase more than 2 mM. Thus cell proliferation rate of K562-D cells was used as a read-out to evaluate the cellular effects of phenylalanine up to 5 mM added to the culture medium.

Cell proliferation was monitored by measuring cell density every 24 h for 5 d following the addition of phenylalanine. K562-D cells, differentiated by hemin and Am80, showed significant sensitivity to phenylalanine at 3 mM compared to the parental K562 cells (Figure 1a, b). Although there was no significant difference between 0–3 mM of phenylalanine in parental K562 cells (Figure 1a), K562-D cells exhibit slow proliferation in concentration dependent fashion after 120 h of inoculation with 3 mM or greater phenylalanine (Figure 1b). However, at 10 mM phenylalanine, parental K562 cells showed slow proliferation that was comparable to K562-D cells treated with 5 mM phenylalanine. Since cell-counting method cannot distinguish the effects of cell death from slow proliferation, LDH activity measurement in cell culture media and counting of pyknotic nuclei were chosen to evaluate cell viability. There was no significant increase on LDH activity or any evidence of changes in nuclear morphology at 5 mM of phenylalanine until 5 d after inoculation (Figure 1c). It suggests that counts of the cell number represent mainly cell proliferation, and has less contribution by cell viability and cell death. In order to increase assay throughput, resazurin method was employed to estimate cell numbers, which quantifies the amount of resazurin metabolite, resorufin (see materials and methods) as a surrogate of cell counts. This resazurin method was highly comparable to standard hemocytometer-based counting and the results were highly reproducible. Since K562 and K562-D cells reach confluence by 120 h after inoculation in the control setup, proliferation rate was compared at 96 h after the addition of phenylalanine (Figure 1d). Although parental K562 cells did not show growth retardation by phenylalanine concentrations less than 3 mM, the K562-D cells were significantly sensitive to phenylalanine at concentrations greater than or equal to 1 mM (Figure 1d). At 3 or 5 mM phenylalanine, the degree of inhibition was 3.4, and 4.8 times higher in K562-D cells than K562 cells, respectively. Of note, increased osmotic pressure by the addition of phenylalanine did not cause similar effect since the control cells with sodium chloride (5 mM, equivalent to 10 mM phenylalanine) added to the culture media.

Figure 1 | Phenylalanine-sensitive slow proliferation phenotype in K562-D cells. (a-b) Cell growth was monitored every 24 h for 5 d. Cells were cultured with various amount of phenylalanine in the culture medium and cell density was calculated by using a counting grid under the microscope (n = 4 for each condition). The parental K562 cells (a) and the differentiated K562-D cells (b) showed marked differences in cell proliferation. K562-D cells were sensitive to more than or equal to 3 mM of phenylalanine; however, the parental K562 cells showed slow proliferation in concentration dependent fashion after 120 h of inoculation with 3 mM or greater phenylalanine (Figure 1b). However, at 10 mM phenylalanine, parental K562 cells showed slow proliferation that was comparable to K562-D cells treated with 5 mM phenylalanine. Since cell-counting method cannot distinguish the effects of cell death from slow proliferation, LDH activity measurement in cell culture media and counting of pyknotic nuclei were chosen to evaluate cell viability. There was no significant increase on LDH activity or any evidence of changes in nuclear morphology at 5 mM of phenylalanine until 5 d after inoculation (Figure 1c). It suggests that counts of the cell number represent mainly cell proliferation, and has less contribution by cell viability and cell death. In order to increase assay throughput, resazurin method was employed to estimate cell numbers, which quantifies the amount of resazurin metabolite, resorufin (see materials and methods) as a surrogate of cell counts. This resazurin method was highly comparable to standard hemocytometer-based counting and the results were highly reproducible. Since K562 and K562-D cells reach confluence by 120 h after inoculation in the control setup, proliferation rate was compared at 96 h after the addition of phenylalanine (Figure 1d). Although parental K562 cells did not show growth retardation by phenylalanine concentrations less than 3 mM, the K562-D cells were significantly sensitive to phenylalanine at concentrations greater than or equal to 1 mM (Figure 1d). At 3 or 5 mM phenylalanine, the degree of inhibition was 3.4, and 4.8 times higher in K562-D cells than K562 cells, respectively. Of note, increased osmotic pressure by the addition of phenylalanine did not cause similar effect since the control cells with sodium chloride (5 mM, equivalent to 10 mM phenylalanine) added to the culture media.
uptake of other amino acids in the cell1,14. Since differentiated amino acids at the transporter level, resulting in the decreased membrane, excess phenylalanine competes with other neutral share LAT as a common transporter through the plasma Since large neutral amino acids (LNAA) including phenylalanine the biochemical constituents of PKU pathogenesis, significant concentration range in PKU.

**LAT2 and 4F2hc expressions are elevated in K562-D cells.** Among the biochemical constituents of PKU pathogenesis, significant attention has paid to the L-type amino acid transporter, LAT. Since large neutral amino acids (LNAA) including phenylalanine share LAT as a common transporter through the plasma membrane, excess phenylalanine competes with other neutral amino acids at the transporter level, resulting in the decreased uptake of other amino acids in the cell114. Since differentiated K562-D cells have higher phenylalanine sensitivity than their parental K562 cells, the expression levels of the gene involved in the large neutral amino acid transport were examined by semi-quantitative real-time PCR method (qPCR). Although there was no significant change on LAT1 (SLC7A5) expressions before and after the differentiation induced by hemin and Am80, the expression level of LAT2 (SLC7A8) and 4F2hc (SLC3A2) genes were increased by 2.36 and 4.87 times higher than parental cells, respectively (Figure 2a). 4F2hc (4F2 heavy chain antigen, also known as CD98) is a type II transmembrane protein that is covalently associated with a number of transport proteins, such as LAT1 and LAT2, to localize these transporter proteins on the plasma membrane and allow amino-acid transport15. Phenylalanine is selectively transported through LAT1 and LAT2 among other LNAAas, the augmentation of phenylalanine sensitivity is induced in conjunction with the functional complex formation of LAT2 and 4F2hc through increases in gene expression. LAT is a bilateral transporter which imports LNAA in exchange with glutamine; thus, glutamine transporter, ASCT2 (SLC1A5), and intracellular glutamine synthase, GLUL, functionally couples with LAT.

**Supplementation of amino acids does not compensate for the cell proliferation inhibited by phenylalanine.** Phenylalanine toxicity may be mediated by the competitive inhibition of the uptake of other essential amino acids by LAT114. Leucine was chosen as a representative LAT substrate to investigate whether supplementation of LNAAas might restore this imbalance in amino acids transport, since leucine has low Km value for LATs19–21. Since the culture medium (RPMI1640) contains >90 μM phenylalanine, the possible beneficial effects of added leucine in K562-D cells were assayed from 100 to 1000 μM leucine in the presence of 3 mM added phenylalanine; however, there was no significant recovery of the cell proliferation (Figure 3a). Other amino acids were then investigated the rescue of growth inhibition in cells treated with 5 mM phenylalanine. Various amino acids (e.g. Asn, Ala, Gly, Lys, Pro, Arg, His, Ser, Met, etc.) were supplemented at 1 mM in the culture medium over phenylalanine (5 mM), and cell proliferation was analyzed after four days. No significant recovery of cell proliferation was observed in this group of assay except for methionine, which further decreased the growth rate by 1.5 times (Figure 3b). These results suggested that competition of other essential amino acid transporter by excess phenylalanine are not be critical for toxicity in this model.

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**Figure 2** | Differentiation induced higher expressions of LAT2 and 4F2hc genes in K562-D cells. (a) Gene expression levels were analyzed by semi-quantitative qPCR with RPL4 as internal standard. (n = 2–3). The expression levels of LAT2 and 4F2hc genes were markedly higher in K562-D cells compared to K562 cells; whereas little difference was found on LAT1. There is little or no expression of LAT2 gene in T24 cells as reported previously18. (b) Contribution of LAT1 to phenylalanine-dependent slow proliferation was not significant in T24 cells. Following a 4-day incubation with different amount of phenylalanine, cell proliferation was assayed by resazurin method. There was a little difference at 5 mM phenylalanine on cell proliferation; however, the normalized cell proliferation was 71% in K562-D cells (Figure 1b) vs. 88% in T24 cells at 5 mM phenylalanine. (n = 12–16 for each group). (**p < 0.01 vs. Phe 0 mM) Data are presented as mean ± S.E.
Valine supplementation over phenylalanine partly restored K562-D cell proliferation. Pyruvate kinase (PK) has been identified as a target of phenylalanine in vivo. Phenylalanine binding maintains PK in an inactive T-state by the allosteric effects. Decreased activity of PK by phenylalanine may contribute to decreased energy metabolism in PKU patients. Thus the PK pathway has drawn attention in K562-D cells. If the inhibition of PK by phenylalanine is key to the reduction in cell growth, enhancement of the TCA cycle by supplying the metabolic intermediates, such as acetyl-CoA or succinyl-CoA, might rescue ATP synthesis, resulting in the recovery of cell proliferation. These intermediates are the metabolic products of branched chain amino acids (BCAA), such as leucine, isoleucine, and valine. Thus, each BCAA at 1 mM was added to the culture medium over 5 mM phenylalanine. As shown previously, leucine had no effect on the recovery of cell growth; however, valine partly restored the cell growth (Figure 4a). Interestingly 0.2 molar equivalents of valine to phenylalanine successfully restored the cell growth by around 50%. At a fixed concentration of valine at 1 mM, cell growth was evaluated over the different concentrations of phenylalanine (Figure 4b). At 3 mM of phenylalanine, 71% of recovery was achieved by 1 mM valine. Collectively, valine at 1 mM can restore the cell growth to normal levels at concentrations of up to 2.2 mM phenylalanine. Of note, addition of valine to the medium did not reduce the proliferation up to 10 mM (Data not shown). Although D-valine showed no effect against phenylalanine (5 mM), even low concentrations of L-valine (0.1–1.0 mM) effectively competed for the uptake of phenylalanine-induced slow proliferation (Figure 4c). There was no significant change with an addition of phenylalanine in the expression level of LAT1/2 and 4F2hc mRNA analyzed by qPCR method (data not shown). As a result of the metabolic process of BCAA in the peripheral tissue, valine and leucine supply succinyl-CoA and acetyl-CoA, respectively. Both metabolic intermediates are necessary for the TCA cycle. As shown in figure 4a, valine significantly restored cell growth inhibited by phenylalanine. This result may indicate the possibility that only succinyl-CoA, but not acetyl-CoA, appears to counteract the effects of phenylalanine. To estimate the involvement of leucine/acetyl-CoA in this process, higher concentrations of leucine (3 or 10 mM) were applied to K562-D cells. Although leucine at 1 mM did not show an inhibitory effect on the K562-D cell growth over phenylalanine (Figure 4a), higher concentrations of leucine actually significantly inhibited the cell growth (Figure 4d). Interestingly, valine successfully restored the growth inhibition by leucine.

Tyrosine deficiency due to the inactivation of PAH could be a cause of PKU symptom. Since PAH expression is rather limited in the liver and kidney, K562-D cells does not have PAH expression in significant level. Since rescue effect of tyrosine in sepiapterin reductase deficient mice has been reported, tyrosine (0.3 or 1.0 mM) was supplemented over phenylalanine to see whether the cell proliferation can be enhanced. However, at least within the concentrations tested, there was no effect by tyrosine on cell proliferation, suggesting that sensitivity to phenylalanine in K562-D cells is not due to tyrosine deficiency.

Excess phenylalanine inhibits mTORC activity in K562-D cells. It has been reported that amino acid deficiency has an impact on cell proliferation through the regulation of mTORC (mammalian target of rapamycin complex) activity. Since mTORC is one of the major regulators of cell proliferation, the activity of mTORC1 was analyzed by measuring phosphorylation at Thr389 of the p70 S6 kinase (p70S6K). Phosphorylation of p70S6K at this site was significantly lower when K562-D cells were cultured with phenylalanine (5 mM) for three days; however, the addition of valine (1 mM) to phenylalanine successfully restored the phosphorylation level of p70S6K back to control levels (Figure 4e).

Discussion
Here, we have presented a new in vitro culture system, K562-D cells, as a tool to investigate biochemical processes induced by phenylalanine overload, and to screen compound which can modify cell proliferation rate against phenylalanine. Our findings demonstrated that the elevated expressions of LAT2 and 4F2hc in K562-D cells allow
phenylalanine influx into the cell, leading to impaired cell proliferation through the inhibition of the mTORC1. The lack of an in vitro model system for the study of amino acid overload has been a significant limitation for the detailed molecular analyses of metabolic diseases including PKU. Animal models and the material obtained from the patients have not so far allowed us to sufficiently test hypotheses or to screen compounds for the potential treatment. This in vitro model system with K562-D cells allowed us to uncover the...
compensative effects of valine, which may have important clinical significance.

It has been implicated that elevated levels of phenylalanine will overwhelm LAT homeostasis and increase its uptake to the detriment of other LNAA's. In this study, however, supplementation of amino acids except valine did not reduce inhibitory effect by phenylalanine. This evidence indicated an alternative pathway which might be responsible for effects induced by excess phenylalanine. It has been reported that glucose metabolism and pyruvate kinase activity in the brain are significantly low in PKU patients due to the elevated phenylalanine in the cytoplasm. Since pyruvate uptake into the mitochondria is the rate-limiting step for ATP synthesis, excess phenylalanine could cause insufficient production of ATP through the TCA cycle, leading to the inhibition of mTORC1 resulting from the activation of AMPK. Since AMP kinase (AMPK) is a negative regulator of mTORC1,24,25, insufficient synthesis of ATP decreases ATP/AMP ratio, which in turn activates AMPK. It may suggest the existence of regulatory process originated from the inhibition of pyruvate kinase and attenuated ATP synthesis and AMPK activation, resulting in the inhibition of mTORC activity, which directly suppresses proliferation of K562-D cells. Even when pyruvate kinase activity is suppressed by phenylalanine, glucose supplementation could increase the substrate availability for pyruvate kinase such as phosphoenolpyruvate (PEP) as a product of glycolysis; however, K562-D cells are highly sensitive to phenylalanine even in normal media, which contains relatively high concentration of glucose (about 11.1 mM in RPMI1640). This evidence suggests that further biochemical evaluation is necessary to clarify relation between glucose metabolism and pyruvate level in K562-D cells.

The therapeutic strategy for PKU is the reduction of blood phenylalanine level. Although dietary restriction is the basic strategy to reduce phenylalanine intake, it is challenging particularly for adults with PKU since the diet is bland and not affordable for all PKU patients. Thus, poor dietary adherence continues to be a major problem. Supplementation with sapropterin or large neutral amino acids (LNAA) partially fulfill the dietary desire by increasing the amino acid concentration was annotated in this study as the amount of added to the normal media, which contains relatively high concentration of glucose (about 11.1 mM in RPMI1640). This evidence suggests that further biochemical evaluation is necessary to clarify relation between glucose metabolism and pyruvate level in K562-D cells.

Amino acid treatments and growth assay. Cells were seeded at the density of 1.7 × 10^4 cells/well in a 24-well plate. For K562-D cells, hemin (10 μM) and Amn80 (1 μM) were kept in the culture medium. Amino acid stock solutions (100 mM in serum-free RPMI1640, with the exception of tyrosine at 3 mM, and tryptophan at 50 mM) were added to each well to increase amino acid levels in the culture medium. These additions were termed as K562-D cells, and used for the further experiments. K562-D cells were prepared fresh for each set of experiments. Erythroid differentiation was assayed by benzidine staining of hemoglobin accumulated in the cells. Following the staining, at least 1000 cells were examined by light microscopy to count the number of benzidine-positive cells. Under typical conditions, at least 96% of total cells were benzidine-positive at the time of amino acid addition. The parental K562 cells under the regular culture condition did not show stained cells.

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Assay for cell death. Pyknotic nucleus and dense stained nucleus was used as a marker of cell death. Following a treatment with amino acid for the indicated period, cells were collected in a tube, declared viability was detected with Hoechst 333358 dye (B2621) for 3 min at room temp, followed by fixation with 4% paraformaldehyde in phosphate buffer (163-20145 Wako, Japan). Nuclei were visualized using a fluorescence microscope (TE300, Nikon, Japan) and digitized. Cells with dense stained nuclei or condensed or discrete fragmented nuclei were counted as dead cells. At least 2500 cells/sample were counted to calculate the ratio of dead cells to total cells.

Lactate dehydrogenase (LDH) activity measurement was also employed as a biochemical indicator of cell death. Culture medium (1 ml) was obtained on day 2, 3, and
5 from the dish prepared for the pyrrolidone assay. Following a centrifugation at 800 g for 3 min at 4 °C to separate cells, the LDH assay was conducted according to the manufacturer’s instructions (LDH CII, Wako, Japan).

Assays for mTORC1 activity. mTORC1 complex (mTORC1) is essential for the phosphorylation and activation of the 70 kDa ribosomal protein S6 kinase (S6K) 1 and 2. Phosphorylation levels were assessed as mTORC1 activity by immunoblotting with a S6K pThr389-specific antibody (#9234 Cell signaling) normalized to total S6K (#2708 Cell Signaling). GAPDH (sc-25778 Santa Cruz) was used as an internal loading control. K562-D cells were cultured in a 10-cm dish for 3 days with amino acid as indicated. Cells were collected by a centrifugation and stored at −80 °C until use. Cells were thawed in hypotonic lysis buffer (20 mM Hepes pH 7.6, 10 mM NaCl, 1.5 mM MgCl2, 0.1% Triton X-100) supplemented with 1 mM EDTA (pH 8.0) and protease inhibitors (1836170, Roche, USA) and phosphatase inhibitors (07575-51, Nakalai Tesque, Japan). Following incubation on ice for 10 min, cell lysate was cleared by centrifugation at 20,000 g for 10 min at 4 °C. Protein quantity was measured using BCA method with BSA as standard. Total cell lysate (20 μg) reduced in sample buffer was resolved by 10% SDS-PAGE for p70S6K.

Proteins were transferred to nitrocellulose membrane, and probed with a specific antibody (1:1000 dilution) overnight at 4 °C. Proteins were visualized using anti-rabbit secondary antibody conjugated to HRP and a chemiluminescence detection system (Immobilon Western Chemiluminescent HRP Substrate, Millipore).

Statistical analysis and data managing. Differences between groups were assessed by one-way or two-way analysis of variance (ANOVA). P values of < .05 were considered significant unless otherwise stated. Statistical analyses were performed with SPSS 21.0.

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