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REGULAR ARTICLE

Glycine regulates proliferation and differentiation of salivary-gland-derived progenitor cells

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Abstract Amino acids have various physiological activities that influence processes such as intestinal regeneration, EGF secretion, protein synthesis, and cell growth. Salivary glands are exposed to nutrients that influence their proliferation and regeneration. Glycine is included in saliva in large quantities and reportedly has important roles in antibacterial activities and the inhibition of tumor growth and as a precursor of nucleotide synthesis in cell proliferation. We have investigated the effects of glycine on the proliferation and differentiation of salivary glands by using mouse salivary-gland-derived progenitor (mSGP) cells. In cultures of mSGP cells, cell proliferation is suppressed in the presence of glycine, whereas it is promoted by its removal. Glycine promotes three-dimensional formations of mSGP cells, which are negative for immature markers and positive for differentiation markers. In cell-cycle analysis, cell-cycle progression is delayed at the S-phase by glycine supplementation. Glycine also suppresses the phosphorylation of p42/p44MAPK. These results suggest that glycine suppresses the proliferation and promotes the differentiation of mSGP cells, and that it has inhibitory effects on growth factor signaling and cell-cycle progression. Glycine might therefore be a physiological activator that regulates the proliferation and differentiation of salivary glands.

Keywords Salivary glands · Glycine · Growth factor · Mouse salivary-gland-derived progenitor (mSGP) cells · Cytokeratin (CK) 19 · Cell cycle

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Introduction

The major components of the gastrointestinal system are the oral cavity, salivary glands, esophagus, stomach, intestine, liver, and pancreas. Since the gastrointestinal tract is directly exposed to external nutrients, it is the component that is the most affected by them. For example, the intestinal epithelium contains subpopulations of cells that exhibit the most rapid turnover and synthesis of proteins (Drucker et al. 1996). The supply of most of the amino acids in the intestine is highly dependent on interstitial absorption. In particular, L-glutamate, L-glutamine, and L-aspartic acid are taken up as an energy source directly from the lumen. In addition, there are numerous reports of the role of L-glutamine as a biologically active factor in the salivary gland and the intestinal epithelium in which it stimulates epidermal growth factor (EGF) secretion, accelerates intestinal immunity by the secretion of EGF, and induces intestinal epithelial regeneration (Wilmore et al.

1988). Therefore, these amino acids may regulate the functions of various organs.

Amino acids are known to have various physiological activities depending on the biological conditions. For example, branched-chain amino acids (BCAAs) such as L-valine, L-leucine, and L-isoleucine have physiological properties such as accelerating protein synthesis and inhibiting protein degradation in skeletal muscle (Goldberg and Chang 1978). BCAAs, which account for approximately 40% of all the free amino acids in serum, are useful energy sources during exercise and for maintaining blood-sugar levels, as mediated by the glucose-alanine cycle (Harper et al. 1984). L-arginine is an intermediate in the urea cycle and is also a precursor for the synthesis of nitric oxide and polyamines (Peranzoni et al. 2007; Rees et al. 1998; Reynolds et al. 1990). L-arginine is known to stimulate the secretion of various hormones (Ghigo et al. 1994). Moreover, L-arginine deprivation can cause a delay in cell-cycle progression in HeLa cells (Wheatley et al. 2000). A high-dose of L-alanine has been reported to improve liver function and the survival ratio in rats with acute liver failure caused by a lethal dose of D-galactosamine (Maezono et al. 1996). Several other amino acids are thought to be vital for cell proliferation and important for maintaining homeostasis as neurotransmitters and as a source of adenosine triphosphate. Glycine is a component of natural products and functions as an inhibitory neurotransmitter (Ghavanini et al. 2005). Glycine also exists in saliva, inhibiting the co-agglutination of oral bacteria (Amano et al. 1997). In addition, glycine prevents tumor growth in vivo (Rose et al. 1999a, 1999b).

In this study, we have analyzed the effect of glycine on cell proliferation and differentiation by using mouse salivary-gland-derived progenitor (mSGP) cells. The salivary glands originate from the endoderm and the ectoderm, both of which participate in organogenesis (Larsen et al. 2001; Denny et al. 1997). mSGP cells were originally isolated from c-kit$^+$/Sca-1$^+$ small epithelial ducts that had proliferated and been detected by a fluorescence-activated cell sorter (FACS) only after duct ligation (Hisatomi et al. 2004; Matsumoto et al. 2007; Okumura et al. 2003; Sato et al. 2007). Isolated immature mSGP cells, which differentiate into hepatic and pancreatic cells, are positive for c-kit, Sca-1, Thy-1, intracellular laminin, CD49f, and alpha-fetoprotein (AFP). These cells are reportedly negative for differentiation markers such as cytokeratin (CK) 19, insulin, and albumin, and they do not exhibit any of the characteristics of duct epithelial cells (Hisatomi et al. 2004). mSGP cells can form cell clusters autonomously on type I collagen and differentiate at the tips of the clusters, which exhibit intracellular-laminin disappearance and CK19 expression. Therefore, since the number of cell clusters, CK19-positive cells, and intracellular laminin-negative cells reflects differentiation, we have employed a three-dimensional (3-D) culture assay to screen and detect the effect of amino acids on the differentiation of mSGP cells.

Materials and methods

Cell isolation and culture

Cells were isolated and cultured as previously described (Hisatomi et al. 2004). Cells isolated from submandibular glands, named mSGP cells, were plated and cultured on type I collagen (Asahi Techno Glass, Tokyo, Japan) at a density of 1×10^6 cells/100-mm dish, in control culture medium. The medium was renewed every 3 days.

The control culture medium was Williams' medium E supplemented with 5% fetal bovine serum (Invitrogen), 20 ng/ml mouse EGF (Chemicon International), 10^{-6} mol/l dexamethasone (Sigma, St Louis, MO., USA), 100 U/ml penicillin G, 100 μg/ml streptomycin (Invitrogen), 1× insulin-transferrin-serenium-X (Invitrogen), and 10 mmol/l nicotinamide (Sigma).

Preparation of media with or without amino acids

Delta media (δ media) were provided by AJINOMOTO (Kawasaki, Japan): the 20 types of δ media consisted of the 20 kinds of amino acids with the exclusion of one amino acid (0 mM) each. The media were prepared based on an amino-acid-free medium of the same composition as Dulbecco's modified Eagle's medium (DMEM) except for the amino acids. The final concentration of amino acids in these media was based on that of DMEM as follows (in mM): 0.4 mM glycine, 0.4 mM L-alanine, 0.4 mM L-arginine, 0.4 mM L-asparagine, 0.4 mM L-aspartic acid, 0.2 mM L-cystine, 4.0 mM L-glutamine, 0.4 mM L-glutamic acid, 0.2 mM L-histidine, 0.8 mM L-isoleucine, 0.8 mM L-leucine, 0.8 mM L-lysine, 0.2 mM L-methionine, 0.4 mM L-phenylalanine, 0.4 mM L-proline, 0.4 mM L-serine, 0.8 mM L-threonine, 0.08 mM L-tryptophan, 0.4 mM L-tyrosine, and 0.8 mM L-valine.

Medium with the addition of glycine, L-alanine, or L-serine at a concentration of 10 mM was called plus (+) medium (+Gly, +Ala, or +Ser, respectively). All media were prepared 24 h before use and kept at 4°C.

In vitro cell proliferation studies

The number of proliferating cells present at 24 h and 48 h was determined by a modified 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT)-based assay (Kishida Reagents Chemicals, Osaka, Japan) and bromodeoxyuridine (BrdU) labeling as in the manufacturer's protocol.

In vitro cell differentiation studies

To induce cell differentiation, mSGP cells were cultured on type I collagen (Asahi Techno Glass) at a density of 2×10^5 cells/60-mm dish in +Gly, +Ala, or +Ser for 2 weeks. The medium was renewed every 3 days. After 2 weeks, the phenotypes of the differentiated cells cultured in these media were estimated by immunofluorescent stains and reverse-transcriptase polymerase chain reactions (RT-PCR).

In vitro cell signal inhibition studies

To inhibit cell proliferation signaling, mSGP cells were cultured in media supplemented with a 5 μM MEK inhibitor (U0126), 20 μM PI3K inhibitor (LY294112), 20 μM Akt inhibitor (Calbiochem Biochemicals, Darmstadt, Germany), or 5 nM rapamycin (Cell Signaling Technology, Danvers, Mass., USA) for 24 h. The cell phenotypes were then estimated, as was cell differentiation.

Analysis for DNA content, cyclin expression, and apoptosis

Cells were plated on type I collagen (Asahi Techno Glass) at a density of 2.5×10^5 cells/60-mm dish and treated with 10 mM glycine for 24 h. The DNA content, cyclin expression, and apoptosis of cells treated with glycine were analyzed by flow cytometry on a Becton Dickinson FACS Caliber (BD Bioscience), as in a previous study (Hisatomi et al. 2004).

Immunostaining of cultured cells

Cells on glass dishes were stained as previously described (Hisatomi et al. 2004). The primary antibodies used were anti-CD49f (BD Bioscience PharMingen), anti-AFP, anti-CK19, anti-E cadherin (Santa Cruz Biotechnology), anti-laminin, or anti-albumin (DAKO Cytomation) diluted at 1:100 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, for 1 h at 37°C. The secondary antibodies used were Alexa488-labeled anti-goat IgG, Alexa488-labeled anti-rabbit IgG, Alexa594-labeled anti-mouse IgG, Alexa594-labeled anti-goat IgG, Alexa594-labeled anti-rat IgG, Alexa594-labeled anti-rabbit IgG (Molecular Probes) diluted at 1:1,000 in PBS containing 1% bovine serum albumin, for 1 h at 37°C. Cells were viewed under a confocal laser-scanning microscope FV500 (Olympus Optical, Tokyo, Japan).

RT-PCR analysis

Total RNA isolation and complementary DNA preparation were as previously described (Hisatomi et al. 2004; Matsumoto et al. 2007; Sato et al. 2007). The resulting complementary DNA was amplified by using GeneAmp PCR 9700 (Perkin-Elmer, Norwalk, Conn., USA) with the following sets of primers: AFP: forward 5′-actcaccc caaccttcctgtc-3′, reverse 5′-cagcagtggctgataccagag-3′; albumin: forward 5′-catgacaccatgcctgctgat-3′, reverse 5′-ctctgatcttcaggaagtgtac-3′; CK19: forward 5′-gtcctacagattga caatgc-3′, reverse 5′-cacgctctggatctgtgacag-3′; GAPDH (D-glyceraldehyde-3-phosphate dehydrogenase): forward 5-ccgccaccaccaactgctta-3′, reverse 5′-tcatgagcccttccacaatg-3′.

Western blot analysis

Cells were grown in culture in the presence of 10 mM glycine or inhibitors or in control medium for the time periods indicated. The cells were lysed in complete RIPA buffer (150 mM NaCl, 50 mM TRIS-HCl pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 1 mM EDTA) containing a protease inhibitor cocktail and phosphatase inhibitor cocktail (Nacalai Tesque). Aliquots containing 20 μg protein were loaded per lane onto 1.5-mm 10% SDS-polyacrylamide gels (ATTO, Tokyo, Japan) for electrophoresis and subsequently transferred to a polyvinylidene difluoride membrane; the expression of each protein was confirmed as previously described (Lianguzova et al. 2007). The primary antibodies used were anti-phospho-p42/p44MAPK, anti-p42/p44MAPK (Cell Signaling Technology) diluted at 1:1,000, and the secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-biotin IgG (Cell Signaling Technology) diluted at 1:2,000.

Results

Short-term glycine addition inhibits cell proliferation

In order to investigate whether glycine affected cell proliferation in vitro, we utilized mSGP cells, i.e., tissue progenitor cells derived from duct-ligated submandibular glands and originating from small epithelia ducts; these cells were able to grow while maintaining their immaturity in the presence of EGF (Hisatomi et al. 2004; Matsumoto et al. 2007; Okumura et al. 2003; Sato et al. 2007). To investigate the effects of each amino acid on cell proliferation, we cultured mSGP cells in media supplemented with one of the 20 amino acids. Cell proliferation in the presence of glycine was reduced in comparison with that of the control, whereas cells treated with L-alanine or L-serine grew as well as the control population. Based on these data, we selected three amino acids, viz., glycine, L-alanine, and L-serine, in order to investigate whether these amino acids affected cell proliferation in vitro. We cultured mSGP cells for 48 h with glycine, L-alanine, or L-serine and observed

the morphology and counted the numbers of mSGP cells every 24 h by a modified MTT assay. The number of cells increased in all cultures. However, glycine suppressed the number of cells at 24 h and 48 h (Fig. 1b). In the media with L-alanine or L-serine, no changes were seen in the cell morphology or the proliferation of mSGP cells (Fig. 1a, b). To confirm the effect of glycine on cell proliferation, we used an assay with BrdU. These results also showed that glycine supplementation suppressed the numbers of BrdU-positive cells at 24 h, similar to the MTT assays (Fig. 1c, d).

Short-term glycine depletion promotes cell proliferation

A decrease in glycine might promote cell proliferation. To determine the effects of a deprivation of glycine, we cultured mSGP cells with various amino acids removed from the medium, for 48 h. We prepared 20 types of medium (δ medium), which consisted of amino acid groupings with the removal of one amino acid each. Glycine-free medium (δGly) demonstrated a promotion of proliferation compared with control at 48 h (Fig. 2b). Depletion of L-cysteine, L-glutamine, L-glutamic acid, L-aspartic acid, L-arginine, or L-proline resulted in a reduction in cell number at 48 h (Fig. 2b), as did depletion of L-threonine, L-methionine, L-valine, L-leucine, L-isoleucine, L-phenylalanine, L-tryptophane, L-lysine, or L-histidine. No changes were observed in the L-alanine, L-serine, L-asparagine, or L-tyrosine depleted media compared with the control. Morphology was maintained in all of the δ media (Fig. 2a). Thus, glycine had unique effects on cell growth; glycine-free medium promoted the proliferation and glycine supplementation suppressed the proliferation of mSGP cells.

To investigate whether glycine inhibition of cell proliferation was associated with amino acid toxicity, we cultured mouse fetal fibroblasts (E13) for 48 h in 0 mM, 2.5 mM, 5 mM, 10 mM, 20 mM, or 50 mM concentrations of glycine. We counted the number of cells every 24 h by

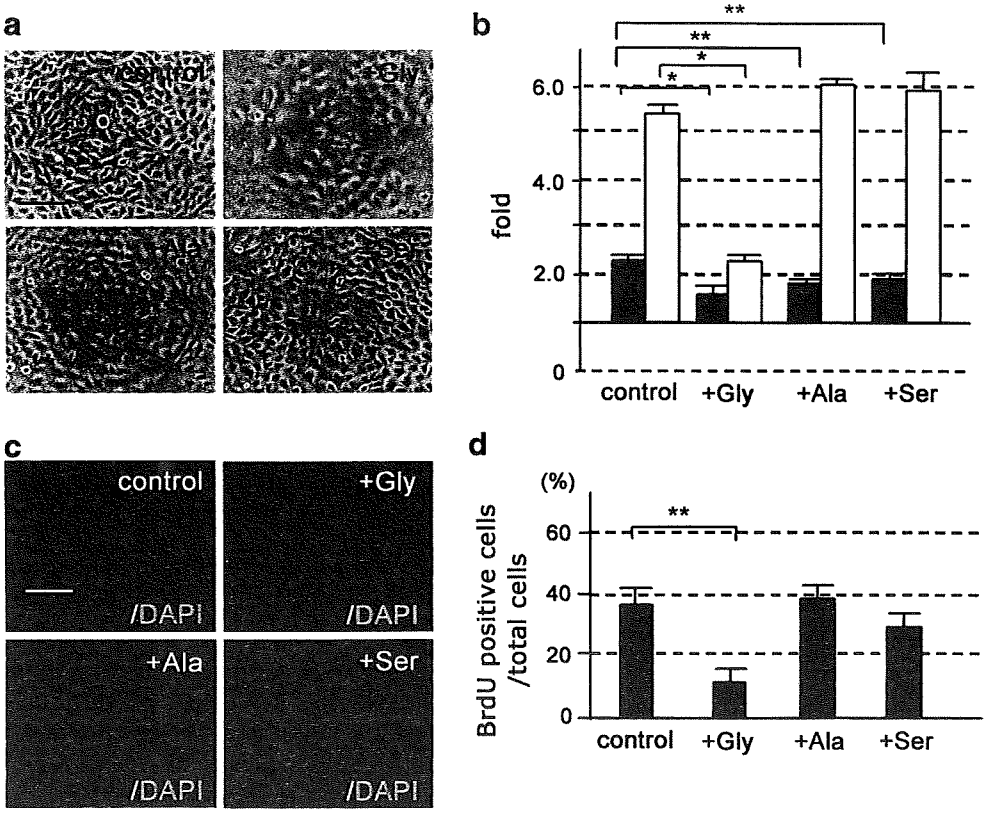


Fig. 1 Glycine inhibits cell proliferation of mSGP cells in short-term culture. mSGP cells were cultured in media supplemented with glycine, L-alanine, or L-serine. The morphology was observed, and the numbers of mSGP cells were counted every 24 h by modified MTT assay. **a** Morphology of mSGP cells at 24 h; this morphology was maintained in all cultures. *Bar* 100 μm. **b** Effects of amino acids on mSGP cell proliferation (*y-axis* numbers of proliferating cells at each time point/numbers of cells at 0 h, *black bars* 24 h, *white bars* 48 h). Glycine suppressed the numbers of cells at 24 h and 48 h (24 h: 2.23-fold in *control*, 1.19-fold in *+Gly* compared with culture at time 0 h; 48 h: 5.67-fold in *control*, 2.27-fold in *+Gly*, compared with culture at time 0 h. $*P<0.001$, $**P<0.05$). **c**, **d** Cell proliferation analysis by BrdU assay. **c** Immunofluorescent staining of mSGP cells. Glycine suppressed the numbers of BrdU-positive cells (*red*). Counterstained with 4,6-diamidino-2-phenylindole (*DAPI*). *Bar* 100 μm. **d** Quantitative analysis of BrdU-positive cells (*y-axis* numbers of BrdU-positive cells/total number of cells). The ratio of BrdU-positive cells was decreased by glycine compared with control (*control* 41.19%, *+Gly* 11.9%, *+Ala* 45.93%, *+Ser* 28.35%, $**P<0.05$)

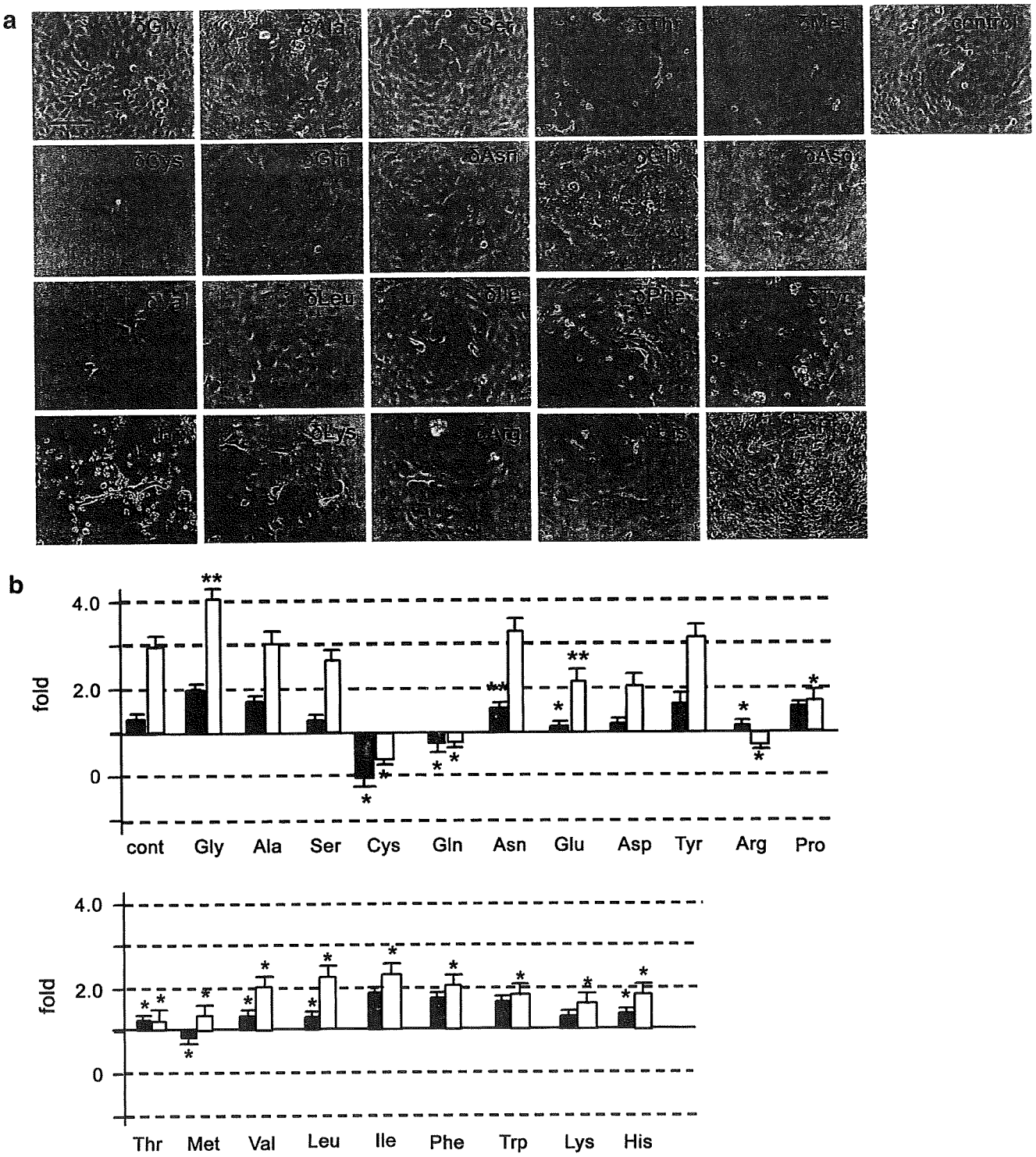


Fig. 2 Glycine depletion promotes cell proliferation of mSGP cells in short-term culture. **a** Morphology of mSGP cells at 48 h; this morphology was maintained in all cultures. *Bar* 100 μm. **b** Effects of amino acid depletion on mSGP cells proliferation (*y-axis* number of proliferating cells at each time point/number of cells at 0 h, *black bars* 24 h, *white bars* 48 h). Glycine-free medium demonstrated proliferation promotion compared with control (3.89±0.24-fold in δGly, 3.17±0.23-fold in control, $^{**}P<0.05$). Depletion of L-cysteine, L-glutamine, L-glutamic acid, L-aspartic acid, L-arginine, or L-proline resulted in a reduction in cell number at 48 h (0.73±0.05-fold in δCys, 0.87±0.04-fold in δGln, 2.29±0.21-fold in δGlu, 2.33±0.35-fold in δAsp, 0.97±0.06-fold in δArg, 1.84±0.09-fold in δPro, $^{*}P<0.001$, $^{**}P<0.05$). Depletion of L-threonine, L-methionine, L-valine, L-leucine, L-isoleucine, L-phenylalanine, L-tryptophane, L-lysine, or L-histidine also resulted in a reduction in cell number (48 h: 1.09±0.11-fold in δThr, 1.39±0.19-fold in δMet, 2.11±0.12-fold in δVal, 2.32±0.20-fold in δLeu, 2.30±0.22-fold in δIle, 1.94±0.16-fold in δPhe, 2.27±0.39-fold in δTrp, 1.89±0.15-fold in δLys, 2.52±0.19-fold in δHis, $^{*}P<0.001$, $^{**}P<0.05$)

modified MTT assay. The results revealed a dose-dependent suppression by glycine in proliferating cells. In addition, the number of proliferating cells increased in glycine-free medium (data not shown). Neither apoptotic cells nor necrotic cells could be detected in any of the media. These results suggested that the suppression of cell proliferation in glycine-supplemented medium was not caused by amino acid toxicity, and that glycine was a regulator of cell proliferation.

Glycine induces cell-cycle arrest/delay in the S-phase

We investigated whether glycine influenced cell-cycle progression. mSGP cells were synchronized in the G0/G1-phases in the presence of glycine, and the cell-cycle distribution pattern was analyzed by quantifying the DNA content with flow cytometry analysis at 24 h after glycine treatment. Treatment with 10 mM glycine increased the percentage of cells in the S-phase (Fig. 3a), whereas the percentage of cells in the G2/M-phase was decreased (Fig. 3a). The percentage of cells in the G1-phase was unchanged (Fig. 3a). To confirm these results, we investigated cyclin expression by flow cytometry analysis. The cyclinA-positive cells, which appeared in the S-phase increased after 24 h glycine treatment, whereas the cyclinD1-positive or cyclinB1-positive cells, which appeared in the G1 or G2/M-phase, significantly decreased (Fig. 3b). These results suggested that glycine inhibited the cell-cycle progression in the S-phase, but not in the G0/G1-phase. These effects of glycine were independent of apoptosis (Supplemental Fig. 1a, b). Therefore, glycine regulated the cell cycle and cell proliferation without toxicity.

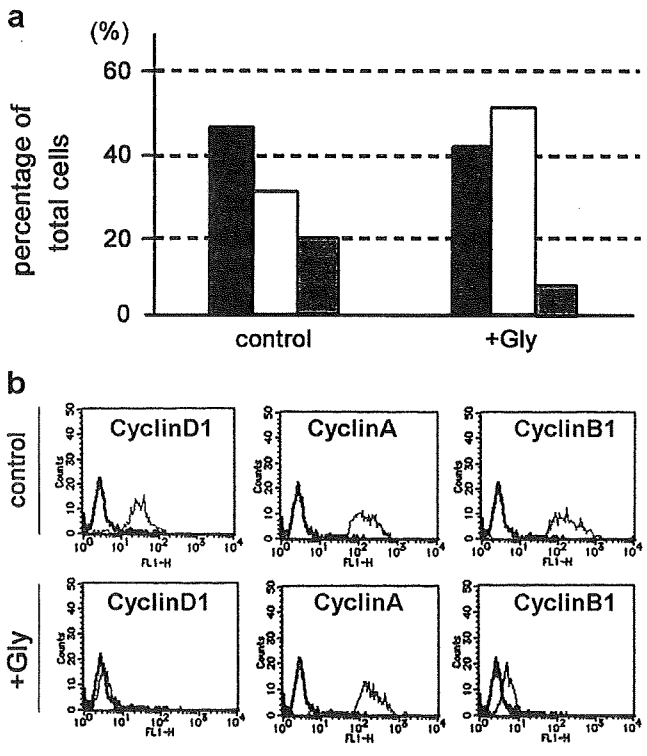
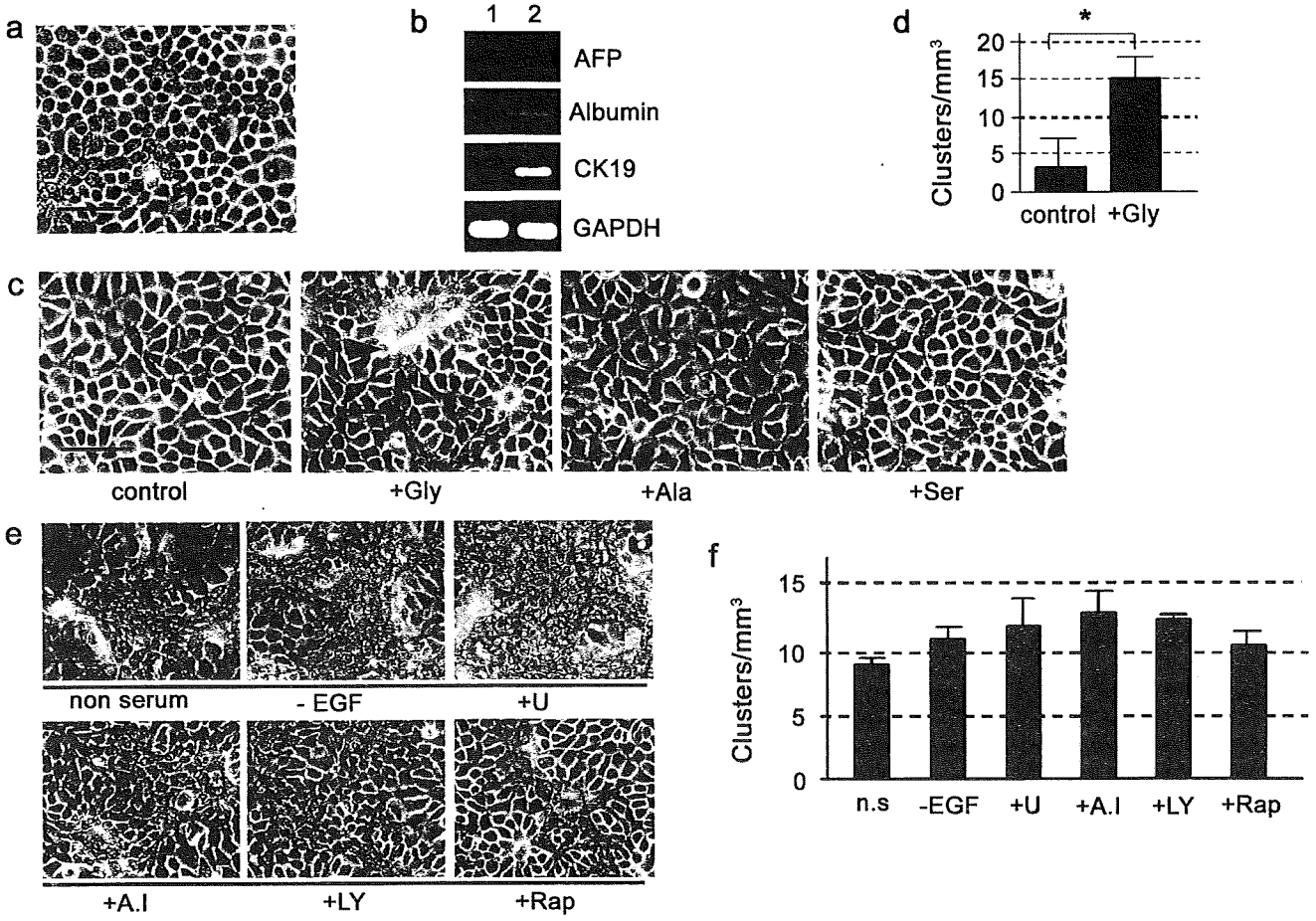


Fig. 3 Glycine affects on cell-cycle progression. Cell-cycle distribution was analyzed by quantification of DNA content and cyclin expression at 24 h after 10 mM glycine treatment. **a** Quantitative analysis of nuclear DNA contents by using Vybrant Dye-Cycle Green Staining. Glycine increased the percentage of cells in the S-phase compared with control (*control*: 45.95% [*black bar* G1-phase], 32.24% [*white bar* S-phase], 21.81% [*gray bar* G2/M-phase]; *+Gly*: 43.51% [*black bar* G1-phase], 49.75% [*white bar* S-phase], 6.75% [*gray bar* G2/M-phase]). **b** Flow cytometry analysis of cyclin expression. The numbers of cyclinA-positive cells increased after glycine treatment, whereas the numbers of cyclinD1-positive or cyclinB1-positive cells decreased (*gray lines*)

Fig. 4 Glycine promotes differentiation of mSGP cells in long-term culture. To investigate the effects of glycine on cell differentiation, mSGP cells were cultured for 2 weeks in medium supplemented with 10 mM glycine, and the cell morphology and gene expression were estimated by immunofluorescent stain and RT-PCR. **a** Morphology of immature mSGP cells. Cells formed uniform polygonal cell populations with large nuclei, assuming the form of an epithelium. *Bar* 100 μm. **b** RT-PCR performed on mRNA obtained from mSGP cells cultured in control medium; the cells expressed alpha-fetoprotein (*AFP*) but were negative for cytokeratin 19 (*CK19*) and albumin (*lane 1* mSGP cells in control medium, *lane 2* positive control, *GAPDH* D-glyceraldehyde-3-phosphate dehydrogenase). **c** Morphology of mSGP cells treated with glycine for 2 weeks. Small cell accumulation, cluster formations (*arrowhead*), and duct-like structures (*arrow*) were increased. The duct-like structures could not be detected in other media. *Bar* 100 μm. **d** Effect of glycine on cluster formation. Numbers of clusters of 1 mm^2 were counted. Clusters comprised differentiated mSGP cells. Glycine promoted the numbers of clusters compared with the control (*control*: 3.6±2.63 clusters/mm^2; *+Gly*: 14.7±4.17 clusters/mm^2, $*P<0.001$). **e** Morphology of mSGP cells in cultures with serum withdrawal (*non serum*), EGF withdrawal (*-EGF*), and signal transduction inhibitors (*+U* 5 μM U0126, *+LY* 20 μM LY294112, *+A.I* 20 μM Akt-inhibitor, *+Rap* 5 nM rapamycin). Cluster formations (*arrowheads*) and duct-like structures (*arrows*) were increased in all cultures. *Bar* 100 μm. **f** The effect of serum withdrawal (*n.s*), EGF withdrawal, and cell proliferation inhibitors on cluster formation. The number of clusters was not statistically different between the media. **g** Effect of glycine on EGF signaling pathway. Western blot analysis was performed on whole-cell lysates from cells cultured in 10 mM glycine or 5 μM U0126 supplementation for 2, 6, or 24 h. Glycine decreased phospho-p42/p44MAPK expression at 6 h in comparison with the control and abolished it at 24 h. Whole cell lysates from HeLa cells were used as a positive control (*+Gly* 10 mM glycine, *+U* 5 μM U0126, *HeLa* HeLa cells). **h, i** Immunofluorescent staining of mSGP cells. Immature mSGP cells cultured in control medium were positive for AFP, CD49f, and intracellular laminin (antigens of immaturity) and negative for CK19 (expressed by duct epithelium). **h** Cells cultured in glycine-containing medium for 2 weeks. Glycine decreased the expression of AFP (*green*), CD49f (*green*), and laminin (*green*), whereas it increased that of E-cadherin (*red*) and CK19 (*red*). *Bar* 100 μm. **i** Cells cultured with signal transduction inhibitors or without EGF. The expression of laminin (*green*) decreased and CK19 (*red*) increased in comparison with the control in **h**. *Bar* 100 μm. **j** RT-PCR performed on mRNA obtained from mSGPs cultured in glycine-containing medium. Glycine increased the gene expression of CK19 (*lane 1* control, *lane 2* +Gly, *lane 3* positive control)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Long-term glycine addition promotes differentiation of mSGP cells

Glycine has been suggested to promote the differentiation of mSGP cells. We investigated the effects of glycine on the differentiation of mSGP cells cultured for 2 weeks. The results showed a significant increase in differentiated clusters in glycine-containing medium (Fig. 4c, d). In addition, epithelial-duct-like structures appeared in mSGP cells cultured in this medium. The duct-like structures could not be detected in other media (Fig. 4c, arrow). The morphology of mSGP cells was unchanged with the other amino acids (Fig. 4c).

To investigate whether the effects of glycine on differentiation were associated with EGF signaling, we cultured mSGP cells in media supplemented with signal transduction inhibitors such as 5 μM MEK inhibitor (U0126), 20 μM PI3K inhibitor (LY294112), 20 μM Akt-inhibitor, or 5 nM rapamycin. The treatment with inhibitors led to an increase in clusters and duct-like structures, similar to the treatment with glycine (Fig. 4e, f). Western blotting analysis revealed that glycine decreased phospho-p42/p44MAPK and its activity at 6 h in comparison with control and completely abolished it at 24 h (Fig. 4g). Increased expression of CK19 was detected in cells cultured in glycine-supplemented medium, serum-free medium, EGF-free medium, or media with inhibitors (Fig. 4h-j). These results suggested that the effects of glycine on the differentiation of mSGP cells were similar to that of EGF-signal inhibition.

Discussion

In this study, we have demonstrated that glycine, which is one of the non-essential amino acids for mammals, suppresses the proliferation and promotes the differentiation of mSGP cells.

To investigate the effect of glycine on cell proliferation in vitro, we have cultured mSGP cells in the presence of glycine. Glycine supplementation suppresses cell proliferation of mSGP cells.

Our previous studies have demonstrated three procedures useful for investigating the differentiation of mSGP cells in three-dimensional culture; autonomous cell-cluster formation on monolayer culture, induced 3-D structure formation in matrigel matrix culture, and spherical culture (Hisatomi et al. 2004; Matsumoto et al. 2007; Okumura et al. 2003; Sato et al. 2007). The clear differences between each procedure are evident in terms of gene expression and function of mSGP cells. Cell clusters are formed autonomously on monolayer culture, and the differentiated mSGP cells in cell clusters are positive for CK19, whereas they are negative for insulin and albumin. This indicates that the cell-cluster formation on monolayer culture represents an early stage of mSGP cell differentiation. The cell culture procedures in matrigel and spherical culture also encourage mSGP cells to form 3-D structures, inducing differentiation. The differentiated cells in matrigel are of the hepatic lineage; they express albumin and antitrypsin and differentiate into hepatocytes after transplantation into the liver (Hisatomi et al. 2004; Okumura et al. 2003). Spherical cultures cause mSGP cells to differentiate into the pancreatic endocrine lineage via artificial 3-D structural formations. The differentiated cells in the spheres are positive for insulin and glucagon. These cells release insulin, when stimulated by glucose and potassium (Hisatomi et al. 2004; Matsumoto et al. 2007). In this study, we have used cell-cluster formation on monolayer culture to analyze the effect of glycine at the early stage of mSGP cell differentiation. Glycine supplementation on monolayer culture enhances cell-cluster formation of mSGP cells; the clusters are CK19 positive. Moreover, glycine supplementation encourages mSGP cells to form duct-like structures. The increase of autonomous cell-cluster formation by glycine suggests that glycine promotes mSGP cell differentiation. These results indicate that glycine exerts an effect on the differentiation of mSGP cells at an early stage.

We have also investigated the effect of signal transduction inhibitors, the removal of EGF, or glycine-supplementation on mSGP cells. Previous studies have shown that the phosphorylation and activation of members of the mitogen-activating protein kinase (MAPK) family such as p42/p44MAPK and p38MAPK, which are downstream effectors of the EGF receptor, occur during the development of several vertebral organs including the salivary glands (Cardoso and Lu 2006; Liu et al. 2008). Inhibition of PI3K, which is a downstream effector of the receptor of fibroblast growth factor (FGF), is reported to suppress the formation of the salivary gland epithelial bud, as shown when the MAPKs are inhibited (Larsen et al. 2003). The inhibitors and deprivation of EGF promote the formation of cell clusters, similar to the finding with glycine. EGF is an essential growth factor for mSGP cells to retain their immaturity. Glycine might therefore function to inhibit the EGF signaling pathway, leading to growth suppression, and to promote the differentiation of tissue progenitor cells.

During cell proliferation, growth factors are well known to affect cell-cycle progression, especially the G0 to G1 transition and the G1-S progression (Cardoso and Lu 2006; Jones and Kazlauskas 2001; Liu et al. 2008; Meloche and Pouyssegur 2007); hence, cell proliferation is suppressed by cell-cycle arrest in the G0/G1-phase (Jones and Kazlauskas 2001; Shackelford et al. 1999). When deprived of serum or growth factors, cells exit into

the G0-phase after they complete mitosis. These cells can be reintroduced into the cell cycle by the re-addition of serum or purified growth factors (Jones and Kazlauskas 2001; Pardee 1974, 1989). Growth factors such as EGF, FGF, and hepatocyte growth factor have recently been reported also to induce G2/M transition delay (Dangi et al. 2006; Nam et al. 2008). We have demonstrated that glycine induces S-phase arrest/delay in mSGP cells. The cell-cycle arrest at S-phase usually indicates a delay of DNA synthesis or DNA repair. A previous study has shown that the chemical inhibition of pyrimidine nucleotide synthesis in cells delays S-phase progression (Liu et al. 2007), indicating that S-phase arrest can indeed occur as a result of a delay in DNA synthesis. In this study, we have shown that glycine inhibits MAPK signaling (Fig. 4), suppresses cell proliferation (Fig. 1), promotes differentiation (Fig. 4), and arrests (or delays) the cell cycle (Fig. 3). Cells with DNA damage caused by radiation or chemical injury initiate cellular recovery mechanisms, such as cell-cycle arrest in the G1-phase and apoptosis (Delia et al. 1997; Gentile et al. 2003; Shackelford et al. 1999). Other reports have revealed that the deprivation of growth factors induces caspase and superoxide activities and cell apoptosis (Lieberthal et al. 1998). Our results provide no evidence of cell death resulting from apoptosis or necrosis after glycine supplementation. Furthermore, glycine-treated cells are induced to proliferate by the removal of glycine from culture media, suggesting that glycine has regulatory functions in cell proliferation, not in cell toxicity. The S-phase delay in non-proliferating cells treated with glycine might be caused by a delay in DNA synthesis or a delay in the G2/M transition accompanying the inhibition of growth factors.

Non-ketotic hyperglycenemia (NKH), which is an inherited deficiency of glycine metabolism, presents severe neurological symptoms and frequently accompanies brain malformations (Ichinohe et al. 2004; Sakata et al. 2001). It is characterized by the accumulation of a large amount of glycine in serum and cerebrospinal fluid (CSF), indicating that the normal glycine concentration in CSF is indispensable for normal brain development. In NKH, the oxidative breakdown of glycine is impaired, causing the deprivation of 5, 10-methylenetetrahydrofolate, which is essential for the synthesis of DNA during cell proliferation (Ichinohe et al. 2004; Sakata et al. 2001). This evidence suggests that a high concentration of glycine might have inhibitory or toxic effects on neurogenesis.

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ORIGINAL ARTICLE

Mutant alleles associated with late-onset ornithine transcarbamylase deficiency in male patients have recurrently arisen and have been retained in some populations

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We performed haplotype analysis using nine single nucleotide polymorphisms in the ornithine transcarbamylase gene to explore the ancestral origins of three mutations associated with late-onset phenotype in male patients: p.R40H, p.R277W and p.Y55D. Overall, 8 haplotypes were defined among 14 families carrying p.R40H, 5 families carrying p.R277W and 2 families with p.Y55D mutations. Of nine Japanese families carrying p.R40H, eight exhibited haplotype (HT)1, whereas the other family harbored HT2. Among three Caucasian families, one Spanish and one Australian family bore HT3; one Austrian family had HT4. Two US patients harbored HT2 and HT4. Among families carrying p.R277W, HT5 was found in one Japanese, one Korean and one US family. Two other US families had HT2 and HT6. Two families carrying p.Y55D, both Japanese, shared HT1. These results indicate that the p.R40H mutation has arisen recurrently in all populations studied, although there is evidence for a founder effect in Japan, with most cases probably sharing a common origin, and to a lesser extent in subjects of European ancestry (HT3). It is evident that p.R277W mutation has recurred in discrete populations. The p.Y55D mutation appears to have arisen from a common ancestor, because this transversion (c.163T>G) occurs rarely.
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INTRODUCTION

Ornithine transcarbamylase (OTC) deficiency (OMIM no. 311250) is the most common inherited disorder of the urea cycle and is transmitted as an X-linked trait.

The locus of the gene encoding OTC is on the short arm of the X chromosome within band Xp21.1.[1] The gene spans 74 kb with an open reading frame of 1062 nucleotides distributed into 10 exons and 9 introns.[2,3] The phenotypes of females heterozygous for a mutant OTC allele vary from asymptomatic carrier state to overt, even fatal disease, depending first on the nature of the gene mutation, second on X-inactivation pattern and third on other genes and environmental factors. In contrast, in hemizygous male patients, the phenotype is determined by the nature of mutation and other yet unknown factors (other genes/environment). Such male patients most commonly develop symptoms of hyperammonemia in the neonatal period or in early infancy and their disease is often fatal.[4] However, there are some male patients in whom the onset of the disease is delayed until the preschool age period[5] through to adulthood.[6,7] Some affected males within the same families may remain asymptomatic for life.[8] Their condition is now recognized as 'late-onset OTC deficiency in male patients', accounting for ~30% of male patients.[9] Such male patients reproduce at a fitness value of 0.49.[10] Although the majority of mutations at human OTC locus are 'private', being observed in single families only,[11] several mutations have been observed repeatedly in discrete families,[12–14] mainly affecting CpG dinucleotides. Among those, the c.119G>A (p.R40H) and c.829C>T (p.R277W) mutations have been repeatedly reported in multiple ethnicities. In our previous series of Japanese families, the c.119G>A (p.R40H) mutation was encountered in a cluster.[7,10,15] In addition, we identified another novel mutation, c.163T>G (p.Y55D) in two discrete families.[10,16] It is not

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known whether or not these mutations share a common ancestral origin or have arisen recurrently.

Polymorphic sites in the human OTC gene having potential for family tracking were reported previously. These include single nucleotide substitutions, insertions and short tandem repeats.[9,17–22] After completion of the HapMap Project, single nucleotide polymorphisms (SNPs) have become available for haplotype analysis on a given gene.

However, SNP-based haplotype analysis of the human OTC locus has not been reported previously. We aimed to determine the haplotypes of these mutant OTC alleles to explore the origins of these mutations.

MATERIALS AND METHODS

Families

A total of 14 families with the c.119G>A (p.R40H) mutation, 5 families with the c.829C>T (p.R277W) mutation and 2 families with the c.163T>G (p.Y55D) mutation were studied. The 14 families with the p.R40H mutation consisted of 9 families from Japan, 1 family each from Spain, Australia and Austria, and 2 families from the United States. The families with p.R277W included 1 family from Japan, 1 family from Korea and 3 families from US. Two families with p.Y55D were both Japanese. The probands were all male, except the proband in family 11, a 13-year-old symptomatic girl who carried the p.R40H mutation, as did her asymptomatic mother. Families 1–9, 15, 20 and 21 were Japanese; family 16, Korean; and families 10–12, Caucasians.

None of the families in the present series were known to be related to each other. Demographic information, including ethnic background, of the US families (13, 14, 17–19) was not available because of the US Personal Data Protection Act. Haplotype analysis was performed on the proband from each family and their relatives, when specimens were available from them. In family 11, the DNA specimens from parents of the proband were also analyzed to determine the mutation-bearing allele. In family 12, the DNA specimen of the proband was prepared from the liver tissue obtained after it had been inadvertently transplanted to a woman.[23]

Haplotype analysis

A total of 9 tagged SNPs were selected on the Haploview[24] with r^2 of 0.80 and minor-allele frequency of 0.05 (Table 1). The haplotype frequencies of Japanese in Tokyo (JPT), Utah residents with Northern and Western European ancestry from CEPH collection (CEU) and Yoruban in Ibadan, Nigeria (YRI) were available on http://hapmap.ncbi.nlm.nih.gov/index.html.en. The nucleotide combinations in *Pan troglodytes* (chimpanzee) were obtained on http://www.ensembl.org/index.html to estimate the human ancestral alleles. The nucleotide in the polymorphic site 6 (rs5963421 for human) for *P. troglodytes* was represented by that in *Pongo pygmaeus abelii* (orangutan), because this nucleotide was not available for *P. troglodytes*.

The SNPs were determined by the TaqMan probe-based real-time PCR on LightCycler LC-480 (Roche Diagnostics GmbH, Mannheim, Germany). The PCR was carried out in a total volume of 20 µl, containing 20 ng genomic DNA, 10 µl LC-480 Probe Master (Roche) and 1 µl 20× Probe/Primer Mix (Applied Biosystems, LLC, Foster City, CA, USA). The probes used were labeled as either FAM or VIC. The temperature was programmed as follows: a pre-incubation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 60 s and extension at 72 °C for 1 s. At the end of the annealing step, the fluorescence signal was measured. After the PCR reaction, the temperature was decreased to 40 °C as a cooling step.

Ethical considerations

The Ethical Committee of Kurume University approved this project and specimens were obtained in accordance with respective institutional bioethical standards and relevant bioethical regulations or guidelines in each country.

RESULTS

Haplotypes defined in the families and their allelic frequency and heterozygosity

The data on the nine SNPs employed and the haplotypes determined in the families by the use of these SNPs are summarized in Table 1. Six discrete haplotypes (HT's) (1–6) were found among the probands. The parents of the female proband in family 11 carried two additional HT's, 7 and 8.

Table 1 Haplotypes generated by single-nucleotide polymorphism in human ornithine transcarbamylase locus

			Allele frequency heterozygosity			*Haplotype*								
Polymorphic sites	*Tagged SNPs*	*Nucleotide*	*JPN*	*CEU*	*YRI*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	Pan troglodytes
1	rs5917576	G/A	0.91/0.09	0.50/0.50	0.83/0.17	G	G	G	A	G	G	A	G	G
			0.164	0.500	0.282									
2	rs17274134	G/C	0.53/0.47	0.08/0.92	0.19/0.81	G	C	C	C	C	C	C	C	C
			0.498	0.147	0.308									
3	rs6417794	G/C	0.77/0.23	0.79/0.21	0.88/0.12	G	C	C	G	G	C	G	G	G
			0.354	0.332	0.211									
4	rs6609709	G/A	0.11/0.89	0.23/0.77	0.30/0.70	A	A	A	A	G	A	A	A	A
			0.196	0.354	0.420									
5	rs2235125	G/A	0.38/0.62	0.63/0.36	0.22/0.78	A	G	G	G	A	G	A	A	A
			0.471	0.454	0.343									
6	rs5963421	A/T	0.82/0.18	0.60/0.40	0.92/0.08	A	A	T	T	A	A	A	A	A
			0.295	0.480	0.147									
7	rs17274141	C/T	0.46/0.53	0.13/0.87	0.08/0.92	C	T	T	T	T	T	C	C	T[a]
			0.488	0.226	0.147									
8	rs5963428	A/T	0.05/0.95	0.24/0.76	0.43/0.47	T	T	T	T	A	T	T	T	T
			0.095	0.365	0.404									
9	rs12557315	C/T	0.82/0.18	0.80/0.20	0.98/0.02	C	T	C	C	C	C	C	C	C
			0.295	0.320	0.039									

Abbreviations: JPT, Japanese in Tokyo; CEU, Utah residents with Northern and Western European ancestry from CEPH collection; YRI, Yoruban in Ibadan, Nigeria.
[a]Surrogated by the nucleotide in this position in *Pongo pygmaeus abelii* (Orangutan) because it was not available for *P. troglodytes*.
Shaded cells indicate nucleotide bases different from those in *P. Troglodytes*. The segments enclosed by frames indicate those which may have been involved in recombinatory event to form HT4 or generation of HT's 3 and 7 from HT4.

Association of the three mutant alleles and particular haplotypes

The results of the haplotype analysis of the 21 families are summarized in Table 2. Among the nine Japanese probands carrying the p.R40H mutation, those belonging to families 1–8, who resided in an area within a radius of 140 km, all had HT1, whereas family 9, who lived 650 km away from that area, had HT2. One Spanish family and one Australian family had HT3. In this Australian family, the proband (a girl) bore HT3/8; her mother, 3/7; and father, HT8. Previous analysis had shown that mother carried the mutant allele. Therefore, it was determined that the mutation was linked with the HT3 allele. The Austrian family and one US family shared HT4. The proband in the remaining one US family had HT2. Thus, in the 14 families carrying the p.R40H mutation, this mutation was associated with four different HT's, indicating that it had appeared anew at least four times in the Japanese and the Caucasian populations. Similarly, the p.R277W mutation was associated with three different HT's in our five families carrying this mutation. Three families that carried p.R277W, one Japanese, Korean and US family, shared HT5 each. One of the remaining two US families carried HT6 and the other, HT2. The two Japanese families that carried the p.Y55D mutation shared HT1.

Evolutional order of the haplotypes

To estimate the evolutional order of these haplotypes, nucleotides in each polymorphic site were compared with those in *P. troglodytes* (Table 1). HT8 differed only in polymorphic site 7 (T-to-G) from that of *P. troglodytes*. HT1, HT5, HT6 and HT7 exhibited nucleotide changes in two polymorphic sites, whereas in HT2, HT3 and HT4, there were nucleotide changes in three polymorphic sites.

DISCUSSION

Although the majority of mutations at the OTC locus are 'private' in patients with neonatal and infantile presentation,[11] some mutations have been found to recur in unrelated families.[12] Among mutations associated with late-onset OTCD in male patients, the two mutations, p.R40H and p.R277W, have been most frequently reported in multiple different families.[7,12–15,25] The p.Y55D mutation has been found only in two unrelated Japanese families.[10,16] It was not known whether or not the recurring mutations shared a common ancestral origin or had arisen independently. The present study suggests that the p.R40H mutation occurred at least four times, or even five times, if the family 14 is non-Japanese American, in the Japanese and in the Caucasian populations. It appears very likely that those eight Japanese families (1–8) share a common ancestral origin and the mutant allele had been retained in the population in this small area. The other Japanese family (family 9) had a distinct haplotype, however, suggesting this mutation had arisen recurrently. Families 10 and 11, both Caucasian, may also have a common ancestral origin. Although the Australian family has no known Spanish ancestry, the proband's maternal grandfather (not studied) was of North Italian descent and could conceivably share ancestry with the Spanish family. It remains possible that the mutation occurred recurrently, but it is noteworthy that the allele in these families has a low haplotype frequency (0.033) among

Table 2 Haplotypes of mutant alleles carrying the three mutations

Family	*1–6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
Mutation	p.R40H								
Haplotype	1	1	1	2	3	3	4	4	2
Reference no.	Harada *et al.*[7a]	Matsuda *et al.*[25]	Present report	Present report	Arranz *et al.*[27]	Pinner *et al.*[28]	Plöchl *et al.*[23]	Tuchman *et al.*,[11] Tuchman *et al.*[12]	
Ethnicity	Japanese	Japanese	Japanese	Japanese	Caucasian	Caucasian	Caucasian	Unknown	Unknown
Residential country	Japan	Japan	Japan	Japan	Spain	Australia	Austria	United States	United States
Allele frequency									
JPT	0.408			0.151	0.031		0.133		0.151
CEU	0.067			0.155	0.033		0.356		0.155
YRI	0.056			0.022	<0.05[b]		0.044		0.022

Family	*15*	*16*	*17*	*18*	*19*	*20*	*21*
Mutation	p.R277W					p.Y55D	
Haplotype	5	5	5	6	2	1	1
Reference no.	Numata *et al.*[10]	Kim *et al.*[29]	McCullough *et al.*[14]			Nishiyori *et al.*[16]	Numata *et al.*[10]
Ethnicity	Japanese	Korean	Unknown	Unknown	Unknown	Japanese	Japanese
Residential country	Japan	Korea	United States	United States	United States	Japan	Japan
Allele frequency							
JPT	0.061			<0.05[b]	0.151	0.408	
CEU	0.175			<0.05[b]	0.155	0.067	
YRI	0.122			0.056	0.022	0.056	

Abbreviations: JPT, Japanese in Tokyo; CEU, Utah residents with Northern and Western European ancestry from CEPH collection; YRI, Yoruban in Ibadan, Nigeria.
[a]Patients 1, 3, 4, 8. 9 and 10 in reference Harada *et al.*[7]
[b]Below minimum-allele frequency.

Caucasians. Families 12 and 13 share a haplotype, but there is insufficient information to say whether this represents the effect of common ancestry or of recurrence on the same haplotype. HT4 is the most common in Caucasians (allele frequency 0.356 in the CEPH Caucasian samples) and there is no information about the ethnicity of family 13.

Among the families with the p.R277W mutation, the Japanese and the Korean families (families 15 and 16) could have stemmed from a common ancestor. Family 18 bore HT6, confirming recurrent origin of this mutation.

The families that harbored the p.Y55D mutation (families 20 and 21) shared HT1. This mutation has been found among the Japanese population alone to date.[10,16] In contrast to the p.R40H and p.R277W mutations, which involve CpG dinucleotides and hence are likely to recur, this transversion (T-to-G) is not common among single base substitutions in X chromosome genes in general[26] and in the OTC gene in particular.[12,14,27] This characteristic of the p.Y55D mutation would again support that the affected families share a common ancestral origin.

The search for polymorphic markers and the determination of their allelic frequency is required for exploring the origin of an allele. As expected, different types of polymorphic markers in the OTC gene or in its vicinity are known and include SNPs in the coding region,[9,17,20] in the 5′ untranslated region[21] and extragenic microsatellite markers.[18,19,22] Four SNPs, A-to-G in codon 46 in exon 2 (E2 46), A-to-T in intron 3 (IVS3-8nt), A-to-G in intron 4 (IVS4-7nt) and A-to-G in codon 270 of exon 8 (E8 270), were found to be informative among a US population,[17] though their informativity varied somewhat in the Iberian population and in the Mozambiquan population.[18] One additional SNP (IVS3-39_insT) was informative in a Spanish population,[20] indicating that informativity of these SNPs varied between populations. The nine tagged SNPs we used in the present study were all highly informative, except rs5963428, which presented 0.095 heterozygosity in JPT (Table 1). These tagged SNPs were all informative in CEU and YRI also, with the exception of rs12557315 in YRI. We thus considered it appropriate to apply these tagged SNPs to define haplotypes in other ethnicities also. There are several advantages of using these tagged SNPs in determining haplotypes. First, these SNPs generally show high heterozygosity frequency, allowing high informativity. Second, the tagged SNPs are located over the entire span of the OTC gene, reducing misdiagnosis due to an extragenic recombination event when diagnosis by linkage analysis is necessary in the absence of an identifiable mutation. Third, it permits easy differentiation between intragenic recombination and mutation of one particular marker single nucleotide (both rare events). Finally, the analytical efficiency of this technique is high as amplification and data acquisition of up to seven specimens can be completed simultaneously within 90 min under the conditions employed.

Both the p.R40H and p.R277W mutations arise in CpG dinucleotides, which represent mutational hot spots, consistent with our finding that they have recurred in different populations. Recurrent point mutations occur evenly among most CpG dinucleotides in the OTC gene.[9,12,14,27] These two mutations arise in arginine codons. It is not surprising that these single nucleotide changes have a higher chance to recur than others, because four of six codons that encode for arginine contain CpG dinucleotides. Indeed, scrutiny of a recent OTC mutation update[9] reveals that 20 discrete mutations have occurred in a total of 15 arginine codons (ratio: 20/15=1.33) in the human OTC gene, whereas this ratio is less than 1.0 in the vast majority of codons encoding for other amino acids.

Haplotype 8 differed by one nucleotide from that in *P. troglodytes*, whereas the other seven HT's exhibited two or three nucleotide differences. This possibly indicates that HT8 is the oldest among the eight HT's identified in the present series. HT's 3 and 4 shared segments consisting of identical nucleotides (polymorphic sites 5–9) and HT's 4 and 7 had jointly another identical segment (polymorphic sites 1–3) (Table 1). It is thus possible that HT3 and HT7 were generated by a recombination of the HT4 allele with another allele, or conversely HT4 was generated by a recombination of HT3 with HT7.

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High frequency of acid α-glucosidase pseudodeficiency complicates newborn screening for glycogen storage disease type II in the Japanese population

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ABSTRACT

To investigate the feasibility of newborn screening for glycogen storage disease type II (GSDII; Pompe disease or acid maltase deficiency) in the Japanese population, we assayed the acid α-glucosidase activity in dried blood spots from 715 Japanese newborns and 18 previously diagnosed patients using a fluorometric procedure. The enzyme activity of apparently healthy newborns showed a bimodal distribution. The median activity of the minor group (31 individuals, 4.3% of the samples) was 6.5 times lower than that of the major group. Four of the 715 control samples (0.56%) fell in the patient range. We then analyzed genomic DNA, extracted from the same blood spots, for the occurrence of two sequence variants, c.1726G>A and c.2065G>A, known to cause "pseudodeficiency". This analysis revealed that 27 of 28 individuals homozygous for c.[1726A; 2065A] belonged to the minor group. One c.[1726A; 2065A] homozygote had just slightly higher activity. Twelve of the 18 patients with GSDII either had one (9 cases) or two (3 cases) c.[1726A; 2065A] alleles. The frequency of this allele was double in the patient compared to the control group (0.42 *vs* 0.19) at the expense of a lower frequency of the c.[1726G; 2065G] and c.[1726G; 2065A] alleles (0.58 *vs* 0.71 and 0 *vs* 0.1). These findings illustrate that c.[1726A; 2065A] homozygosity among apparently healthy individuals (3.9 per 100) complicates newborn screening for GSDII in Japan, and further that one or more pathogenic mutations are associated with the c.[1726A; 2065A] allele.

Enzyme replacement therapy for lysosomal storage diseases has focused attention on the need for early diagnosis in order to optimize the therapeutic outcome. Along this line several initiatives have been taken to develop methods for newborn screening. Most methods are based on the direct measurement of lysosomal enzyme activities in dried blood spots (DBSs) [1–5]. Other procedures include antibodies to increase the specificity of the assay, or to determine the amount of enzyme protein rather than activity, or to probe lysosomal disease markers [6–9]. Multiplex assays with the measurement of several lysosomal enzyme activities are aimed to improve the cost effectiveness of newborn screening [10–13].

From several clinical trials since 1999, the picture emerges that patients with glycogen storage disease type II (GSDII) can benefit from enzyme replacement therapy [14–18]. GSDII, also known as Pompe disease or acid maltase deficiency (OMIM No. 232300) is an autosomal recessive disorder of glycogen metabolism resulting from a generalized deficiency of the lysosomal enzyme acid α-glucosidase (AαGlu[1]; EC 3.2.1.20/3). The enzyme deficiency causes intralysosomal glycogen storage in numerous tissues, but predominantly in muscle. The disorder exhibits a broad clinical spectrum with regard to age of onset, cardiac involvement and progression of skeletal muscle dysfunction. The effect of therapy in severely affected infants is readily recognized by regression of the cardiomegaly, prolonged survival and acquirement of motor skills. Beneficial effects of enzyme replacement therapy in children, adolescents and adults with GSDII also have been reported and are promising, but the crucial outcomes of larger clinical trials is still to be awaited as well as the long term effects [17–20]. Further, it appears that infants with rather well preserved muscle morphology respond better to therapy than those who are diagnosed late and have severe muscle damage at start of treatment. Early diagnosis seems a must in GSDII to optimize any form of therapeutic intervention [21].

Recently, we have established an assay procedure for the reliable diagnosis of GSDII in mixed leukocytes whereby acarbose is used to inhibit the interfering α-glucosidase activity of

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[1] *Abbreviations used:* AαGlu, acid α-glucosidase; GSDII, glycogen storage disease type II; 4MU-αGlc, 4-methylumbelliferyl α-D-glucopyranoside; DBS, dried blood spot.

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maltase-glucoamylase [22]. Of note, acarbose was introduced earlier to eliminate the interfering maltase-glucoamylase activity in DBS assays [10] and is more suitable for that purpose than maltose [3]. Given the recent interest in newborn screening and the awareness that the diagnosis of GSDII in Japan and in other Asian populations might be complicated by the existence of a "pseudodeficiency" allele [23,24], we performed a pilot experiment investigating the feasibility of newborn screening in Japan. "Pseudodeficiency" of AαGlu is associated with two SNPs, c.1726G>A (p.G576S) and c.2065G>A (p.E689K) that have a different distribution in Asian compared with Caucasian populations [23–25]. Substitution p.E689K caused by c.2065G>A characterizes the "GAA4" allozyme, which is found in Chinese and Japanese populations with frequencies of 0.27–0.28 and 0.27–0.31, respectively, and reduces the AαGlu activity by 50% at most [26–28; JSNP, http://snp.ims.u-tokyo.ac.jp/map/cgi-bin/aa_XM.cgi?NM_000152.2]. On the contrary, substitution p.G576S caused by c.1726G>A reduces the activity to such extent that it falls into the patient range [24]. Recently, it was shown that the structural changes brought about by each of the two substitutions are small and do not affect the active site of AαGlu [23].

Here, we present the results of the experiment in which we measured the AαGlu activity in DBSs with a fluorometric procedure while we performed in parallel haplotype analysis on DNA extracted from the same spot. Based on our findings we conclude that the high frequency of the "pseudodeficiency" allele in the Japanese population complicates the finding of an enzymatic screening procedure that is both sensitive and specific.

Subjects, materials and methods

Subjects and DBS collection

Seven hundred and fifteen Japanese newborns (second to fifth day postpartum) and 18 Japanese patients with GSDII were enrolled in this study. The patient group included one patient with classic infantile form, 6 with juvenile form, 10 with adult form and one with unknown phenotype. The DBSs on filter paper were obtained with the standard heel-stick for collecting newborn screening samples, or prepared by drop-wise addition of EDTA-blood samples on the filter paper (filter paper #510AD01, Advantec, Tokyo, Japan) that is routinely used for newborn screening in Japan. DBSs were dried at room temperature for at least 3 h but no more than 16 h, and were subsequently stored at −20 °C in sealed plastic bags until use. Written informed consent was obtained from all subjects, and all samples from these subjects were prepared and analyzed in accordance with the protocols approved by the institutional responsible committee.

Chemicals and reagents

4-Methylumbelliferyl α-D-glucopyranoside (4MU-αGlc) was purchased from Sigma-Aldrich (St. Louis, MO). Acarbose, 4-methylumbelliferone and Proteinase K were from Toronto Research Chemicals (North York, Canada), Nacalai Tesque (Osaka, Japan) and Roche (Basel, Switzerland), respectively. Ampdirect™ Plus with NovaTaq™ Hot Start DNA polymerase was obtained from Simadzu (Kyoto, Japan). Other chemicals were of reagent grade and from Sigma-Aldrich or Nacalai Tesque.

Enzymatic assay

A 3.2-mm diameter disk was punched out from the DBS on the filter paper and incubated in a well of a 96-well clear microwell-plate (Corning, New York, NY) with 100 μL distilled water by mixing gently for 1 h at room temperature. The water extract was used for the enzymatic assay. The disk was recovered for DNA extraction and genotype analysis. The AαGlu activity was measured fluorometrically with 4MU-αGlc as substrate according to our previous report with minor changes [22]. Briefly, 20 μL of the extract was added to 40 μL of the substrate solution containing 2.0 mmol/L 4MU-αGlc in 0.2 mol/L citrate/0.4 mol/L sodium-phosphate buffer, at pH 4.0 with 4.5 μmol/L acarbose (3.0 μmol/L in final concentration), in a 96-well black microwell-plate (PerkinElmer, Boston, MA). The reaction mixture was incubated at 37 °C for 24 h, and the reaction was stopped by addition of 190 μL of 0.2 mol/L glycine-NaOH buffer at pH 10.7 containing 0.1% Triton X100. The fluorescence intensity was measured with the CORONA spectrofluorometer (MTP-600F, Colona Electric, Hitachinaka, Japan) at excitation and emission wave-lengths of 360 nm and 450 nm, respectively, and corrected for substrate blank. We used a stock solution of 100 μmol/L 4-methylumbelliferone in 20 mmol/L sodium-phosphate buffer (pH 7.0) to calibrate the measurement of liberated 4-methylumbelliferone. The enzyme activity was expressed as pico moles 4-methylumbelliferone released per hour per 3.2 mm diameter disk (pmol/h/disk). Each assay was performed in duplicate. The measured values per group are expressed as means ± SD unless otherwise indicated.

Disk clean-up and genotype analysis

After extraction with distilled water for assay of enzyme activity, the disk was recovered, washed with a 100 μL solution of 0.1% Triton X100 in water and incubated in 100 μL digestion buffer containing 0.2 mg/ml Proteinase K, 0.5% sodium dodecyl sulfate, 5 mM EDTA, 400 mM NaCl and 20 mM Tris–HCl (pH 8.0), in a 1.5 ml reaction tube, at 55 °C for 1 h. The reaction was terminated by heating for 10 min on a heat block at 95 °C. The disk was rinsed twice with 500 μL of 10 mM Tris–HCl containing 1 mM EDTA (pH 8.0), once with 400 μL isopropanol, and then dried on a heat block at 70 °C for 60 min. The cleaned-up disk was stored at 4 °C until use for genotype analysis. Genotype analysis was performed by PCR-based detection (amplification refractory mutation system; ARMS). Each cleaned-up disk was cut into 4 pieces with scissors or with a scalpel, and the pieces were placed into 4 PCR tubes each containing 10 μL Ampdirect™ Plus (including PCR buffer and dNTPs), 0.5 units NovaTaq™ Hot Start DNA polymerase, and a set of specific primers (each 0.5 μmol/L), in a total volume of 20 μL reaction mixture. We designed 4 different oligonucleotide primers for 4 PCR sets including either, 5′-TACAACCTGCACAACCTCAACG-3′ (F1) or 5′-TACAACCTGCACAACCTCAACA-3′ (F2) as forward primer and either, 5′-GGCCTGCTGGGCCGACTC-3′ (R1) or 5′-GGCCTGCTGGGCCGACTT-3′ (R2) as reverse primer for the amplification of 4 different *GAA* alleles characterized by different SNPs: the combination F1 + R1 for c.[1726G; 2065G], F1 + R2 for c.[1726G; 2065A], F2 + R1 for c.[1726A; 2065G], and F2 + R2 for c.[1726A; 2065A]. Each oligonucleotide primer was designed to have a one-base mismatch nucleotide at the −4 base position from the 3′ terminal end to improve the selectivity for allele detection. PCR was performed under the following conditions; an initial denaturation at 96 °C for 10 min; 40 cycles amplification with denaturation at 96 °C for 20 s, annealing at 64 °C for 20 s and extension at 72 °C for 90 s; and extra extension at 72 °C for 7 min. The PCR products (1209 bp fragments) were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. This method allows determining 10 different diplotypes, which were constructed from the combination of the 4 haplotypes (Tables 1 and 2). To confirm the reliability of the present method, DNA sequencing analysis was performed according to the procedure described elsewhere [24] for all diplotypes from 18 individuals.

Table 1
Frequency of the *GAA* haplotype in the controls and the patients.

Haplotype[a]	Controls		Patients	
	Number	Frequency	Number	Frequency
GG	1017	0.71	21	0.58
AA	274	0.19	15	0.42
GA	139	0.10	0	0.00
AG	0	0.00	0	0.00
Total	1430	1.00	36	1.00

Statistically significant differences between controls and patients in each haplotype; GG, $p = 0.145$; AA, $p = 0.004$; GA, $p = 0.053$.

[a] Abbreviations for haplotype are depicted as follows; GG, c.[1726G; 2065G]; AA, c.[1726A; 2065A]; GA, c.[1726G; 2065A]; AG, c.[1726A; 2065G].

Statistical analysis

The measured values are expressed as means ± SD unless otherwise indicated. Two-sample independent-groups *t*-test was used for data comparison between the two groups. Data comparison among three or more groups was based on analysis of variance (ANOVA) with respective all pair-wise multiple comparison post-hoc analysis utilizing the Bonferroni's method. Categorical variables were compared with chi-square analysis. Results were considered to be significant at $p < 0.05$.

Results

Validation of the measurement of AαGlu activity in DBSs

The within-imprecision of the present method was estimated by repeated analysis of some DBSs with different activities. The within-run CVs ($n = 12$) were 1.9–5.6%. To estimate between-day imprecision, the DBSs in sealed plastic bags were stored at −20 °C and then assayed with the present method over 12 days. The between-day CVs ($n = 12$) were 3.4–6.8%. The stability of the enzyme activity in DBSs was assessed by determining the average activity of 5 different DBSs stored at room temperature, at 4 °C and at −20 °C for 2, 4, 8 and 16 weeks. The activity change was less than 10% for 16 weeks at either 4 or −20 °C.

AαGlu activity in DBSs from healthy newborns and patients with GSDII

The AαGlu activity in DBSs from 715 healthy Japanese newborns (controls) and 18 patients with GSDII was measured with 4MU-αGlc in the presence of acarbose. As shown in Fig. 1 there was no normal Gaussian distribution of activities in the control group, instead there appeared to be a bimodal distribution: a minor group with less than 9.0 pmol/h/disk of enzyme activity and a major group with activities of more than 9.0 pmol/h/disk. Four individuals in the minor control group fell into the range of activities measured in the patient group (0–2.8 pmol/h/disk). The poor separation between the minor control group and the patient group was not improved by taking the ratio of acarbose-inhibited over uninhibited activity into account (data not shown).

Genotyping with DBSs from healthy newborns and patients with GSDII

We then examined the allele frequencies of 4 *GAA* haplotypes and 10 diplotypes as determined by two SNPs (c.1726G>A and c.2065G>A) using DNA extracted from the same blood spots that were used to measure the AαGlu activity (Tables 1 and 2). Significant correlation was observed between the enzyme activity in the DBS and the diplotype of the DNA from the same spot. Twenty-seven of the 28 individuals homozygous for c.[1726A; 2065A] (3.9% of the total number of controls) belonged to the minor group with low enzyme activity, whereas individuals heterozygous for c.[1726G; 2065G] and c.[1726A; 2065A] formed together a broad range of activities overlapping with those from c.[1726A; 2065A] or c.[1726G; 2065G] homozygotes (Table 2). The mean activity of c.[1726G; 2065A] homozygotes was only slightly lower (82%) than that of c.[1726G; 2065G] homozygotes, but c.[1726A; 2065A] homozygotes had markedly lower activity (12%).

The c.[1726A; 2065A] allele had a significantly higher frequency (42%) in the patient group than in the control group (19%). Twelve of the 18 patients had either one (9 cases) or two (3 cases) c.[1726A; 2065A] allele(s) indicating linkage disequilibrium of the two SNPs between controls and patients. None of the controls nor patients had a c.[1726A; 2065G] allele.

Validation of genotyping with DBSs

Six of the diplotypes that we identified with the present method were confirmed by sequence analysis of genomic DNA from 18 DBS samples (six diplotypes, $n = 3$ each). There was no discrepancy between the results obtained by either method (data not shown).

Table 2
Frequency of the *GAA* diplotype and AαGlu activity in the controls and the patients.

Diplotype[a]	Controls				Patients			
	Number	Frequency	Enzyme activity[b]		Number	Frequency	Enzyme activity[b]	
			Mean ± SD	Range			Mean ± SD	Range
GG/GG	360	0.503	36.4 ± 11.2	12.4–102.1	6	0.333	0.5 ± 0.5	0–1.1
GG/AA	194	0.271	19.5 ± 6.9	7.7–47.4	9	0.500	1.0 ± 0.9	0–2.8
AA/AA	28	0.039	4.4 ± 1.7	1.4–10.1	3	0.167	0.5 ± 0.3	0.2–0.8
GG/GA	103	0.144	32.8 ± 9.6	6.7–58.0	0	0	–	–
GA/GA	6	0.008	29.8 ± 9.3	16.0–41.2	0	0	–	–
GA/AA	24	0.034	17.4 ± 4.1	11.3–28.6	0	0	–	–
AG/AG	0	0	–	–	0	0	–	–
GG/AG	0	0	–	–	0	0	–	–
GA/AG	0	0	–	–	0	0	–	–
AG/AA	0	0	–	–	0	0	–	–
Total	715	1.000	29.4 ± 13.2	1.4–102.1	18	1.000	0.8 ± 0.8	0–2.8

[a] Abbreviations for diplotype are depicted as the combination of the haplotypes described in Table 1.

[b] AαGlu activity in a 3.2-mm diameter disk from DBSs of Japanese newborns (controls) and Japanese patients with GSDII was measured in duplicate with 4-methylumbelliferyl α-D-glucopyranoside as substrate in the present of 3 μmol/L acarbose. The activity was expressed as pmol methylumbelliferone/h/disk. Statistically significant differences between GG/GG and other diplotypes; GG/AA, $p < 0.001$; AA/AA, $p < 0.001$; GG/GA, $p = 0.052$; GA/GA, $p = 0.057$; GA/AA, $p < 0.001$.

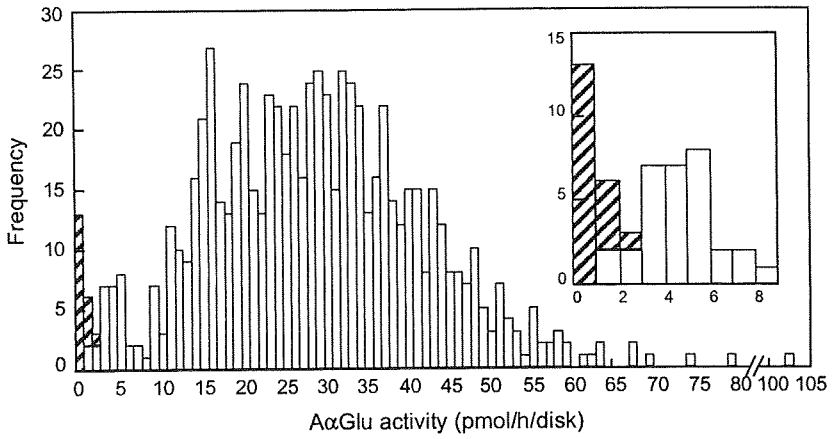

Fig. 1. Distribution of AαGlu activity in DBSs from controls and patients. The AαGlu activity was measured with 4MU-αGlu as substrate in the presence of acarbose using DBSs from 715 Japanese healthy newborns (controls) and 18 Japanese patients with GSDII. Open and hatched bars represent the controls and the patients, respectively. The enzyme activities (mean ± SD, pmol/h/disk) were 29.4 ± 13.2 for the controls and 0.8 ± 0.8 for the patients, and the range of the activities were 1.4–102.1 for the controls and 0–2.8 for the patients. The measurement was performed as described in Subject, materials and methods, and the data were expressed as an average of duplicate determinations. Inset indicates the area of low activities enlarged.

Discussion

In this study, we measured the AαGlu activity in 715 randomly collected DBSs from Japanese newborns, and obtained a bimodal distribution of the activities (a major group with 684 individuals and a minor group with 31 individuals). We could then demonstrate by ARMS that 27 of 31 control individuals (minor group) with an activity of less than 9.0 pmol/h/disk (30.6% of total mean) were c.[1726A; 2065A] homozygotes. Three other individuals with very low activity (7.7, 7.9 and 8.8 pmol/h/disk) were c.[1726G; 2065G]/c.[1726A; 2065A] heterozygotes and one individual (6.7 pmol/h/disk) was c.[1726G;2065G]/c.[1726G;2065A] heterozygote. One c.[1726A; 2065A] homozygote had slightly higher activity (10.1 pmol/h/disk) (Table 2). The 28 homozygotes with c.[1726A; 2065A] representing 3.9% of the study group (Table 2) had on average a markedly lower AαGlu activity (12%) than the homozygotes with c.[1726G;2065G] forming approximately 50% of the study group. Thus, we observed a close correlation between the enzyme activities and the genotypes of the donors. The actual diplotype frequency of c.[1726A; 2065A]/c.[1726A; 2065A] in our sample collection (3.9%) comes close to what we could calculate from the published c.[1726A; 2065A] allele frequency in the Japanese population based on the allele frequencies in the NCBI and in our own sample set [24]. Four of the 28 (14%) homozygotes with c.[1726A;2065A] had an AαGlu activity that fell in the patient range (0–2.8 pmol/h/disk). This poses a serious problem for newborn screening in Japan. If 3.9% of the population is homozygote with c.[1726A; 2065A] and 14% of them has very low activity it means that newborn screening potentially results in 0.56% false positive cases, which is too many to handle. Heterozygotes with genotype c.[1726G; 2065G]/c.[1726A; 2065A] had about half the activity (54%) of c.[1726G; 2065G] homozygotes. None of the DBSs from these heterozygotes or those from individuals with other diplotypes overlapped with the patient range.

The AαGlu activity distribution curves depicted in Fig. 2 are based on the frequencies of the three *GAA* genotypes, c.1726G/G, c.1726G/A and c.1726A/A, in the Japanese population and illustrate the problem encountered in newborn screening. Obviously, the high number of false positives is caused by the high frequency of the c.1726A/A allele in the Japanese population. The same problem will be encountered in other Asian countries [24]. Especially, in Taiwan higher frequency of c.1726A/A (14% of normal individuals) was reported as compared to that in Japan (3.9%) [29]. In practice, the very first large scale newborn screening for GSDII in Taiwan

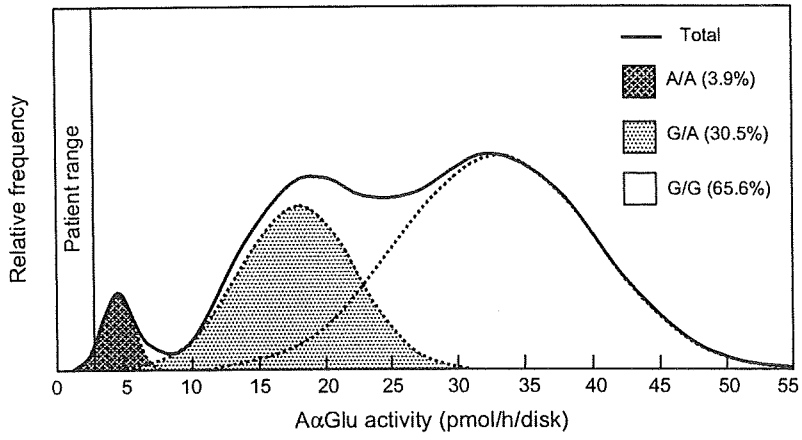

Fig. 2. Estimated distribution of AαGlu activity in DBS in Japan based on the frequency of the three genotypes, c.1726G/G, c.1726G/A and c.1726A/A. Gaussian curves illustrating the distribution of AαGlu activities in DBS were drawn for the Japanese populations based on the frequencies of the three relevant *GAA* genotypes, and their mean and SD of the enzyme activity; c.1726G/G, 33.0 ± 10.0 (n = 469); c.1726G/A, 18.5 ± 5.5 (n = 218); and c.1726A/A, 4.4 ± 1.7 pmol/h/disk (n = 28).