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

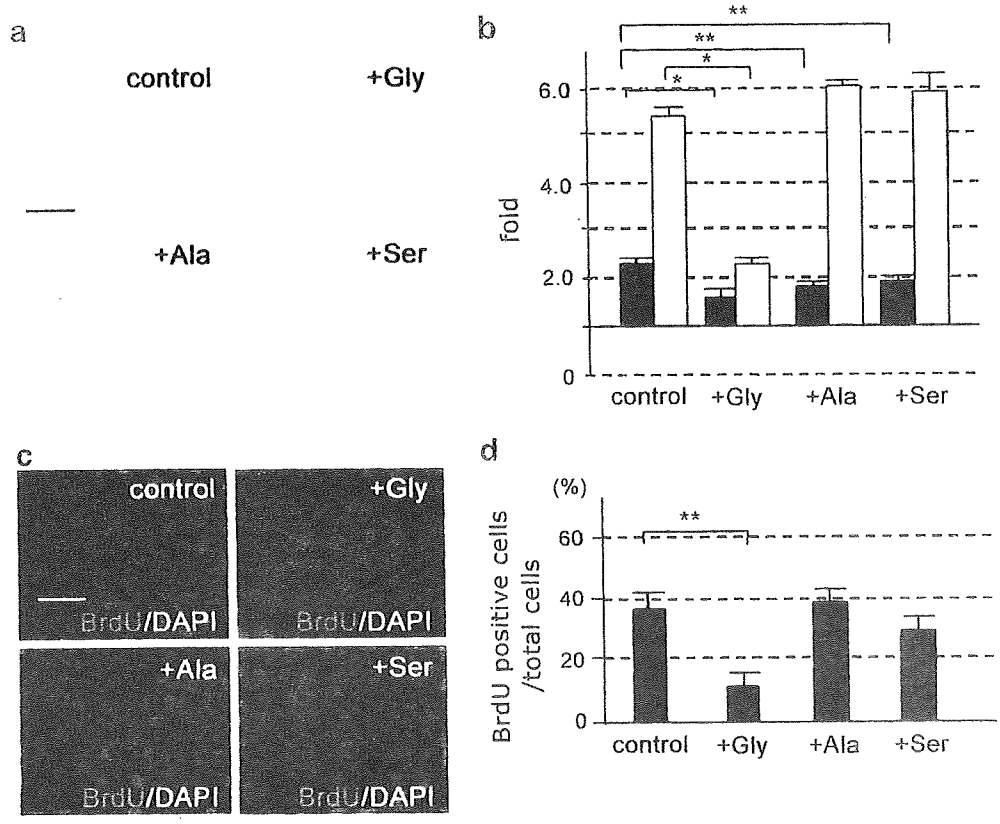


Fig. 1 Glycine inhibits cell proliferation of mSGP cells in short-term culture. mSGP cells were cultured in media supplemented with glycine, L-alanine, or L-serine. The morphology was observed, and the numbers of mSGP cells were counted every 24 h by modified MTT assay. **a** Morphology of mSGP cells 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)

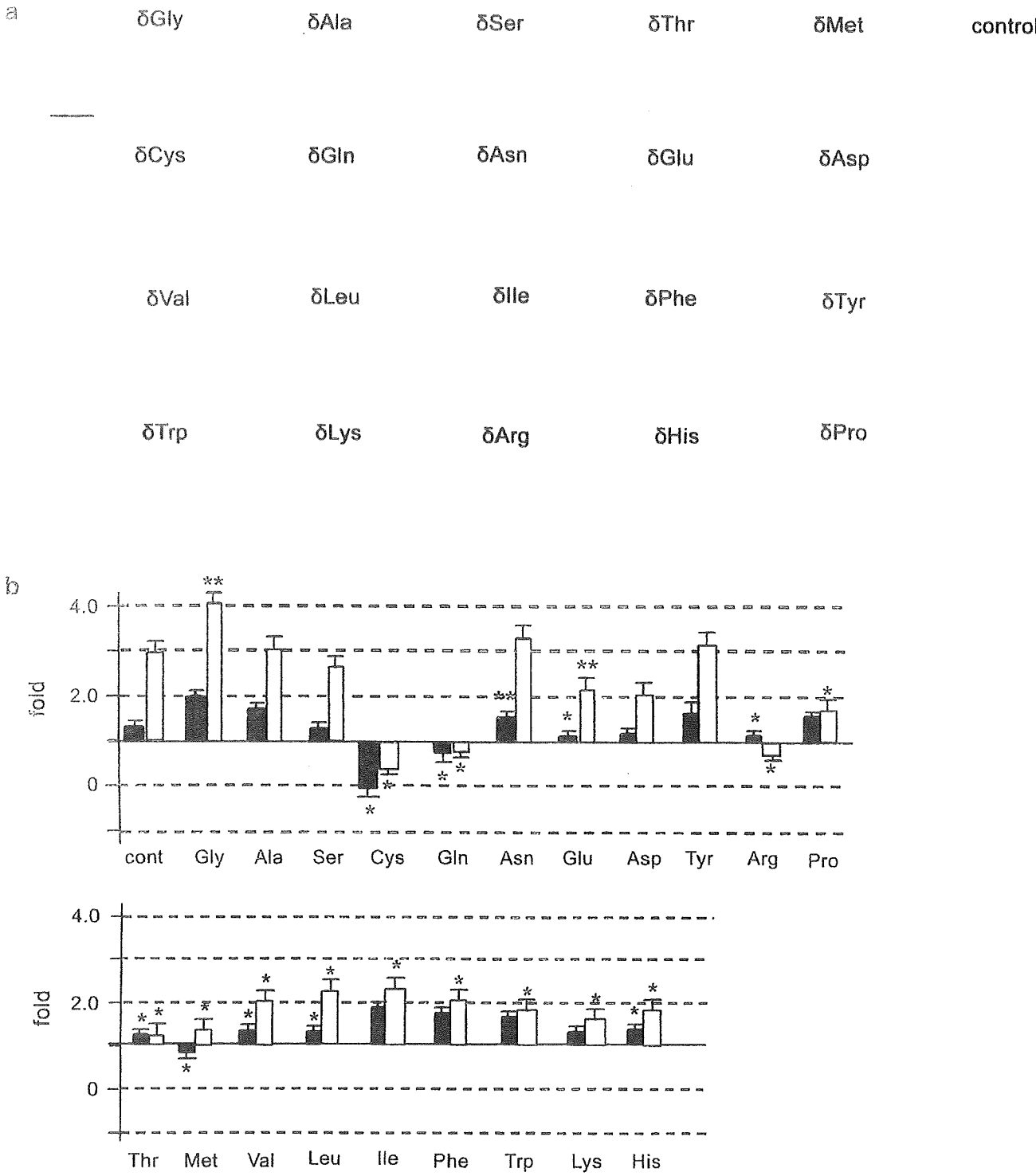


Fig. 2 Glycine depletion promotes cell proliferation of mSGP cells in short-term culture. a Morphology of mSGP cells at 48 h; this morphology was maintained in all cultures. *Bar* 100 μm. b Effects of amino acid depletion on mSGP cells proliferation (*y-axis* number of proliferating cells at each time 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.

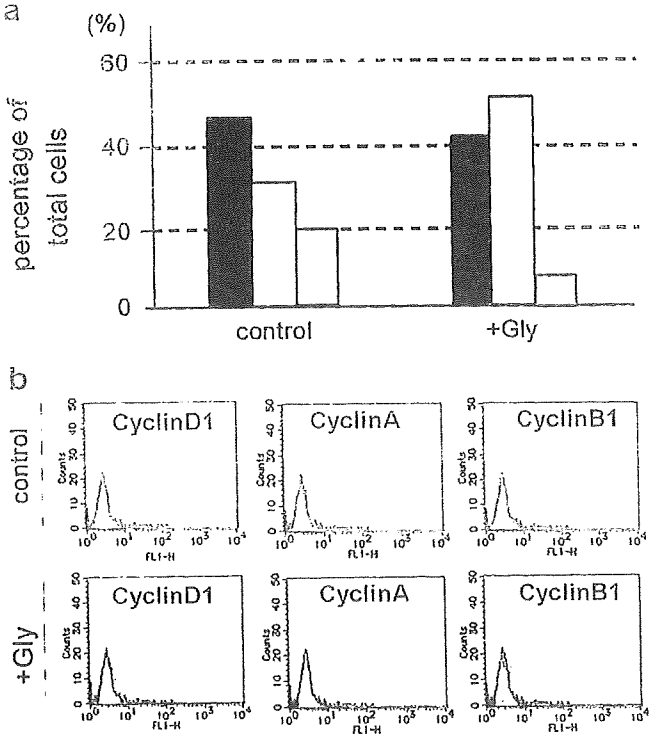
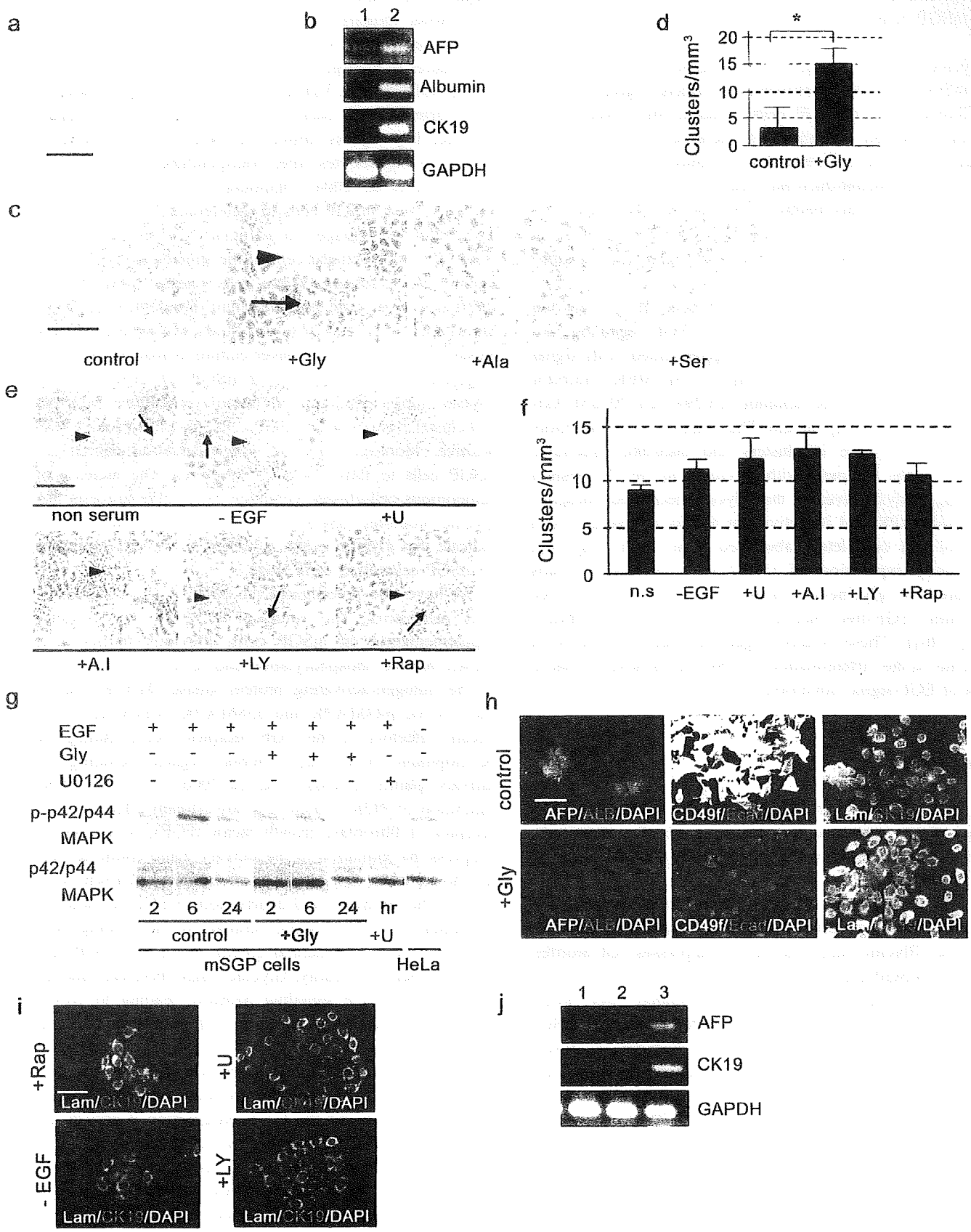


Fig. 3 Glycine affects on cell-cycle progression. Cell-cycle distribution was analyzed by quantification of DNA content and cyclin expression at 24 h after 10 mM glycine treatment. **a** Quantitative analysis of nuclear DNA contents by using Vybrant Dye-Cycle Green Staining. Glycine increased the percentage 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, +*AI* 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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