Carcinogenesis vol.20 no.5 pp.793–798, 1999

Dietary glycine inhibits the growth of B16 melanoma tumors in mice

Michelle L.Rose[1,2], Johnathan Madren[2], Hartwig Bunzendahl[3] and Ronald G.Thurman[1,2,4]

[1]Curriculum in Toxicology, Laboratory of Hepatobiology and Toxicology, [2]Department of Pharmacology and [3]Department of Surgery, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365, USA

[4]To whom correspondence should be addressed
Email: thurman@med.unc.edu

Dietary glycine inhibited hepatocyte proliferation in response to the carcinogen WY-14,643. Since increased cell replication is associated with hepatic cancer caused by WY-14,643, glycine may have anti-cancer properties. Therefore, these experiments were designed to test the hypothesis that dietary glycine would inhibit the growth of tumors arising from B16 melanoma cells implanted subcutaneously in mice. C57BL/6 mice were fed diet supplemented with 5% glycine and 15% casein or control diet (20% casein) for 3 days prior to subcutaneous implantation of B16 tumor cells. Tumor volume was estimated from tumor diameter for 14 days. Tumors were excised, weighed and sectioned for histology post-mortem. B16 cells and endothelial cells were cultured *in vitro* to assess effects of glycine on cell growth. Statistical tests were two-sided and a *P*-value of 0.05 was defined as a significant difference between groups. Weight gain did not differ between mice fed control and glycine-containing diets. B16 tumors grew rapidly in mice fed control diet; however, in mice fed glycine diet, tumor size was 50–75% less. At the time of death, tumors from glycine-fed mice weighed nearly 65% less than tumors from mice fed control diet ($P < 0.05$). Glycine (0.01–10 mM) did not effect growth rates of B16 cells *in vitro*. Moreover, tumor volume and mitotic index of B16 tumors *in vivo* did not differ 2 days after implantation when tumors were small enough to be independent of vascularization. After 14 days, tumors from mice fed dietary glycine had 70% fewer arteries ($P < 0.05$). Furthermore, glycine (0.01–10 mM) inhibited the growth of endothelial cells *in vitro* in a dose-dependent manner ($P < 0.05$; $IC_{50} = 0.05$ mM). These data support the hypothesis that dietary glycine prevents tumor growth *in vivo* by inhibiting angiogenesis through mechanisms involving inhibition of endothelial cell proliferation.

Introduction

Previous studies have shown that dietary supplementation with the simple amino acid glycine prevents increases in hepatocyte replication caused by a potent peroxisome proliferator and tumor promoter (1). Specifically, the hypolipidemic drug WY-14,643 causes hepatic tumors in 100% of rats fed the compound at 0.1% in the diet for 1 year (2). The tumors have been characterized as both adenoma and carcinoma (2), and considerable evidence supports the hypothesis that increased rates of hepatocyte proliferation are central to the mechanism by which WY-14,643 causes liver cancer in rodents (2,3). Rates of hepatocyte proliferation in rats fed WY-14,643 are increased 5- to 8-fold above basal rates, which are generally <1% in liver (2). However, in rats fed dietary glycine for either 24 h or 3 weeks, WY-14,643 did not induce cell proliferation above control levels (1). Therefore, it was hypothesized that dietary glycine may be an effective anti-cancer agent.

WY-14,643-induced liver cancer requires 1 year of dietary feeding; therefore, a more rapid model of tumor growth was chosen to test the hypothesis that dietary glycine would inhibit tumor development. The B16 mouse melanoma model has been used extensively to test possible chemotherapeutic agents, and involves following tumor size for only 2 weeks after implantation of the cells (4). B16 melanoma arose spontaneously in the skin at the base of the ear of a C57BL/6 mouse (5). Originally, the tumor was used in trochar passage to produce an experimental model of melanoma that was 100% fatal in 3–5 weeks (5). Later, it was demonstrated that B16 cells cultivated *in vitro* and then transplanted subcutaneously into C57BL/6 mice produced an experimental model with characteristics similar to those of the tumor of origin (6). Therefore, these studies were designed to test the hypothesis that glycine would inhibit tumor growth *in vivo* in mice with B16 melanoma cells implanted subcutaneously.

Materials and methods

Animals and treatments

C57BL/6 mice were purchased from Jackson Laboratories (Raleigh, NC) at 6 weeks of age (18–20 g). Three days prior to implantation of tumor cells, mice were placed on pelleted semi-synthetic AIN 76 diets (Novartis Nutrition, Minneapolis, MN) containing 5% glycine (glycine diet; Table I) or casein for nitrogen balance (control diet; Table I). Animals were maintained on tap water and diets *ad libitum*, and food consumption and body weights were measured throughout the study. Animals remained on these diets for 14 days after tumor implantation (total of 17 days on diets). The B16F1 murine melanoma cell line was purchased from the University of North Carolina Tissue Culture Facility (original source: American Type Culture Collection no. CRL 6322) and was maintained in RPMI 1640 (Gibco BRL, Grand Island, NY) + 10% fetal bovine serum (FBS; Sigma, St Louis, MO) + 20 mM HEPES (Gibco BRL) + penicillin and streptomycin (100 IU/ml and 100 μg/ml, respectively). Cells in log-phase growth (24–48 h after plating) were harvested by brief trypsinization and 7×10^5 cells were injected subcutaneously in 0.15 ml phosphate-buffered saline. The B16F1 variant was established by Fidler through *in vivo* selection (7). Subcutaneous implantation of both B16 and B16F1 cells yields tumors that are similar in gross and histological features (8).

Palpable tumors were measured in two perpendicular diameters using digital calipers, and the radius was estimated by dividing the mean diameter by two. Tumor volume, which correlates well with tumor weight (9), was calculated assuming spherical growth, by the formula $4/3(\pi r^3)$ (10). Fourteen days after implantation of the cells, mice were killed under pentobarbital anesthesia (120 mg/kg) and tumors were excised and weighed. Some mice were killed 2 or 3 days after implantation of the cells to determine tumor size and mitotic index. Animals were killed at 2 and 3 days after implantation of cells because at this time tumors were <2 mm diameter and therefore were not yet dependent on angiogenesis (11). All animal treatments were in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee. Sections of tumors were formalin fixed or frozen in liquid nitrogen for further study.

Table I. Composition of control and glycine diets

Component	Control (% w/w)	Glycine (% w/w)
Casein	20.0	15.0
Glycine	0.0	5.0
Sucrose	50.0	50.0
Corn oil	5.0	5.0
Cellulose	5.0	5.0
Mineral mix	3.5	3.5
Vitamin mix	1.0	1.0
DL-methionine	0.3	0.3
Choline bitartrate	0.2	0.2
Corn starch	15.0	15.0

Histology and immunohistochemistry

Formalin-fixed tumor sections were stained with hematoxylin and eosin, and the mitotic index was determined. The number of cells with mitotic figures was counted in 10 high-power (400×) fields (1000 cells/slide). Formalin-fixed tumor sections were stained with elastic van Gieson to identify arteries based on the presence of elastin in the intima of the artery (12).

B16F1 and endothelial cell growth in vitro

B16F1 cells were plated at a density of 10 000 cells/ml in glycine-free RPMI (Gibco BRL) + 10% FBS + 20 mM HEPES + penicillin and streptomycin in 24-well plates. After 24 h, fresh media was added to the cultures containing various concentrations of glycine (0–10 mM, Sigma) for 24 h. During the last 4 h of incubation, 1.0 μCi of [^{3}H]thymidine (Amersham, Piscataway, NJ) was added to each well to measure DNA synthesis and estimate cell growth. Cells were harvested in lysis buffer (1% SDS, 200 mM NaOH) and protein precipitated with 3 M sodium acetate. Samples were centrifuged to pellet the protein, and the supernatant containing DNA was applied to glass filters to remove free radioactivity. Filters were washed three times with 3 M sodium acetate, dried and added to 5 ml scintillation fluid. Radioactivity incorporated into DNA was determined using a Beckman LS5000 scintillation counter. Independent experiments were defined as cultures established from different passages of the cell line.

Endothelial cells (CPA cells) were purchased from the University of North Carolina's Tissue Culture Facility (original source: American Type Culture Collection no. CCL 207) and plated at a density of 50 000 cells/3 ml of glycine-free MEM media (Gibco BRL) + 10% FBS + non-essential amino acids (except glycine; Sigma) + penicillin and streptomycin in 60 mm dishes (Costar). Glycine (0–10 mM) was added to the culture medium and the non-essential amino acid mix was used as a nitrogen balance. Twenty-four hours after plating, some cultures were harvested using brief trypsinization, and the number of cells counted using a hemocytometer to determine the plating efficiency. For experimental cultures, endothelial cells were grown for 4 days; fresh media was added every 48 h and cells were harvested and counted as above. Similar to experiments with B16 cells, independent experiments were defined as cultures established from different passages of the cell line.

Statistics

ANOVA with post-hoc tests, Repeated Measures ANOVA with post-hoc tests or Student's *t*-tests were used, as appropriate, for data analysis and are reported in the figure legends for each data set. Two-sided tests were used in all cases and a *P*-value <0.05 was defined as a significant difference between groups.

Results

Dietary glycine inhibits the growth of subcutaneous B16F1 tumors

C57BL/6 mice were fed semi-synthetic control or glycine diets (Table I) for 3 days prior to subcutaneous implantation of B16 melanoma cells, and continued on diets for 14 more days. Food consumption and body weights were measured throughout the study. Food consumption (data not shown) did not differ between the two dietary treatment groups at any time. Furthermore, body weight changes were similar for both control and glycine-fed animals (Figure 1) demonstrating that both groups of mice received adequate nutrition. Six-week-old male mice weighed ~19 g at the beginning of the study and gained 3–4 g over 2 weeks (Figure 1). There were no statistical differences in body weight between the two groups at any time.

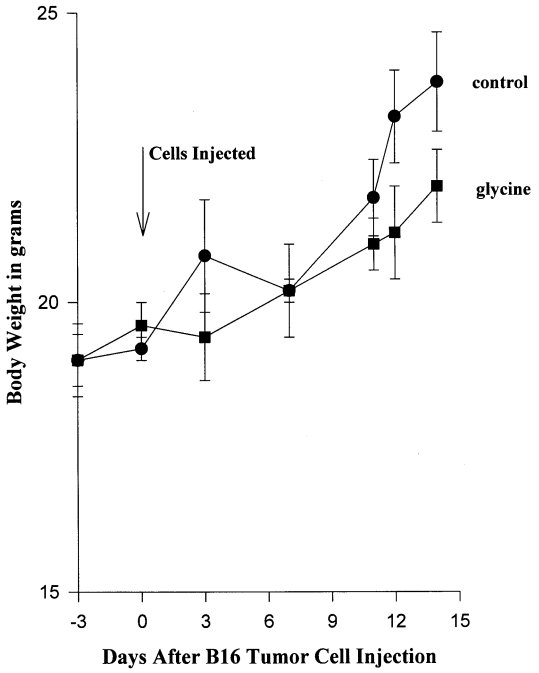

Fig. 1. Effect of diets on body weight gain in mice with implanted tumor cells. Male C57BL/6 mice were fed control or glycine-containing semi-synthetic diets (Table I) for 3 days prior to injection (–3 days on *x*-axis) with B16F1 melanoma cells on day 0 as described in Materials and methods. Animals were continued on diets for 14 days after injection of cells (total of 17 days on diets). Body weights were monitored throughout the study, and data shown are means ± SEM for $n = 5$ in each group. Body weights were not statistically different at any time ($P < 0.05$, Repeated measures ANOVA).

Tumor volume was estimated from the diameter of the tumors on days 2, 3, 7, 11, 12 and 14 after implantation of B16 cells (Figure 2). As early as 7 days after injection of melanoma cells, tumor volume in mice fed dietary glycine was blunted significantly by 50% (Figure 2). Glycine continued to blunt tumor size with the final tumor volume (day 14) in the glycine-fed group reaching only 642 ± 105 mm^3 compared with 2853 ± 497 mm^3 for mice on control diet ($P < 0.05$; Figure 2). This represents a 77% inhibition in tumor size by glycine. The sizes of tumors in mice fed control diet were similar to those reported previously where the time for the tumor volume to increase 10-fold was ~10 days (13). After 14 days, mice were killed and the tumors excised. Representative tumors from control and glycine-fed mice are shown in Figure 3A. The tumor's gross appearance was soft, smooth, glossy and dark black as described previously (8). Tumors from control mice weighed 2.6 ± 0.2 g while tumors from glycine-fed mice weighed significantly less, at 0.9 ± 0.2 g, a reduction of 65% ($P < 0.05$, Figure 3B).

To determine if glycine inhibited growth of B16F1 cells directly, they were cultured in glycine-free media and the effect of added glycine on [^{3}H]thymidine incorporation into newly synthesized DNA was assessed. Interestingly, glycine (0.01–10 mM) had no effect on basal rates of growth of B16 cells *in vitro* (Table II). Furthermore, the mitotic index in tumors from control and glycine-fed animals did not differ after 2 days of tumor growth (Figure 4), a time when tumor volume did not differ between the groups (Figure 2). Since it is possible that decreased tumor growth could be caused by

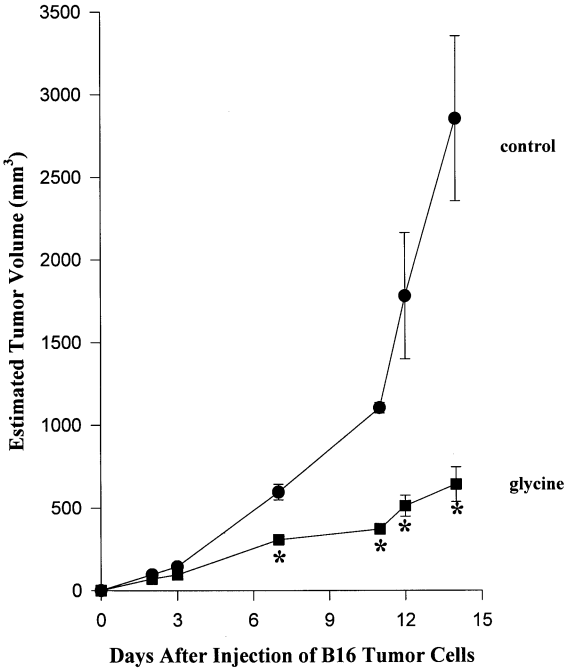

Fig. 2. Effect of dietary glycine on estimated tumor volume. Tumor diameter was measured using digital calipers and volume was estimated as described in Materials and methods. Data shown are means ± SEM for $n = 5$ in each group. Asterisks (*) denote statistical difference from the control ($P < 0.05$, Repeated measures ANOVA on ranks with Student–Newman–Keuls post-hoc tests.)

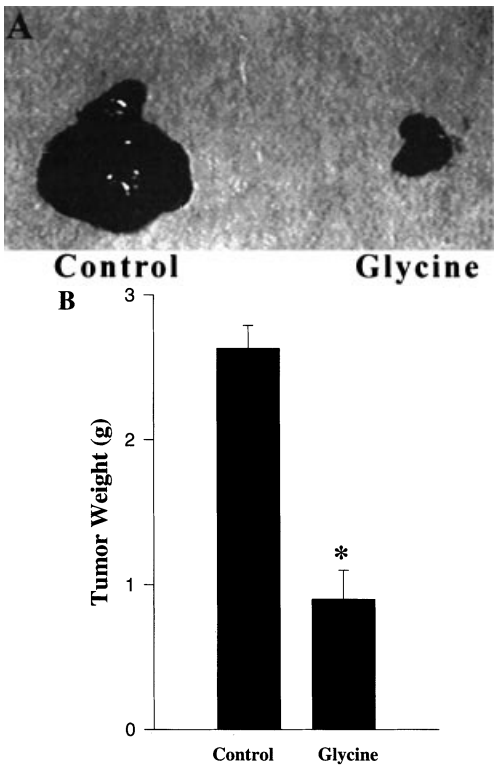

Fig. 3. Effect of dietary glycine on tumor size. (**A**) Mice were killed 14 days after implantation of tumor cells as described in Materials and methods. Photographs of representative tumors from mice fed control diet (Control) and diet containing 5% glycine (Glycine) are shown. (**B**) At time of death, tumors were excised and weighed. Data shown are means ± SEM for $n = 5$ in each group. Asterisk (*) denotes significant difference from control ($P < 0.05$, Student's *t*-test).

Table II. Effect of glycine on B16 cell growth *in vitro*

[Glycine] mM	% B16 cell growth (CPMs)
0.0	100.0 ± 5.8
0.01	113.0 ± 6.1
0.1	92.2 ± 4.5
1.0	100.2 ± 3.9
3.0	115.0 ± 6.2
10.0	123.0 ± 4.0

B16 cells were grown in glycine-free RPMI-1640. Glycine was added at the concentrations indicated and cell growth was assessed as incorporation of [^{3}H]thymidine into newly synthesized DNA as described in Materials and methods. Data shown are means ± SEM for $n = 4$ different passages of cells. None of the treatment groups was statistically different from control ($P < 0.05$, ANOVA).

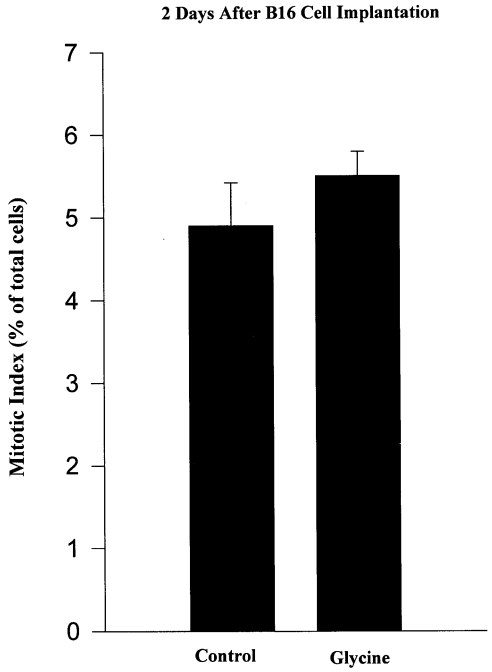

Fig. 4. Effect of dietary glycine on mitotic index of B16 tumor cells. Mitotic index was determined in hematoxylin and eosin stained sections of formalin-fixed tumors 2 days after implantation. Mitotic index is defined as the percentage of cells with mitoses per 1000 cells counted. Data shown are means ± SEM for $n = 5$ in each group. Mitotic index did not differ between control and glycine-fed mice ($P < 0.05$, Student's *t*-test).

an increase in the rate of apoptosis in the tumor, it was evaluated. However, rates were <0.5% and did not differ between the two groups (data not shown).

Glycine inhibits artery formation

Since tumor growth is dependent on formation of new blood vessels for its supply of oxygen and nutrients (14), the effect of glycine on artery development was assessed. Tumor sections were stained with elastic van Gieson to identify arteries from the elastin content of the vessel wall. Four arteries are shown in the tissue surrounding the denser, dark black B16 tumor excised from a mouse fed control diet in the photomicrograph in Figure 5A (marked by arrows) while only one artery is present in the surrounding tissue of the tumor excised from the mouse fed dietary glycine. On average, tumors from mice fed glycine-containing diet had 70% fewer vessels than mice fed glycine-free diet ($P < 0.05$; Figure 5B).

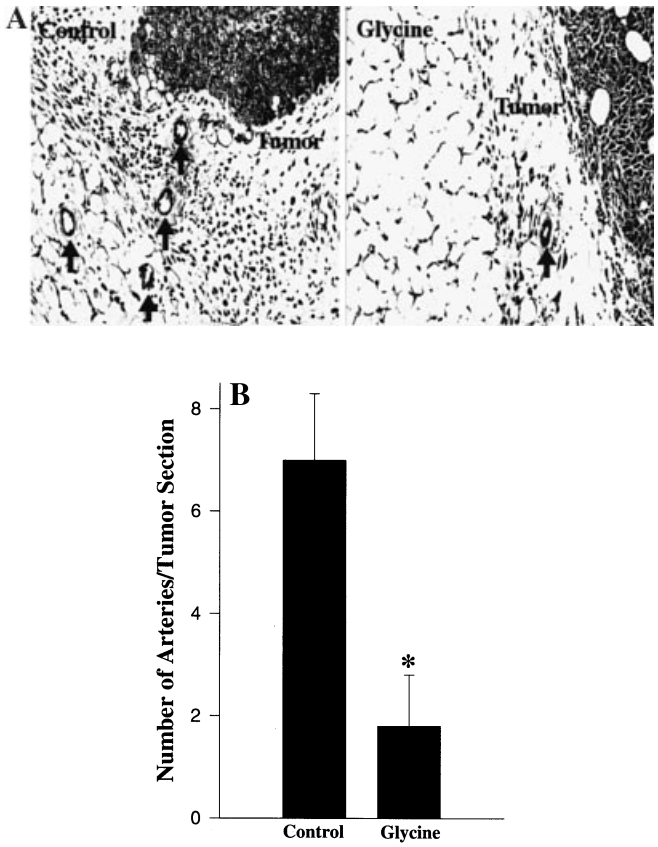

Fig. 5. Effect of dietary glycine on artery number in tumors. (**A**) Tumor tissue was stained with elastic van Gieson and arteries were identified by the black elastin content of the vessel wall (see arrows). The melanoma tumor is dark black (Tumor) while the surrounding tissue containing the arteries is less dense. (**B**) The average number of arteries per tumor was determined in the surrounding tissue of two serial sections per tumor. Data shown are means ± SEM for $n = 5$ mice in each group. Asterisk (*) denotes significant statistical difference from control ($P < 0.05$, Student's *t*-test).

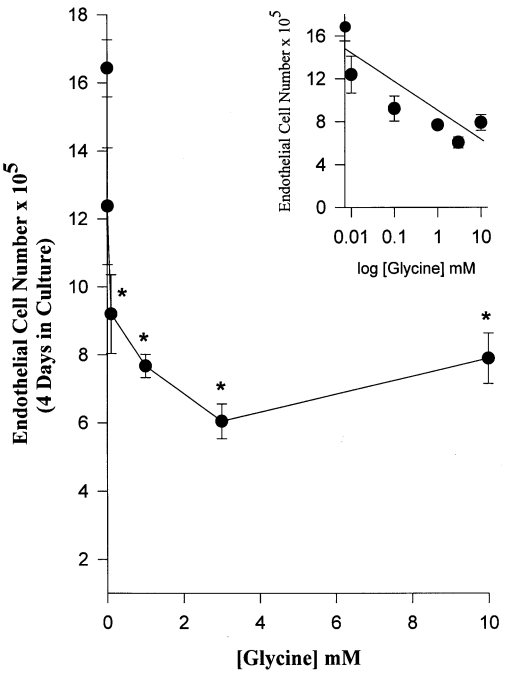

Fig. 6. Effect of glycine on endothelial cell proliferation *in vitro*. Endothelial cells were cultured as described in Materials and methods. Glycine was added at the concentrations indicated and cell growth was assessed by counting the number of cells per plate with a hemocytometer as described in Materials and methods. Data shown are means ± SEM for $n = 4$ different cell passages in each group. Asterisks (*) denote significant difference from control (ANOVA with Bonferroni's post-hoc tests).

In order to determine whether the mechanism by which glycine inhibits artery development involves inhibition of endothelial cell proliferation, cells were treated with glycine *in vitro* and the effect on cell proliferation was determined. About 90% of the cells plated were adherent after 24 h for all concentrations of glycine tested, demonstrating that glycine did not affect endothelial cell plating efficiency (data not shown). Four days after seeding 10^5 endothelial cells, cultures which did not contain glycine had ~16×10^5 cells corresponding to a doubling time of ~24 h (Figure 6). However, the addition of glycine to endothelial cell cultures inhibited cell proliferation in a dose-dependent manner by nearly 65% ($P < 0.05$; Figure 6). The IC_{50} for inhibition of endothelial cell growth by glycine was ~0.05 mM.

Discussion

Working hypothesis: glycine prevents tumor growth via inhibition of angiogenesis

Dietary glycine prevented increases in cell proliferation, a key event in cancer development, caused by the liver carcinogen WY-14,643, suggesting that it may be an effective anti-cancer agent (1). Data presented here demonstrate clearly that dietary glycine inhibits the growth of the experimental tumor B16F1 implanted subcutaneously in mice (Figures 2 and 3). However, the mechanism of growth inhibition was not via a direct action of glycine on B16F1 cell growth since glycine did not inhibit the proliferation of these cells *in vitro* (Table II) or blunt the mitotic index *in vivo* 2 days after injection (Figure 4). Two days after injection all tumors were <2 mm in diameter, which is small enough for tumor cells to rely on diffusion of oxygen and nutrients for cell growth (11). Small tumors, such as these, are not yet dependent on angiogenesis (11); however, after 14 days, the tumors were large enough to require neovascularization to supply oxygen and nutrients (11). It is suggested, therefore, that glycine prevented the growth of large tumors by inhibiting angiogenesis *in vivo*. While it is possible that the smaller tumor size may be responsible for the presence of fewer vessels, this seems unlikely since glycine did not inhibit growth of B16 cells directly either *in vivo* (Figure 4) or *in vitro* (Table II). Therefore, the inhibition of tumor growth was probably not due to a direct effect of glycine to decrease the size of the tumor, and it is hypothesized that it works by inhibiting angiogenesis which indirectly prevents the growth of the tumor.

In support of this hypothesis, B16 melanoma tumor growth *in vivo* has been shown to be dependent on vascularization of the implanted tumor (15). Treatments that inhibit tumor vascularization also prevent tumor growth. For example, urokinase receptor antagonists prevented angiogenesis and inhibited growth of B16 tumor cells *in vivo* (16). In these studies, glycine blocked the formation of arteries in B16 tumors by 70% (Figure 5) and tumor growth was inhibited by 65% *in vivo*. Moreover, glycine directly inhibited the proliferation of endothelial cells *in vitro* (Figure 6) which is a necessary event in the development of new blood vessels (14). Specifically, DNA synthesis in endothelial cells begins as early

as 2 days after addition of the angiogenic stimulus, and mitoses are also present in endothelial cells. Therefore, endothelial cell replication is a critical step early in the angiogenic process. These data are consistent with the hypothesis that dietary glycine inhibits tumor growth by blocking angiogenesis.

At the concentration of glycine approximating normal blood levels (100 μM), glycine inhibited endothelial cell proliferation *in vitro* by ~45% in these studies (Figure 6). When animals are placed on 5% glycine diet, blood glycine levels reach ~1 mM (17), a concentration that resulted in a 63% inhibition of endothelial cell growth *in vitro* (Figure 6). This finding is consistent with *in vivo* studies which demonstrated that dietary glycine did not completely block angiogenesis but resulted in 70% fewer arteries in the tissue surrounding the tumor (Figure 5). Interestingly, the IC_{50} for inhibition of endothelial cell replication *in vitro* was only 50 μM in these studies (Figure 6). Normal blood concentrations of glycine are near 150 μM (17) suggesting that angiogenesis is inhibited under normal physiological conditions. This hypothesis is supported by studies showing that adult endothelium has a low turnover and expresses organ-specific characteristics (18). Because adult endothelium does not proliferate rapidly, it was proposed that the normal adult organism has adjusted its vascular system to its need and may be refractory to new blood vessel formation (19). Consistent with this hypothesis, data presented in Figure 6 suggest that endothelial cell proliferation is inhibited by physiological blood glycine concentrations. However, if local concentrations of glycine decrease due to poor tissue perfusion or increased utilization of nutrients by a rapidly growing tumor, endothelial cells may be more likely to proliferate. These data are consistent with the hypothesis that blood glycine levels play a role in regulating endothelial cell proliferation *in vivo*.

Considerable evidence supports the hypothesis that tumors are dependent on angiogenesis for growth (14). Tumors grown in systems where blood vessels do not proliferate, such as isolated perfused organs, reach a size of only ~2 mm, but expand quickly to nearly 2 cm following transplantation *in vivo* where vascularization occurs (11). In the studies reported here, the initial growth of the tumor *in vivo* 2 days after implantation of cells was not different between control and glycine-fed animals (Figures 2 and 4). At this time, the tumors were <2 mm in diameter, which is small enough to rely on diffusion for the delivery of oxygen and nutrients (14). Angiogenesis associated with B16 tumors has been shown to be directed toward the growing tumor as early as 72 h after implantation of B16 cells (20). Specifically, blood vessels grew in a centripetal pattern around the tumor (20). The tumors are probably supplied by diffusion, since blood vessels have not been identified within the tumor itself in this or other studies (20). Furthermore, studies have shown that the growth rate of tumors is slow and linear before vascularization and becomes rapid and almost exponential following vascularization (21). In support of the hypothesis that dietary glycine inhibits angiogenesis, tumors from control mice entered log-phase growth quickly while those in glycine-fed mice did not (Figure 2). Moreover, angiogenesis inhibitors often are not cytostatic to tumor cells *in vitro*, but they prevent the growth of tumors *in vivo* (22). Consistent with those findings, glycine did not inhibit growth of B16 cells *in vitro* (Table II); however, dietary glycine effectively prevented growth of B16 tumors *in vivo* (Figures 2 and 3). Taken together, these data support the hypothesis that dietary glycine inhibits tumor growth by preventing neovascularization.

How does glycine inhibit angiogenesis?

The mechanism by which glycine inhibits angiogenesis is unclear; however, several possibilities exist. Proliferation of endothelial cells is a key step in the process by which new blood vessels grow from established ones (14), and glycine inhibited endothelial cell proliferation in a dose-dependent manner in these studies (Figure 6). These findings suggest that glycine may inhibit angiogenesis by preventing endothelial cell proliferation.

The mechanisms by which glycine inhibits endothelial cell proliferation, however, are unknown. It is possible that glycine prevents intracellular calcium signaling important for this process. Previous work has shown that glycine inhibits agonist-induced increases in intracellular calcium in other cell types by activating a chloride channel. When glycine binds and activates the channel, the influx of chloride into the cell causes hyperpolarization of the cell membrane and prevents increases in intracellular calcium (23). It is possible, therefore, that endothelial cells express a glycine-gated chloride channel which prevents increases in intracellular calcium when activated. Since regulation of the cell cycle is calcium dependent (24), glycine may inhibit endothelial cell proliferation via inhibition of calcium signaling.

The data presented here demonstrate, for the first time, that dietary glycine inhibits tumor growth *in vivo*. Glycine did not affect the proliferation of B16 cells directly; however, glycine inhibited tumor vascularization and endothelial cell proliferation. Taken together, these data are consistent with the hypothesis that dietary glycine inhibits tumor growth by preventing angiogenesis, most likely via mechanisms involving endothelial cell proliferation. Therefore, this simple, non-toxic amino acid may be a clinically effective anti-cancer agent.

Acknowledgements

This work was supported, in part, by a grant from NIEHS (ES-04325) and a gift from Novartis Nutrition

References

1. Rose,M.L., Germolec,D.R., Arteel,G.E., Schoonhoven,R. and Thurman, R.G. (1997) Dietary glycine completely prevents the increase in hepatocyte proliferation caused by the peroxisome proliferator WY-14,643. *Chem. Res. Toxicol.*, **10**, 1198–1204.
2. Marsman,D.S., Cattley,R.C., Conway,J.G. and Popp,J.A. (1988) Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators di(2-ethylhexyl)phthalate and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (Wy-14,643) in rats. *Cancer Res.*, **48**, 6739–6744.
3. Cattley,R.C., Marsmann,D.S. and Popp,J.A. (1991) Age-related susceptibility to the carcinogenic effect of the peroxisome proliferator WY-14,643 in rat liver. *Carcinogenesis*, **12**, 469–473.
4. Griswold,D.P. (1972) Consideration of the subcutaneously implanted B16 melanoma as a screening model for potential anticancer agents. *Cancer Chemother. Rep.*, **3**, 315–324.
5. Green,E.L. (1962) *Handbook of Genetically Standardized JAX Mice.* Bar Harbor Times Publishing Company, Bar Harbor, Maine.
6. Hagmar,B. and Norby,K. (1973) Influence of cultivation, trypsinization and aggregation on the transplantability of melanoma B16 cells. *Int. J. Cancer*, **11**, 663–675.
7. Fidler,I.J. (1973) Selection of successive tumor lines for metastasis. *Nature New Biol.*, **242**, 148–149.
8. Wosko,T.J., Ferrara,D.T. and Sartori,L.S. (1984) Histological comparison of the B16 melanoma and its F1 variant. *Cancer Lett.*, **24**, 57–63.
9. Gibson,M.H.L. and Bertalanffy,F.D. (1972) The *in vivo* cell kinetics of transplantable B16 mouse melanoma. *Growth*, **36**, 263–274.

10. Simon,S.R. and Ershler,W.B. (1985) Hormonal influences on growth of B16 murine melanoma. *J. Natl Cancer Inst.*, **74**, 1085–1088.
11. Folkman,J., Cole,P. and Zimmerman,S. (1966) Tumor behavior in isolated perfused organs: *In vitro* growth and metastasis of biopsy material in rabbit thyroid and canine intestinal segment. *Ann. Surg.*, **164**, 491–502.
12. Ross,M.H., Romrell,L.J. and Kaye,G.I. (1995) *Histology. A Text and Atlas*. Williams and Wikins, Baltimore.
13. Hill,G.J. and Littlejohn,K. (1971) B16 melanoma in C57BL/6J mice: Kinetics and effects of heterologous serum. *J. Surg. Oncol.*, **3**, 1–7.
14. Folkman,J. (1982) Angiogenesis: Initiation and control. *Ann. NY Acad. Sci.*, 212–227.
15. Gersten,D.M. (1980) Control of growth and vascularity of B16 melanoma by syngeneic lymphocytes. *Cell Biol. Int. Rep.*, **4**, 407–414.
16. Min,H.Y., Doyle,L.V., Vitt,C.R., Zandonella,C.L., Stratton-Thomas,J.R., Shuman,M.A. and Rosenberg,S. (1996) Urokinase receptor antagonists inhibit angiogenesis and primary tumor growth in syngeneic mice. *Cancer Res.*, **56**, 2428–2433.
17. Ikejima,K., Iimuro,Y., Forman,D.T. and Thurman,R.G. (1996) A diet containing glycine improves survival in endotoxin shock in the rat. *Am. J. Physiol.*, **271**, G97–G103.
18. Risau,W. (1995) Differentiation of endothelium. *FASEB J.*, **9**, 926–933.
19. Risau,W. (1997) Mechanisms of angiogenesis. *Nature*, **386**, 671–674.
20. Kreisle,R.A., Stebler,B.A. and Ershler,W.B. (1990) Effect of host age on tumor-associated angiogenesis in mice. *J. Natl Cancer Inst.*, **82**, 44–47.
21. Algire,G.H., Chalkley,H.W. and Legallais,F.Y. (1945) Vascular reactions of mice to wounds and to normal and neoplastic transplants. *J. Natl Cancer Inst.*, **6**, 73–85.
22. Folkman,J. (1985) Angiogenesis and its inhibitors. In DeVita,V.T., Hellman,S. and Rosenberg,S.A. (eds) *Important Advances in Oncology.* Lippincott, New York, NY, pp. 42–62.
23. Ikejima,K., Qu,W., Stachlewitz,R.F. and Thurman,R.G. (1997) Kupffer cells contain a glycine-gated chloride channel. *Am. J. Physiol.*, **272**, G1581–G1586.
24. Whitfield,J.F., Boynton,A.L., MacManus,J.P., Rixon,R.H., Sikorska,M., Tsang,B. and Walker,P.R. (1980) The roles of calcium and cyclic AMP in cell proliferation. *Ann. NY Acad. Sci.*, **1**, 216–240.

Received December 7, 1998; revised February 3, 1999; accepted February 5, 1999