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Original Contribution

PLASMA MEMBRANE γ-GLUTAMYLTRANSFERASE ACTIVITY FACILITATES THE UPTAKE OF VITAMIN C IN MELANOMA CELLS

ALESSANDRO CORTI, CHIARA RAGGI, MARIA FRANZINI, ALDO PAOLICCHI, ALFONSO POMPELLA, and ALESSANDRO F. CASINI

Department of Experimental Pathology B.M.I.E., University of Pisa Medical School, Via Roma 55, 56126 Pisa, Italy

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Abstract—Adequate cellular transport of ascorbic acid (AA) and its oxidation product dehydroascorbate (DHA) is assured through specific carriers. It was shown that vitamin C is taken up as DHA by most cell types, including cancer cells, *via* the facilitative GLUT transporters. Thus, AA oxidation to DHA can be considered a mechanism favoring vitamin C uptake and intracellular accumulation. We have investigated whether such an AA-oxidizing action might be provided by plasma membrane γ-glutamyltransferase (GGT), previously shown to function as an autocrine source of prooxidants. The process was studied using two distinct human metastatic melanoma clones. It was observed that the Me665/2/60 clone, expressing high levels of membrane GGT activity, was capable of effecting the oxidation of extracellular AA, accompanied by a marked increase of intracellular AA levels. The phenomenon was not observed with Me665/2/21 cells, possessing only traces of membrane GGT. On the other hand, AA oxidation and stimulation of cellular uptake were indeed observed after transfection of 2/21 cells with cDNA coding for GGT. The mechanism of GGT-mediated AA oxidation was investigated in acellular systems, including GGT and its substrate glutathione. The process was observed in the presence of redox-active chelated iron(II) and of transferrin or ferritin, i.e., two physiological iron sources. Thus, membrane GGT activity—often expressed at high levels in human malignancies—can oxidize extracellular AA and promote its uptake efficiently.

Keywords—Vitamin C oxidation, Dehydroascorbate, Membrane γ-glutamyltranserase, Vitamin C oxidation, Melanoma cells, Free radicals

INTRODUCTION

Ascorbic acid (AA) plays essential roles in living organisms as effective antioxidants in scavenging reactive oxygen species (ROS) and as an important cofactors in various enzymatic reactions [1]. Such physiological functions of ascorbic acid are associated with its oxidation, leading to the formation of ascorbate free radicals which are then converted to the divalent oxidation product dehydroascorbate (DHA) through disproportionation or further oxidation. Several dehydroascorbate reductase activities have been described, capable of reducing DHA again to ascorbic acid, the physiologically active form of vitamin C [2–6]. Two such activities have been purified and characterized in our laboratories, using either GSH [3,6] or NADPH [4] as donors of reducing equivalents. DHA reduction represents a crucial mechanism to maintain steady state concentrations of ascorbic acid within the cell, despite a constant loss of the reduced molecule following oxidation by free radicals. This role becomes even more important in conditions of oxidative stress, when large amounts of free radicals are produced.

At the plasma membrane level two systems—Na^+-dependent cotransport of ascorbate and facilitated diffusion of dehydroascorbate—have been described [7,8]. In particular, it was shown that vitamin C is taken up by most cell types, including cancer cells, in the oxidized form as DHA, *via* the facilitative hexose transporters (GLUTs) [9]. Once in the intracellular

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Address correspondence to: Alfonso Pompella, Dipartimento di Patologia Sperimentale B.M.I.E., Scuola Medica dell'Università di Pisa, Via Roma 55, 56126 Pisa, Italy. Fax: (39) 050 2218557; E-mail: apompella@biomed.unipi.it.

compartment, DHA can undergo reduction by DHA reductases; facilitated DHA transport therefore represents an important mechanism for cellular vitamin C supply [9,10]. In this perspective, processes leading to the oxidation of ascorbic acid to DHA in the cellular microenvironment can be considered mechanisms favoring vitamin C uptake and intracellular accumulation. A rapid and efficient AA uptake has been described in several cancer cell lines in vitro [10,11], though the phenomenon has not yet been explained. It has been suggested that in tumor stroma some activities capable of favoring the transformation of ascorbate to DHA and its subsequent uptake through GLUT transporters may exist [12].

Against this background and based on previous results obtained in our laboratories, the present study was aimed to determine whether such an AA-oxidizing action might be provided by plasma membrane γ-glutamyltranspeptidase (GGT). We have in fact previously shown that membrane GGT activity is an autocrine source of ROS and other free radicals, capable of promoting prooxidant reactions in the cellular microenvironment [13,14]. GGT, having its active site oriented toward the outer cell surface, is the only enzymatic activity capable of cleaving extracellular glutathione (GSH), thus originating precursor amino acids for the intracellular resynthesis of GSH [15]. In so doing, GGT participates in a sort of "GSH cycling" across the plasma membrane, since a continuous efflux of reduced GSH has been described in most cell types through ubiquitously expressed, specific GSH transporter proteins [16]. GGT activity is often present in high levels in many human spontaneous neoplasms, both primitive and metastatic (carcinomas of ovary, colon, liver; sarcoma; leukemias; melanoma) [17–19]. Previous work in our laboratory has shown that GGT can exert a "prooxidant" action in the extracellular space [13,20–22]. At the extracellular level, GGT-mediated catabolism of GSH in fact leads to the formation of the dipeptide cysteinyl-glycine, whose –SH group is provided with high reactivity in the first place with transition metal ions, e.g., ferric iron ions:

$$\text{GSH---GGT-->glutamic acid} + \text{gly-cys-S}^- + \text{H}^+ \quad (1)$$

$$\text{gly-cys-S}^- + \text{Fe}^{3+}\text{--->gly-cys-S}^{\bullet} + \text{Fe}^{2+} \quad (2)$$

At neutral pH, this "iron-reducing" ability is rather low in GSH while it is high in cysteinyl-glycine and cysteine [23,24]. Since it indeed transforms GSH into these molecules, GGT activity is thus a factor favoring the reduction of transition metal ions present in the extracellular microenvironment. Studies in our laboratories have documented that the "iron-reducing" ability of GGT can in turn give rise to radical processes, leading to the formation of reactive oxygen species (superoxide anion, hydrogen peroxide) and other radicals:

$$\text{Fe}^{2+} + \text{O}_2\text{--->Fe}^{3+} + \text{O}_2^{-\bullet}\,(\text{superoxide anion}) \quad (3)$$

$$\text{O}_2^{-\bullet\cdot} + 2\ \text{H}_2\text{O--->1/2}\ \text{O}_2 + 2\ \text{H}^+ + \text{H}_2\text{O}_2\ (\text{hydrogen peroxide}) \quad (4)$$

Thus, in the presence of GSH fluxes which normally occur across the plasma membrane of cells, extracellular GGT activity is continuously supplied with substrate (GSH) and originates reactive GSH metabolites, reactive oxygen species, and other radicals in the extracellular microenvironment.

The data reported in the present study indicate that membrane GGT activity can oxidize extracellular AA and promote its cellular uptake efficiently. The expression of membrane GGT appears thus to represent an (additional) mechanism for vitamin C replenishment in cancer cells, a phenomenon liable to result in increased resistance to oxidative injury induced by a number of agents, including some important anticancer drugs.

EXPERIMENTAL PROCEDURES

Chemicals

Unless otherwise indicated, all reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture and treatments

Two different cell clones derived from the same subcutaneous metastasis of human melanoma, Me665/2/60 ("*c60 cells*") and Me665/2/21 cells ("*c21 cells*"), expressing high and low to trace levels of GGT activity, respectively, were used. Cells were grown in RPMI 1640 medium supplemented with 5% (v/v) heat-inactivated fetal calf serum and 2 mM L-glutamine, at 37°C in a 5%/95% CO_2/air atmosphere.

For treatments, RPMI medium was replaced with Hanks' buffer, pH 7.4, and the following agents were added (final concns.): ascorbic acid (0.5 mmol/L), GSH (0.5 mmol/L), glycyl-glycine (20 mmol/L), and ADP-chelated $FeCl_3$ (200–20 μmol/L). Incubations were started by adding ADP-Fe(III) to incubation mixtures, in the presence or absence of glycyl-glycine; the latter served as acceptor for transpeptidation and was added to stimulate GGT activity [25]. Inhibition of GGT activity was obtained by treating cells with noncompetitive inhibitor AT125 (1 mmol/L) for 60 min prior to incubation.

In selected experiments, Hanks' buffer was replaced with a 10 mmol/L Hepes/phosphate buffer, pH 7.4, containing 147 mmol/L NaCl, 5 mmol/L KCl, 1.9 mmol/L KH_2PO_4, 1.1 mmol/L Na_2HPO_4, 5.5 mmol/L glucose, 0.3 mmol/L $MgSO_4{:}7H_2O$, 1 mmol/L $MgCl_2{:}6H_2O$, and 0.3 mmol/L $CaCl_2{:}2H_2O$. For sodium-free buffer, NaCl and Na_2HPO_4 were replaced by choline chloride and K_2HPO_4.

In GGT-poor c21 cells, GGT activity was increased by transiently transfecting cells with a vector (pcDNA; Invitrogen) containing GGT cDNA. A lipid transfection according to Lipotaxi mammalian transfection kit protocol (Stratagene) was used.

AA concentration was determined by high-pressure liquid chromatography, according to Rose and Bode [26]. At the established times, incubation mixtures were extracted with 5% (w/v) trichloroacetic acid (Fluka) and then diluted in 50 mM phosphoric acid (J.T. Baker); 20-µl aliquots were injected in a C-18 reverse-phase column (Resolve; Waters, USA). The mobile phase (0.2 M KH_2PO_4/H_3PO_4, pH 3.0) was delivered at a flow rate of 1 mL/min, and ascorbate was detected by an electrochemical detector (ESA Coulochem III) with a Model 5011 analytical cell. Determinations were performed at potentials of –0.35/0.2 V applied on upstream and downstream electrode, respectively.

GSH determinations were performed using the enzymatic method described by Baker et al. [27] adapted to the microtiter plate reader. Other experimental details are given in legends to individual figures.

Preparation of holo-transferrin and ferritin

In experiments including human holo-transferrin or ferritin as sources of redox-active iron, prior to use both proteins were purified through a column of Chelex-100 resin to remove loosely/aspecifically bound Fe^{3+}[28].

Mechanism of ascorbic acid oxidation

Incubations (2 mL) were carried out in 100 mM Tris–HCl buffer, pH 7.4, at 37°C and contained ascorbic acid (5 mmol/L), GSH (0.5 mmol/L), ADP-chelated $FeCl_3$ (final concn.: 200 µmol/L chelator–20 µmol/L $FeCl_3$; stock solution was freshly prepared at least 15 min prior to use), and GGT (100 mU/mL). Glycyl-glycine was also present in the incubations (20 mmol/L), serving as acceptor for the GGT-mediated transpeptidation reaction. Nonenzymatic oxidation of AA was started by adding ADP-Fe(III) to the incubation mixtures. Enzymatic oxidation was started by adding ADP-Fe(III) plus GGT

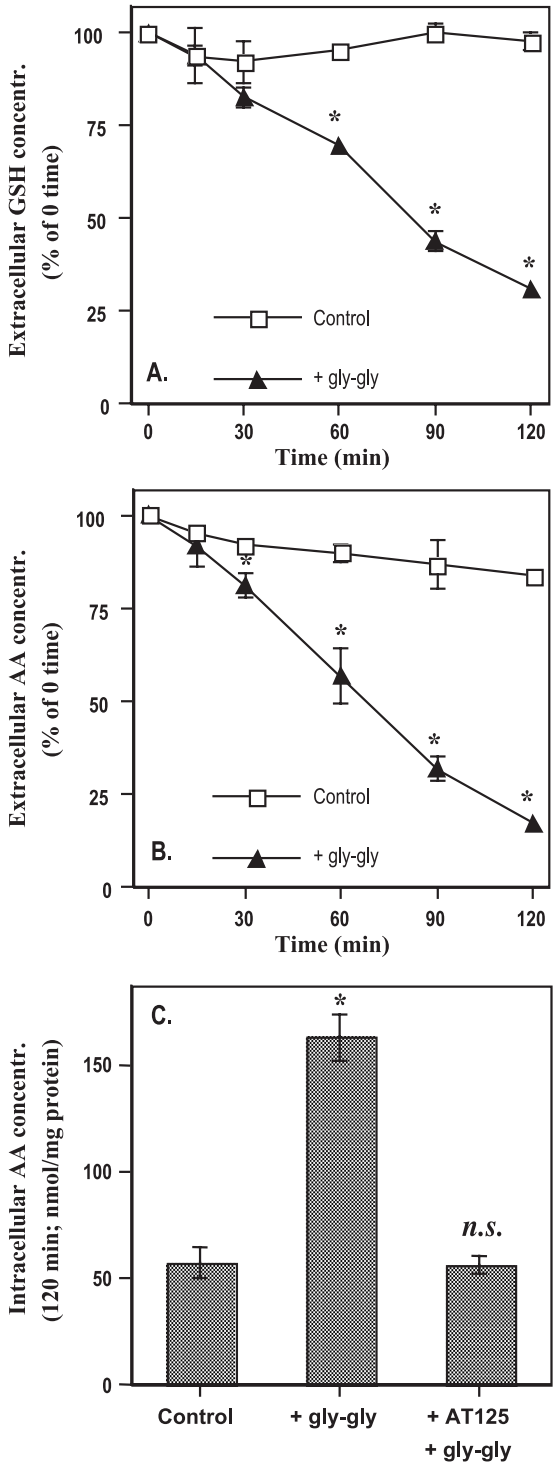

Fig. 1. Role of GGT activity in the uptake of ascorbic acid (AA) by GGT-rich Me665/2/60 human melanoma cells. Cells were incubated up to 120 min at 37°C in Hanks' buffer (pH 7.4) containing AA (0.5 mmol/L), GSH (0.5 mmol/L), and ADP-Fe(III) (200 µmol/L chelator–20 µmol/L $FeCl_3$). Cell-associated GGT activity was ≈26 mU/mg protein. Reactions were started by adding ADP-Fe, in the presence or absence of glycyl-glycine (20 µmol/L). Where indicated, cells were treated prior to incubation with the irreversible GGT inhibitor, AT125 (1 mmol/L), for 60 min. (A) Time course of extracellular GSH concentration; (B) time course of extracellular AA concentration; (C) intracellular AA concentrations after 120 min of incubation. Results are means ± SD of three separate experiments. Data were analyzed by two-way ANOVA. (*n.s.*) Not statistically different and (*) statistically different from control, $p < 0.001$.

at the same time. All components were freshly prepared and kept on ice until used. AA and GSH determinations were performed as described above.

In separate sets of experiments the following antioxidants were used: erythrocyte CuZn/SOD (500 U/mL), thymol-free liver catalase (400 U/mL), mannitol (10 mM). Where indicated, the iron chelator deferoxamine mesylate (DFO; 3 mM) was also employed. Inhibition of GGT activity was obtained with the competitive inhibitor serine/boric acid (20/20 mM) complex (SEB), added to incubations simultaneously with GGT.

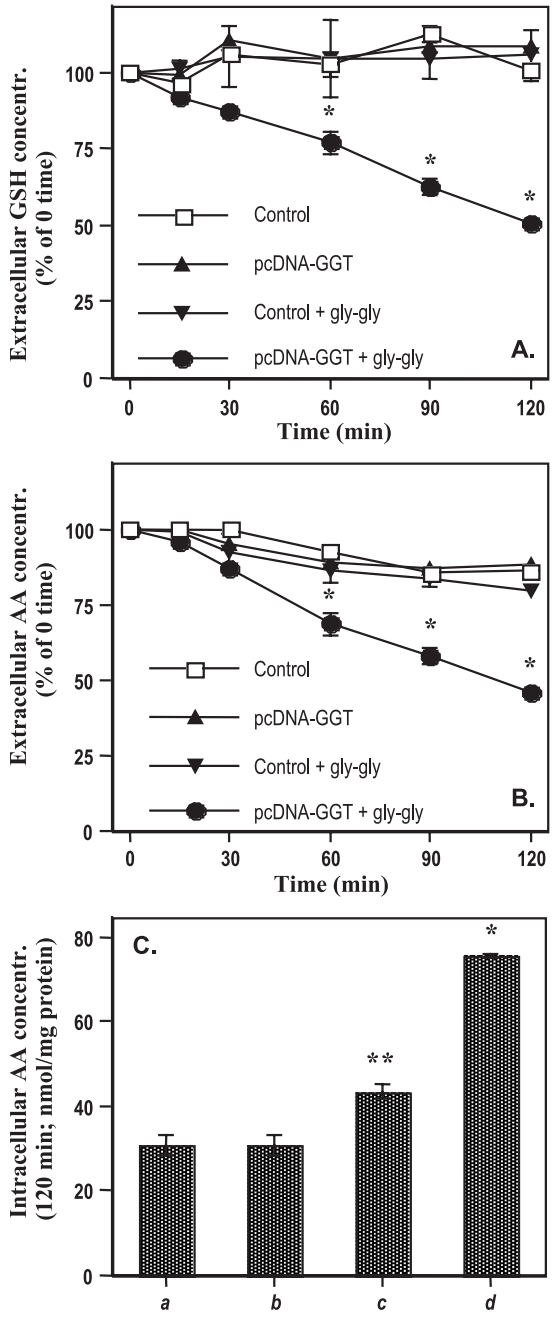

Fig. 2. GSH consumption, extracellular oxidation of ascorbic acid (AA), and AA uptake after transient transfection of GGT in GGT-poor Me665/2/21 human melanoma cells. Cells were transiently transfected with a vector containing GGT cDNA (pcDNA3-GGT), while controls were obtained by transfecting cells with the vector without GGT cDNA (pcDNA3). At 48 h from transfection, GGT activity was ≈0.2 mU/mg protein in control *vs* ≈30 mU/mg protein in pcDNA-GGT transfected cells. Cells were incubated up to 120 min at 37°C in Hanks' buffer (pH 7.4) containing AA (0.5 mmol/L), GSH (0.5 mmol/L), ADP-Fe (III) (200 μmol/L chelator–20 μmol/L $FeCl_3$). Reactions were started by adding ADP-Fe (III), in the presence or absence of glycyl-glycine (20 mmol/L). (A) Time course of extracellular GSH concentration; (B) time course of extracellular AA concentration; (C) intracellular AA concentrations after 120 min of incubation: *a*, controls (cells transfected with empty vector); *b*, cells transfected with pcDNA-GGT vector; *c*, GGT-stimulated controls; *d*, GGT-stimulated transfected cells. Results are means ± SD of three separate experiments. Data were analyzed by two-way ANOVA. (*) $p < 0.001$ compared with pcDNA-GGT; (**) $p < 0.001$ compared with control.

Other determinations

Protein content was determinated by the method of Bradford (Bio-Rad protein assay). Cell viability was routinely controlled by the trypan blue exclusion test. GGT activity was determinated spectrophotometrically using γ-glutamyl-*p*-nitroanilide as substrate, according to Huseby and Strömme [25]. Statistical analysis of data was performed by ANOVA, with Neuman–Keuls test for multiple comparisons.

RESULTS

GGT-dependent facilitation of AA uptake in Me665/2 human melanoma cells

Two distinct clones of human melanoma cells, exhibiting largely different intrinsic GGT activities, were compared; in addition, GGT-coding cDNA was transfected in the low-GGT clone. Fig. 1 reports results obtained with GGT-rich Me665/2/60 cells, presenting with ≈26 mU/mg cellular protein. As can be seen, the stimulation of cellular GGT activity by adding the transpeptidation substrate glycyl-glycine to the incubation medium produced a marked acceleration of GSH consumption (Fig. 1A), along with a marked stimulation of AA oxidation (Fig. 1B). When the intracellular concentrations of AA were assayed, at the latest time of incubation (120 min), it was indeed found that AA uptake was much higher in cells stimulated with glycyl-glycine; the effect was totally prevented in cells whose GGT activity had been irreversibly inhibited with AT125 (Fig. 1C). Accordingly, AT125 pretreatment of cells was observed to suppress both GSH consumption and AA oxidation (not shown).

In experiments with GGT-poor c21 cells, i.e., cells exhibiting ≈0.2 mU GGT/mg protein, the addition of glycyl-glycine essentially produced none or minor effects on GSH consumption, AA oxidation rate, or AA intracellular concentrations (Figs. 2A–2C). The c21 cells were

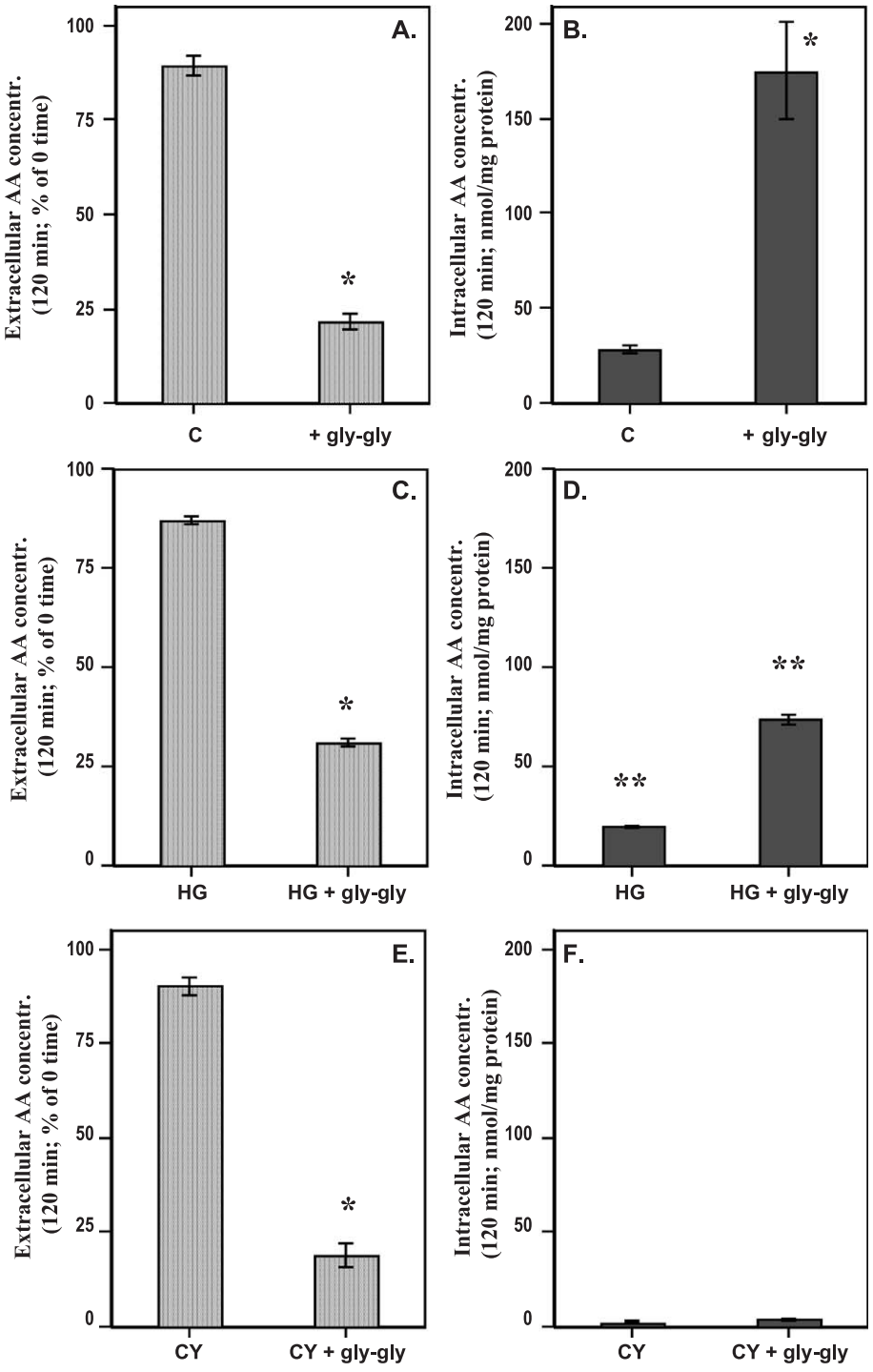

Fig. 3. GSH/GGT-dependent oxidation and cellular uptake of ascorbic acid (AA): effects of glucose and cytochalasin. Melanoma Me665/2/60 cells were incubated for 120 min at 37°C in Hanks' buffer (pH 7.4) containing AA (0.5 mmol/L), GSH (0.5 mmol/L), and ADP-Fe(III) (200 μmol/L chelator–20 μmol/L $FeCl_3$). Glycyl-glycine (20 μmol/L) was added to stimulate GGT activity. (A,B) Control incubations; (C,D) incubations performed in the presence of high glucose concentrations (5 g/L); (E,F) incubations performed in the presence of cytochalasin B (20 μmol/L). Results are means ± SD of three separate determinations. Data were analyzed by ANOVA with Student–Newman–Keuls multiple comparisons test. (*) $p < 0.05$ compared with corresponding control; (**) $p < 0.05$ compared with corresponding low-glucose samples (B).

then transfected with GGT. At 48 h after transfection with pcDNA-GTT vector, GGT activity rose to ≈30 mU/mg protein, as compared with only ≈0.2 mU/mg in control (sham-transfected) cells. The stimulation of GGT-transfected c21 cells with glycyl-glycine indeed produced a facilitation of AA oxidation in the culture medium, while extracellular GSH was progressively consumed (Figs. 2A and 2B). At later times of incubation, the intracellular content of AA after glycyl-glycine stimulation was nearly twice higher in GGT-transfected cells, as compared to the parent cell line transfected with an empty vector (Fig. 2C).

Membrane transporters involved in GGT-facilitated cellular AA uptake

Experiments were carried out to verify the involvement of GLUT transporters in GGT-stimulated uptake of AA in Me665/2/60 cells (Fig. 3). When incubations were performed in the presence of high glucose concentration (5 g/L), while extracellular oxidation of AA was unaffected, a significantly lower AA uptake was observed both before and after GGT activation by glycyl-glycine (Fig. 3D), indicating competition for transport by glucose. In the presence of cytochalasin B, added as an inhibitor of GLUT-mediated transport, complete suppression of AA uptake was observed, despite extracellular AA oxidation not being affected (Figs. 3E and 3F).

Further experiments were aimed to verify the possible involvement of sodium-dependent vitamin C transporters. As reported in Table 1, incubation of cells in sodium-free conditions did not produce significant modifications of the intracellular AA uptake.

Mechanisms of GGT-mediated AA oxidation

Table 2 reports data obtained in acellular systems including purified GGT, glutathione, and glycyl-glycine. Efficient oxidation of AA was observed only when iron ions were also present in the incubation, added in the form of complexes with ADP. The GGT inhibitor SEB produced a marked inhibition of AA oxidation, along with inhibition of GSH consumption (not shown). The iron chelator DFO completely prevented AA oxidation, both in the absence (not shown) and in the presence of GGT activity. GSH consumption was unaffected by DFO, indicating that GGT activity itself was not disturbed by DFO addition (not shown). A marked inhibition of GGT-induced AA oxidation was obtained with SOD, CAT, and a mixture of the two enzymes (Table 2). On the other hand, mannitol, a scavenger of hydroxyl radicals, allowed no protection on AA oxidation (data not shown), indicating that hydroxyl radicals are not involved.

The role of iron in GGT-facilitated cellular uptake of AA was further investigated in experiments reported in Table 3. In the absence of exogenously added iron GGT activation by glycyl-glycine did not significantly stimulate cellular AA uptake. On the other hand, GGT stimulation of cellular AA uptake was well evident after the addition of ADP-chelated iron; the latter also induced an increase of AA uptake in control cells, in the absence of glycyl-glycine.

The possibility that iron ions required by the reaction could be provided by physiological sources of iron was investigated in experiments including human holo-transferrin or ferritin (Fig. 4). Indeed, AA oxidation was also observed when ADP-Fe was substituted with human

Table 1. GSH/GGT-Dependent Oxidation and Cellular Uptake of Ascorbic Acid (AA)–Incubations in Sodium-free Buffer

Samples	Extracellular AA (% of 0-time concentration)	Intracellular AA (nmol/mg protein)
Control	83.0 ± 0.6	28.7 ± 2.3
Control+ gly-gly	37.7 ± 1.4	128.0 ± 3.5
Sodium-free	94.1 ± 2.5	25.6 ± 3.2 *(n.s.)*
Sodium-free+ gly-gly	39.8 ± 0.3	135.1 ± 11.2 *(n.s.)*

Note. Me665/2/60 melanoma cells were incubated for 120 min at 37°C in Hepes/phosphate buffer, pH 7.4. For sodium-free incubations, NaCl and Na_2HPO_4 were replaced with choline chloride and $KHPO_4$. Incubations contained AA (0.5 mmol/L), GSH (0.5 mmol/L), and ADP-Fe(III) (200 μmol/L chelator–20 μmol/L $FeCl_3$). Where indicated, glycyl-glycine (20 μmol/L) was added to stimulate GGT activity. Data are means ± SD of three separate determinations. Data were analyzed by ANOVA with Student–Newman–Keuls multiple comparisons test. (*n.s.*) Not statistically different from corresponding control samples.

Table 2. Effects of GGT Inhibition, Iron Chelation, and Reactive Oxygen Scavenging on GSH/Iron(III)-Dependent Oxidation of Ascorbic Acid (AA) in Acellular Systems

Samples	AA (% of 0-time concentration)
Control	97.0 ± 0.8
+GGT	95.8 ± 5.9 (*n.s.*)
+ADPFe	57.2 ± 1.2 (†)
+GGT+ ADPFe	14.9 ± 1.5 (‡)
+GGT+ ADPFe+ SEB	61.1 ± 4.0 (*)
+GGT+ ADPFe+ DFO	98.9 ± 3.0 (*)
+GGT+ ADPFe+ SOD	34.8 ± 0.1 (*)
+GGT+ ADPFe+ CAT	27.6 ± 0.1 (*)
+GGT+ ADPFe+ SOD+ CAT	42.2 ± 3.2 (*)

Note. Incubations (2 mL final volume) were carried out for 60 min at 37°C in Tris–HCl pH (7.4) and contained AA (0.5 mmol/L), GSH (0.5 mmol/L), glycyl-glycine (20 mmol/L). Reactions were started by adding ADP-Fe (final concn.: 100–10 μmol/L, of chelator–$FeCl_3$, respectively), in presence or absence of purified GGT (100 mU/mL). Where indicated, serine/boric acid (SEB; 20/20 mmol/L), deferoxamine mesylate (DFO; 3 mmol/L), superoxide dismutase (SOD; 500 U/mL), or catalase (CAT; 400 U/mL) were also added to incubations. Data are means ± SD of three separate determinations. Data were analyzed by ANOVA with Student–Newman–Keuls multiple comparisons test. *(n.s.)* Not statistically different from control; (†) $p < 0.001$ compared with control; (‡) $p < 0.001$ compared with ADPFe; (*) $p < 0.001$ compared with +GGT+ADPFe.

Table 3. The Role of Iron in GSH/GGT-Dependent Cellular Uptake of Ascorbic Acid (AA) in GGT-rich Me665/2/60 Melanoma Cells

Samples	Extracellular AA (% of 0-time concentration)	Intracellular AA (nmol/mg protein)
Control	84.4 ± 3.5	6.4 ± 0.3
+gly-gly	84.8 ± 1.6	7.7 ± 1.5 *(n.s.)*
+ADP-Fe	84.5 ± 3.2	21.4 ± 3.0 (*)
+ADP-Fe+ gly-gly	35.4 ± 0.2	150.2 ± 1.1 (**)
+DFO	87.8 ± 1.1	6.0 ± 0.2 *(n.s.)*
+DFO+ gly-gly	88.6 ± 0.8	6.5 ± 0.2 *(n.s.)*

Notes. Cells were incubated for 120 min at 37°C in Hanks' buffer (pH 7.4) containing AA (0.5 mmol/L) and GSH (0.5 mmol/L). Where indicated, ADP-Fe(III) (200 μmol/L chelator–20 μmol/L $FeCl_3$), glycyl-glycine (20 μmol/L), or deferoxamine mesylate (DFO; 3 mmol/L) were also added. Data are means ± SD of three separate determinations. Data were analyzed by ANOVA with Student–Newman–Keuls multiple comparisons test. (n.s.) Not statistically different from control; (*) $p < 0.05$ compared with control; (**) $p < 0.05$ compared with +gly-gly sample.

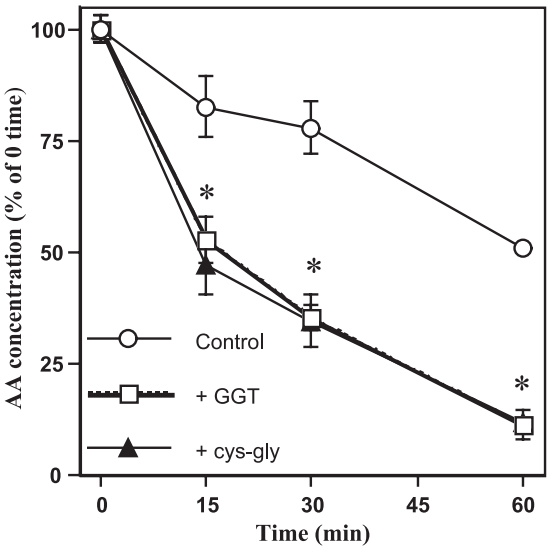

Fig. 5. Oxidation of ascorbic acid (AA) in the presence of the GGT-derived GSH catabolite, cysteinyl-glycine. Experiments were performed at 37°C in Tris–HCl (pH 7.4) and mixtures (2 mL final volume) contained AA (0.5 mmol/L), GSH (0.5 mmol/L), glycyl-glycine (20 mmol/L), and ADP-Fe (III) (200 μmol/L chelator–20 μmol/L $FeCl_3$). Reactions were started by adding ADP-Fe (III). Where indicated, GGT (100 mU/mL) and cysteinyl-glycine (0.5 mmol/L) were added. Results are means ± SD of three separate experiments. Data were analyzed by two-way ANOVA. (*) $p < 0.001$ compared with control.

holo-transferrin as the sole source of redox-active iron, and the addition of GGT to incubations caused a marked enhancement of this effect (Fig. 4A). When human ferritin was used as iron source, this was enough to promote AA oxidation, confirming data previously reported by others [29]; as already observed with holo-transferrin, the presence of GGT resulted in a marked stimulation of AA oxidation (Fig. 4B).

The observations above suggested that the facilitation effect on AA oxidation is produced by (thiol) metabolites originating from GSH cleavage effected by GGT. This was verified in experiments in which soluble GGT was replaced with cysteinyl-glycine, i.e., the dipeptide thiol originated by GGT after the removal of glutamic acid from GSH. As reported in Fig. 5, the addition to incubation mixtures of cysteinyl-glycine was by itself capable of mimicking the facilitation effect observed with GGT activity, suggesting that cysteinyl-glycine may

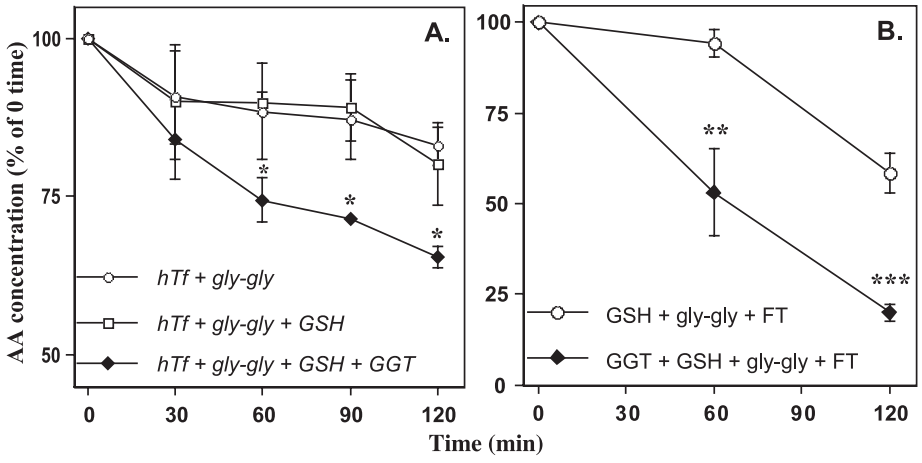

Fig. 4. Role of holo-transferrin (hTf) and ferritin (FT) as sources of iron for the facilitation effect of GGT activity on ascorbic acid (AA) oxidation. Incubations (0.5–2 mL final volumes) were carried out at 37°C in Tris–HCl (pH 7.4) and contained AA (0.5 mmol/L). Prior to use, hTf and FT were prepared as reported under Experimental procedures. Incubation mixtures contained GSH (0.5 mmol/L), glycyl-glycine (20 mmol/L), and GGT (100 mU/mL), as indicated. Reactions were started by addition of (A) hTf, 5 μM or (B) FT, 1 μM. Results are means ± SD of three separate experiments. Data were analyzed by two-way ANOVA. (*) $p < 0.05$ compared with hTf+gly-gly+GSH; (**) $p < 0.05$ compared with GSH+gly-gly+FT; (***) $p < 0.001$ compared with GSH+gly-gly+FT.

be the molecular species responsible for the effect observed in incubations containing ADP-Fe, GSH, and GGT activity.

DISCUSSION

Several reports suggest that ascorbic acid can contribute to the malignant phenotype of tumor cells, by increasing their resistance to prooxidant agents and chemotherapeutic drugs. Several tumors can readily take up vitamin C *in vitro* [10–12], and a higher vitamin C concentration has been demonstrated in breast cancer cells than in the adjacent normal tissue [30]. In cultured Nb2 rat lymphoma cells, tumor progression seems to be associated with an enhanced ability to maintain ascorbate in its reduced form [31]. In LY-as cells, deriving from a mouse B cell lymphoma, the development of chemoresistance is associated with the overexpression of a protein showing an elevated homology (81%) with rat DHAR [32]. AA levels were found to be significantly higher in adriamycin-resistant MCF-7 breast tumor cells than in their adriamycin-sensitive counterparts [33].

The acquirement by tumor cells of additional mechanisms capable of facilitating their AA uptake could provide them with a further selective advantage and features of increased malignancy. The results presented in this study indicate that one such additional mechanism can coincide with the prooxidant reactions originated from the activation of GGT activity at the surface of cells. Such reactions in fact produce a facilitating effect on the iron-dependent oxidation of ascorbic acid present in the extracellular environment. The effect was dependent on the amount of GGT enzyme present in the incubation system and was suppressed by agents capable of inhibiting enzyme activity. The facts that addition of glucose to incubations caused a decrease in AA uptake and that cytochalasin B completely suppressed it indicate that indeed GLUT transporters are involved in the process. On the other hand, no role for sodium-dependent transport in incubations carried out in sodium-free media was observed.

The results of experiments with mannitol and the antioxidant enzymes SOD and CAT point to an involvement of ROS—superoxide anion, hydrogen peroxide, but not hydroxyl radical—in the process and suggest that GGT may act by enhancing thiol-dependent ADP-iron reduction and redox interactions with molecular oxygen (redox cycling). Oxidation of AA in the medium was promoted also by cell-associated GGT activity and in this case the phenomenon was accompanied by a facilitation of cellular uptake of ascorbic acid, allegedly in the form of DHA produced during GGT-mediated extracellular oxidations. In this respect, the characteristics of GGT-dependent AA oxidation and subsequent cellular uptake make it fully comparable with analogous phenomena described with activated phagocytes. It has in fact been shown that the reactive oxygen species produced during the respiratory burst of neutrophils can efficiently promote vitamin C uptake and recycling in the activated cells [34,35] and, importantly, in neighboring cells *via* a bystander effect [35].

Chelated iron was also required for the GGT effect, and this raises the question whether redox-active iron may be available to cells for the described reactions to take place. In this respect, the results of experiments carried out with transferrin or ferritin as sources of catalytic iron document that both storage forms of iron can efficiently sustain GGT-dependent oxidation of ascorbic acid. Concentrations of transferrin used were below the range of those occurring in serum. The higher concentrations used in the case of ferritin may be easily reached at sites of cellular destruction, a condition usually associated with invasive tumors. The catalytic action of transferrin and ferritin in prooxidant reations is a known phenomenon [36,37], and ferritin in particular has been reported to stimulate ascorbate oxidation [29]. Indeed, previous work from our laboratory verified that GGT-dependent reactions are able to produce the delocalization of redox-active iron(II) ions from the transferrin-bound pool [14]. It can be hypothesized that a catalytic role of the same kind may be played by other divalent cations, e.g., copper contained in ceruloplasmin. Indeed, involvement of copper ions in GGT-dependent generation of prooxidants has been described [38].

It has been shown that tumor cells can obtain vitamin C from the medium by inducing the oxidation of AA to DHA, i.e., the molecule capable of crossing plasma membrane through the GLUT transporters, and such oxidation has been ascribed to NADPH oxidase activities present in nonmalignant stromal cells [12]. In addition to phagocytic cells, fibroblasts and endothelial cells in fact are known to express constitutive NADPH oxidase activity [39,40]. On the other hand, an endogenous production of reactive oxygen species has been observed in several cancer cell types [41,42], including melanoma [43], in which it was attributed to prooxidant reactions taking place during melanogenesis [44]. To these recognized sources of prooxidant equivalents, our present data allow the addition of membrane GGT activity. GGT-mediated prooxidant reactions appear to represent an additional mechanism by which tumor cells expressing significant levels of enzyme can access an improved supply of ascorbate from the medium. This so far unrecognized ability could contribute to malignant phenotype, by increasing resistance of cancer cells to prooxidant agents and chemotherapeutic drugs.

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ABBREVIATIONS

AA — ascorbic acid
CAT — catalase
DHA — dehydroascorbic acid
DHAR — dehydroascorbate reductase
FT — ferritin
hTf — holo-transferrin
GGT — γ-glutamyltransferase
gly-gly — glycyl-glycine
GSH — reduced glutathione
ROS — reactive oxygen species
SEB — serine/boric acid complex
SOD — superoxide dismutase