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Membrane gamma-glutamyl transferase activity promotes iron-dependent oxidative DNA damage in melanoma cells

Alessandro Corti[a,*], Tiago L. Duarte[b,c], Chiara Giommarelli[d], Vincenzo De Tata[a], Aldo Paolicchi[a], George D.D. Jones[c], Alfonso Pompella[a]

[a] Dipartimento di Patologia Sperimentale, Biotecnologie Mediche, Infettivologia ed Epidemiologia, University of Pisa, Italy
[b] Radiation and Oxidative Stress Group, Department of Cancer Studies and Molecular Medicine, University of Leicester, UK
[c] Instituto de Biologia Molecular e Celular, Universidade do Porto, 4150-180 Porto, Portugal
[d] Istituto Nazionale Tumori, via Venezian 1, 20133 Milan, Italy

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ABSTRACT

A number of recent observations have suggested a potential role for membrane-bound gamma-glutamyltransferase (GGT) in tumor progression and appearance of more aggressive and resistant phenotypes, through redox interactions leading to production of reactive oxygen species. The present study was aimed to evaluate whether such pro-oxidant activity of GGT can promote oxidative DNA damage, thus contributing to cancer genomic instability. Human GGT-transfected melanoma cells were studied, and DNA damage was measured by using the alkaline comet assay. Our results indicate that higher levels of GGT activity are associated with higher levels of background DNA damage and oxidized bases. This association cannot be explained by differences in cell cycle distribution or apoptotic rates. GGT-over-expressing cells also presented with a markedly higher glucose uptake, a phenomenon potentially leading to higher metabolic rate and oxidative DNA damage. Anyway, when GGT-over-expressing cells were incubated in the presence of GGT substrates and a source of catalytic iron, increased levels of DNA damage and oxidized bases were observed, an effect completely prevented in the presence of GGT inhibitors or various antioxidants.The findings reported indicate that GGT activity is able to promote iron-dependent DNA oxidative damage, thus potentially representing an important mechanism in initiation/progression of neoplastic transformation.

1. Introduction

Gamma-glutamyltransferase (GGT; EC 2.3.2.2) is a plasma membrane-bound enzyme expressed in a wide number of cell types. Under physiological conditions, high enzyme levels are expressed in only a few tissues of the body, e.g. kidney tubules, biliary epithelium and brain capillaries. GGT catalyzes the first step in the degradation of extracellular glutathione (GSH), i.e. the hydrolysis of the gamma-glutamyl bond between glutamate and cysteine, thus facilitating the recovery of GSH aminoacids, in particular cysteine (reviewed in 1). Interestingly, dysregulated expression of GGT has been described in a number of human tumors and tumor-derived cell lines [2–4], and several recent observations suggested a potential role for GGT in tumor progression towards more aggressive and resistant phenotypes. The increased cell resistance against pro-oxidant drugs and other potentially lethal oxidant challenges was generally interpreted as a result of GGT-dependent facilitation of cysteine uptake, a factor favouring the increase of intracellular antioxidant GSH [1]. However, recent studies have documented that GGT-mediated metabolism of extracellular GSH in the presence of extracellular transition metal ions can also exert *pro-oxidant effects* at the membrane surface level, with generation of reactive oxygen species (ROS) [5,6]. This phenomenon is explained with the high reactivity of GGT-product cysteinyl-glycine, which is in fact able to reduce extracellular transition metal cations (Fe^{3+} and others) more efficiently than does GSH itself. Such metal ion 'redox cycling' was shown to produce ROS and other free radicals, i.e. reactive species capable of promoting several intra- and extra-cellular biomolecular effects [7]. On this basis, GGT was suggested to be an additional source of (low levels of) endogenous ROS that could contribute to the "persistent oxidative stress" repeatedly described in genomic instability and carcinogenesis [8,9]. It

Abbreviations: ABBA, L-2-amino-4-boronobutanoic acid; BHT, butylated hydroxytoluene; CAT, catalase; DFO, deferoxamine mesylate; Fpg, formamidopyrimidine-DNA-glycosylase; GGT, gamma-glutamyltransferase; gly-gly, glycyl-glycine; GSH, reduced glutathione; ROS, reactive oxygen species; SBC, serine/boric acid complex; SOD, superoxide dismutase.

* Corresponding author at: Department of Experimental Pathology BMIE, University of Pisa Medical School, Via Roma 55, 56126 Pisa, Italy. Tel.: +39 050 2218 538; fax: +39 050 2218 557.
E-mail address: a.corti@biomed.unipi.it (A. Corti).

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is proposed that such low oxidative stress could be involved in the activation of several intracellular signal transduction pathways. GGT was in fact shown to modulate the redox state of cell surface receptors (e.g. TNFR-1) as well as intracellular transcription factors (e.g. NF-kB, AP-1), thus being potentially involved in modulating cell functions, such as proliferation, apoptosis, adhesion and gene expression, which are of primary importance in cancer and other disease conditions [7–10]. It is conceivable that the described pro-oxidant effects of GGT are normally balanced by its established antioxidant role in favouring the cellular GSH supply. On the other hand, in cells overexpressing the enzyme, conditions could be in favour of a higher extracellular production of cysteinyl-glycine, thus favouring the occurrence of the oxidative reactions described above.

The persistent oxidative stress observed in cancer has been proposed to contribute to genomic instability. The interaction of endogenous ROS with the cellular genome often results in appearance of DNA base and sugar modifications, DNA-protein cross-links, abasic sites and single- and double-strand breaks [11,12]. It is well established that normal oxidative cellular metabolism is an endogenous source of ROS, and that such 'physiological' cellular levels of oxidants are responsible for the background levels of oxidative DNA damage normally detected in tissues. The genome of cancer cells is indeed more prone to oxidative damage: the high metabolic rate associated with increased cellular proliferation produces large amounts of H_2O_2 and significant alterations of antioxidant levels within the cell [13,14]. Increased oxidative DNA damage in cancer can thus be regarded as a factor favouring accumulation of mutations and chromosomal aberrations, that contribute to transformation and cancer progression [8].

Accumulation of DNA damage can also result from alterations of cellular antioxidant defences or DNA repair mechanisms. Reduced activities of antioxidant enzymes with concomitant increased levels of oxidative DNA damage were described in acute lymphoblastic leukemia [15], and a direct association between endonuclease hOGG1 deficiency and 8-OH-dG accumulation in HCC1937 breast cancer cells has been shown [16]. Several studies reported increased DNA damage in different human malignancies as compared to the surrounding non-malignant tissues [17–20], and increased GC > TA transversions potentially deriving from formation of 8-OH-dG have been observed *in vivo* in the *ras* oncogene and the *p53* tumor suppressor gene in lung and liver cancer. Nevertheless, wide variations are present in published estimates of oxidative DNA damage in cancer as well as non-cancer cells. This is likely due to the artifactual oxidation of guanine in methods requiring DNA extraction and derivatisation, i.e. procedures liable to produce overestimated baseline levels. The European standards committee on oxidative DNA damage (ESCODD) has finally established standard protocols and quality control steps [21]. The repeated observation of apparently higher levels of DNA damage in cancer as compared to non-cancer cells is anyway in support of the hypothesis that oxidative DNA lesions can increase genomic instability [14].

The pro-oxidant activity of GGT was previously shown to produce oxidative modifications in cellular lipids and proteins [6,22]. The aim of the present study was to verify whether GGT activity can also promote oxidative damage to DNA, thus contributing to cancer genomic instability. Our results indicate that GGT expression in melanoma cells is associated with increased basal levels of DNA damage and oxidized bases, and that these effects are enhanced when the cells are incubated in the presence of GGT substrate GSH and ADP-chelated iron (III). These observations indicate that GGT activity can indeed increase (metal ion-dependent) oxidative DNA damage, thus pointing to a potential role of GGT in tumor progression.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, all reagents were from Sigma Chemical Co.

2.2. Cell lines and culture conditions

Two human melanoma cell clones expressing different GGT activity were obtained by stable transfection of low-expressing GGT activity Me665/2/21 clone (c21) with the full-length cDNA of human GGT, as previously described [23]. The c21/GGT clone shows high GGT activity (90.78 ± 3.40 mU/mg of cellular protein). A transfected clone in which no increase of GGT activity was observed, i.e. presenting with the same activity of parental line (0.34 ± 0.13 mU/mg of cellular protein), was chosen as control ("c21/basal" cells) [23]. Cytochemical analysis, performed as previously described [24], confirmed that GGT expressed in c21/GGT cells was localized on the outer cell surface (data not shown). Cells were routinely grown in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine (L-Gln) and 0.5 mg/mL G418 (Gibco), at 37 °C in a 5%/95% CO_2/air atmosphere.

2.3. Cell treatments

For basal levels of DNA damage, cells were harvested 24 h after seeding. Where indicated, the GGT competitive inhibitor L-2-amino-4-boronobutanoic acid (20 μM; ABBA) was sterile filtered and added to incubation media. ABBA was kindly provided by Dr. R.E. London (Natl. Inst. Environ. Health Sci., NC, USA); in preliminary experiments a 20 μM concentration of ABBA caused a strong inhibition of GGT activity but no significant effects on cell proliferation (data not shown).

For stimulation of GGT activity, complete culture medium was replaced with RPMI medium containing GSH (2 mmol/L), ADP-chelated $FeCl_3$ (2–0.2 mmol/L) and glycyl-glycine (20 mmol/L). Incubations were started by adding ADP-Fe^{3+} 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]. Incubations were performed for 60 min at 37 °C in a 5%/95% CO_2/air atmosphere. At the end of this time, cells were washed two times and harvested for comet assay. In separate sets of experiments, inhibition of GGT activity was obtained by adding to incubation mixtures GGT competitive inhibitors serine/boric acid (20/20 mM) complex (SBC) or ABBA (20 μM). Where indicated, soluble GGT (100 mU/mL), deferoxamine mesylate (DFO; 3 mM), erythrocyte CuZn/SOD (500 U/mL), thymol-free liver catalase (400 U/mL), α-tocopherol (200 μmol/L), butylated hydroxytoluene (BHT; 200 μmol/L) or Trolox C (1 mmol/L) were added to incubation mixtures.

2.4. Single-cell gel electrophoresis (comet assay)

DNA damage was measured using the alkaline comet assay as described by Duarte et al. [26]. Briefly, cells were harvested by trypsinisation and suspended in 0.6% low melting point agarose. Eighty microliters of the agarose gel (containing approximately 10^4 cells) were dispensed onto glass microscope slides previously coated with 1% normal melting point agarose. The agarose was allowed to set on ice under a coverslip and the slides left overnight in ice-cold lysis buffer (100 mM disodium EDTA, 2.5 M NaCl, 10 mM Trizma® base, adjusted to pH 10 with NaOH 10 M and containing 1% Triton X-100 (v/v) added fresh). Slides were washed with distilled water and then placed in a horizontal electrophoresis tank containing ice-cold alkaline electrophoresis solution (300 mM NaOH, 1 mM disodium EDTA) for 20 min to allow DNA unwinding. Electrophoresis was conducted for 20 min (30 V, 300 mA) at 4 °C. Slides were neutralised with 0.4 M Tris–HCl, pH 7.5 for 20 min and washed with double-distilled water, then allowed to dry. All procedures were carried out under subdued light to minimise background DNA damage. For staining, slides were re-hydrated in distilled water, incubated with a freshly made solution of 2.5 μg/mL propidium iodide for 20 min, washed again for 30 min and allowed to dry.

Comets were visualised with a fluorescence microscope (Leica) at 200× magnification using a 20× objective. Images were captured by an on-line Leica DFC320 camera and subsequently analyzed with the CometScore™ software (TriTek Corporation). A total of 100 cells were analyzed per sample, 50 per duplicate slide. The percentage of DNA in the tail of the comet (% tail DNA) was calculated for each cell by the CometScore™ software.

2.5. Fpg-modified comet assay

Oxidative DNA damage was evaluated by Fpg-modified comet assay as previously described [27]. This assay uses the formamidopyrimidine-DNA-glycosylase (Fpg) enzyme (New England Biolabs), a glycolase that recognizes and specifically cleaves the oxidized bases principally 8-oxoguanine from DNA, producing apurinic sites which are then converted into breaks by the associated AP-endonuclease activity. These additional breaks can be detected by comet assay and give a measure of oxidative DNA damage. The comet assay was carried out as described above, with the exception that after lysis the slides were washed three times for 15 min with the enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine

serum albumin, adjusted to pH 8 with KOH 1 M). After this time, slides were incubated with 100 μL of Fpg enzyme. Enzyme dilution (1:10^3 from a stock solution of 8000 U/mL) was prepared just before use, according to the manufacturer's instructions. Control slides were treated with 100 μL of enzyme reaction buffer only. Slides were placed horizontally in humidity chamber at 37 °C for 45 min. DNA unwinding and electrophoresis were then performed as described above.

2.6. Cell cycle analysis

For cell cycle analysis, 24 h after the various treatments, cells were washed, fixed in ice-cold 70% ethanol, and stored at −20 °C. Subsequently, samples were rehydrated with PBS and cellular DNA was stained with 10 μg/mL propidium iodide in PBS, containing RNase A (66 U/mL). Cell cycle distribution was determined by flow cytometry, and data were analyzed by Cell Quest® software; for each sample 40,000 events were collected.

2.7. Determination of apoptosis

Apoptosis was determined by TUNEL assay 24 h after drug exposure. Treated cells were fixed in 4% paraformaldehyde for 45 min at room temperature, washed and resuspended in ice-cold PBS. The in situ cell death detection kit (Roche, Germany) was used according to the manufacturer's instructions, and samples were analyzed by flow cytometry.

2.8. Glucose uptake

For determination of glucose uptake complete culture medium was replaced with Hanks' buffer, pH 7.4, containing 2 μCi of 2-deoxy-D-[1-^{3}H]glucose (Amersham Biosciences, UK), and cells were incubated for 30 min at 37 °C in a 5%/95% CO_2/air atmosphere. GLUT inhibitor cytochalasin B (20 μM) was added to a subset of wells to determine specific carrier-mediated glucose uptake. The medium was then removed and the cells were washed twice with ice-cold PBS. Lysis was induced by adding 600 μL of 0.1N NaOH. Aliquots of 450 μL of lysates were then mixed with a liquid scintillator (Beckman Ready Safe cocktail) and the radioactivity was measured by liquid scintillation counting (Beckman LS-6500 Multi Purpose Scintillation counter). Finally, the remaining part of samples was used for determination of protein content.

In a separate set of experiments, c21/GGT cells were pre-treated for 24 h with GGT competitive inhibitor ABBA (20 μM) before incubation with radiolabeled glucose.

Data are expressed as glucose uptake in the absence of cytochalasin B minus uptake in the presence of cytochalasin B.

2.9. Determination of GGT activity

Confluent cell monolayers were harvested with hypotonic lysis buffer (10 mM Tris–HCl, pH 7.8) and disrupted by a tight-fitting glass–glass Dounce homogeniser (30 strokes, 4 °C). Determination of GGT activity was performed according to Huseby and Strømme [25] using γ-glutamyl-p-nitroanilide as substrate and glycylglycine as transpeptidation acceptor. The amounts of p-nitroaniline formed were measured by reading the absorbance at 405 nm and using a molar extinction coefficient of 9200 mol/L cm. One unit of GGT activity was defined as 1 μmol of substrate transformed/mL/min. The results were expressed as mU/mg protein.

2.10. Other determinations

Protein content was determinated by the method of Bradford (Bio-Rad protein assay). For GSH determinations, aliquots of incubation mixtures were collected, acidified with 5-sulfosalicylic acid (1%, w/v) and stored to −20 °C until GSH determinations that were performed according to Baker et al. [28]. Statistical analysis of data was performed by ANOVA, with Newman–Keuls test for multiple comparison.

3. Results

3.1. Basal levels of DNA damage and oxidized bases

Two distinct human melanoma cell clones were used: the c21/basal clone, exhibiting low GGT activity (~0.3 mU/mg protein), and the c21/GGT clone, presenting with high enzyme activity (~90 mU/mg protein).

To evaluate the potential role of GGT in inducing DNA damage, basal levels of DNA damage were analyzed in both melanoma clones by alkaline comet assay. Under normal culture conditions, c21/GGT cells showed higher levels of basal DNA damage as compared to c21/basal cells, and this difference was accompanied by higher levels of oxidized bases, as detected by Fpg treatment (Fig. 1A and B). Interestingly, when cells were treated with the GGT-specific inhibitor ABBA, the difference in basal DNA damage between the two clones was suppressed (Fig. 1C).

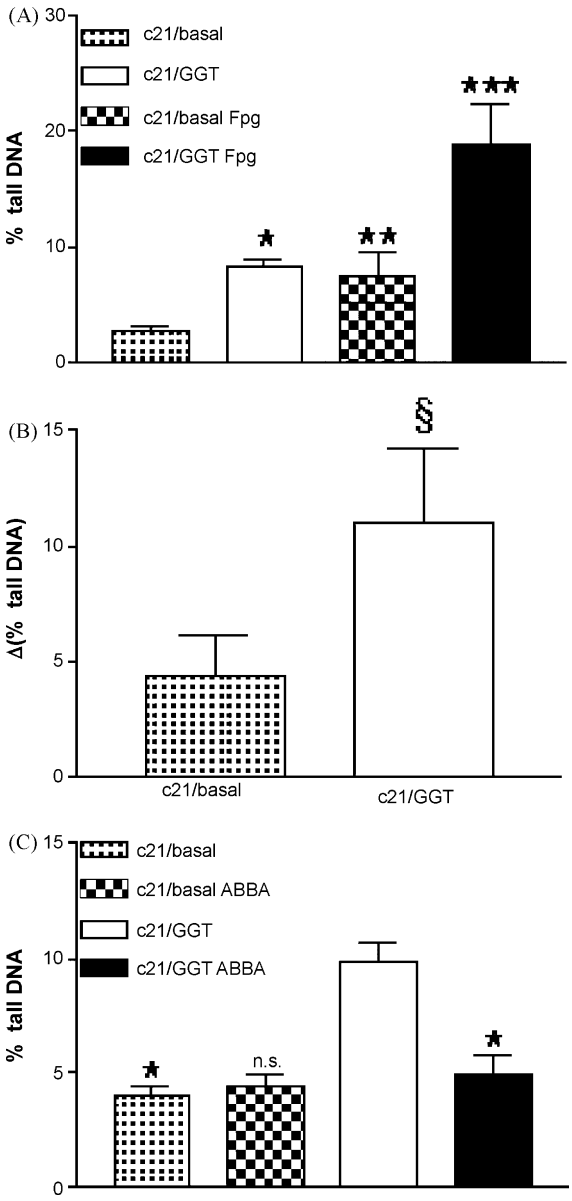

Fig. 1. Basal levels of DNA damage and oxidized bases in c21/basal and c21/GGT cells. Cells were collected after 24 h from seeding and processed for the comet assay. Where indicated cells were incubated with GGT competitive inhibitor ABBA. Data in Fig. 1B were obtained by subtracting the level of DNA damage observed for no treated samples from Fpg treated ones. Each value represents the mean ± S.D. from three independent experiments. Data were analyzed by one-way ANOVA with Newman–Keuls multiple comparisons test. (A) (*) $p < 0.001$, (**) $p < 0.05$ compared with "c21/basal"; (***) $p < 0.001$ compared with "c21/GGT"; (B) (§) $p < 0.05$; *(C)* (*) $p < 0.001$ compared with "c21/GGT"; (n.s.) not statistically different from "c21/basal".

3.2. Cell proliferation and apoptosis

The possibility that the differences observed might depend on different proliferation or apoptotic rates between the two clones was investigated. No differences in cell cycle distributions between the two clones were observed by flow cytometry, neither in basal conditions nor after a 24 h treatment with GGT-inhibitor ABBA (Fig. 2A). Moreover, when apoptosis was analyzed by TUNEL assay,

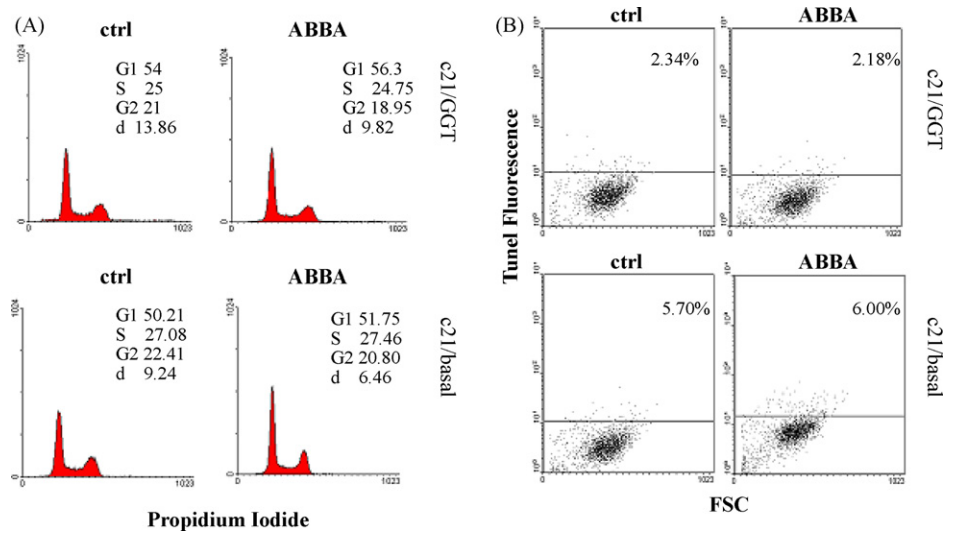

Fig. 2. Cell cycle distribution and apoptotic rate in basal and ABBA treated melanoma clones. Cells were incubated for 24 h in the presence/absence of GGT competitive inhibitor ABBA. (A) Cell cycle distribution was determined by flow cytometry. The percentages of cells in G1, S or G2 phases are reported in each histogram. (B) Apoptosis was detected by TUNEL assay. Numbers in the dot-plots indicate the percentage of tunel positive cells. One representative experiment out of three was reported.

in basal conditions both clones presented with very low, negligible percentages of apoptotic cells, and inhibition of GGT by ABBA did not produce any effect (Fig. 2B).

3.3. GGT expression and oxidative metabolism

A correlation has been reported to occur between DNA oxidative damage and cell metabolic rate [29]. Previous studies in our laboratories showed that the *in vivo* growth rate of tumors obtained from c21/GGT cells (after transplantation in immunodeficient mice) was higher than tumors derived from c21/basal cells [23]. Interestingly, c21/GGT cells presented with a higher glucose uptake than c21/basal (Fig. 3A)—an observation in agreement with their significantly higher proliferation rate *in vitro* (data not shown). Higher glucose uptake in c21/GGT cells was partially prevented by a 24 h pre-treatment with GGT-inhibitor ABBA (Fig. 3B).

3.4. Stimulation of GGT activity and DNA damage

The possibility that the differences observed in basal DNA damage could be related to the pro-oxidant activity of GGT was investigated in more detail. As can be seen in Fig. 4A and B, when the c21/GGT clone was incubated in the presence of substrate GSH, transpeptidation acceptor glycyl-glycine ("GGT stimulation"), and ADP-chelated iron, a marked increase in DNA damage levels was observed (Fig. 4A) along with a marked acceleration of GSH consumption (Fig. 4B). In the absence of glycyl-glycine such effects were markedly lower. Conversely, when GGT activity was stimulated in c21/basal cells, no differences in GSH hydrolysis nor DNA damage – despite the presence of ADP-chelated iron – were observed (Fig. 4C and D). On the other hand, when purified GGT was exogenously added to incubation mixture, GSH consumption and increased DNA damage were concomitantly observed (Fig. 4C and D).

In agreement with what was observed in the case of basal DNA damage levels, significantly higher levels of oxidized bases (oxidized purines) were detected by Fpg treatment in GGT-stimulated c21/GGT cells (Fig. 5A and B).

The relationships between GSH hydrolysis and DNA damage were further investigated by plotting GSH consumption data against DNA damage levels. Data were analyzed in order to isolate the effects truly depending on the sole GGT activity, i.e. by subtracting control GSH hydrolysis from GSH hydrolyzed in conditions of GGT stimulation. Fig. 6 shows the graph obtained by plotting "net" GSH hydrolysis values in c21/GGT cells *vs.* "net" DNA damage levels. As can be seen, a strong correlation between the two parameters was found ($R^2 = 0.867$), further confirming the role of enzyme activity in iron-dependent DNA damage. Such a role was also supported by data independently obtained with two GGT inhibitors, ABBA and serine–boric acid complex (SBC). As shown in Fig. 7, both ABBA and

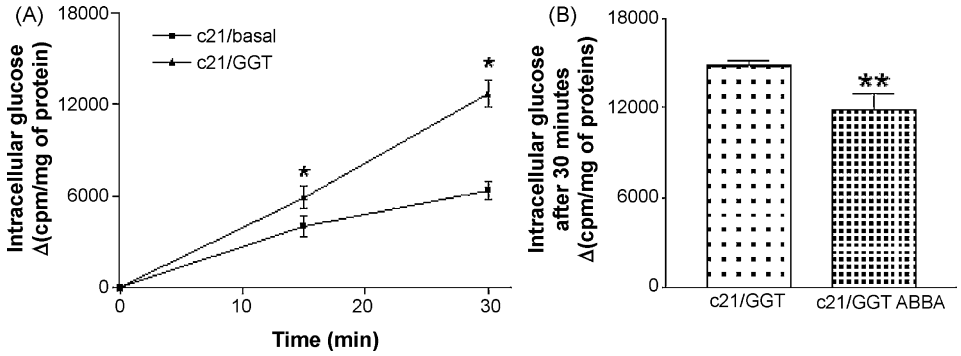

Fig. 3. Deoxy-glucose transport in melanoma clones. (A) Time course of glucose uptake in melanoma clones; (B) effect of a 24 h ABBA pre-incubation on c21/GGT glucose uptake. Data are expressed as glucose uptake in the absence of cytochalasin B minus uptake in the presence of cytochalasin B. Each value represents the mean ± S.D. from five independent experiments. Data were analyzed by two-way ANOVA (A) or Student's *t* test (B). (*) $p < 0.0001$; (**) $p < 0.001$.

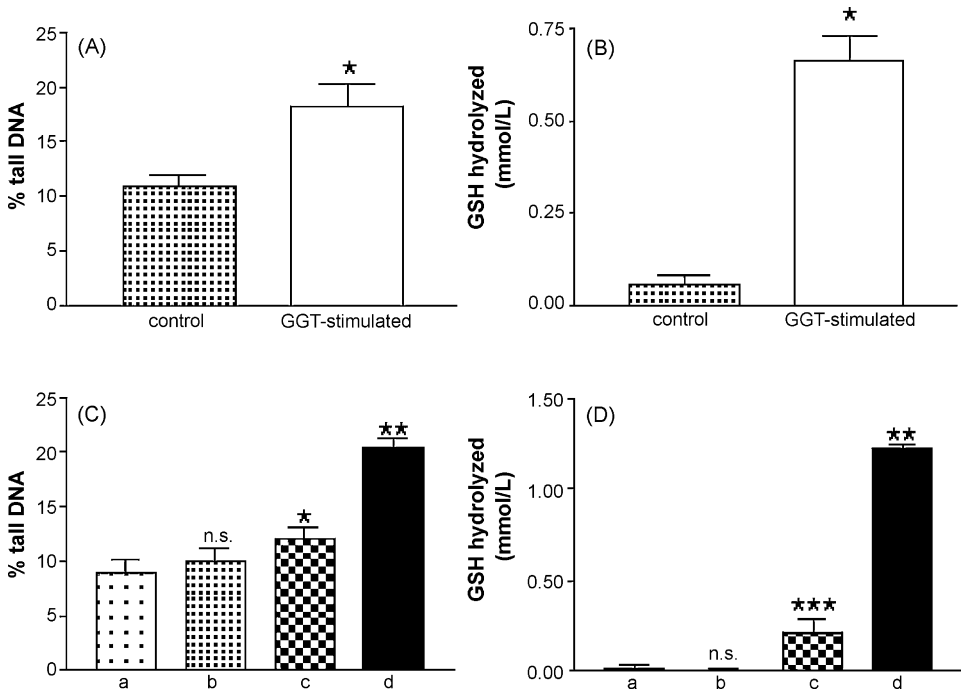

Fig. 4. Effects of GGT stimulation on DNA damage and GSH hydrolysis in melanoma clones. (A and B) c21/GGT cells were incubated in RPMI medium containing GSH, ADP-chelated $FeCl_3$ in the presence ("GGT-stimulated") or absence ("control") of transpeptidation acceptor glycyl-glycine. Each value represents the mean ± S.D. from five independent experiments. Data were analyzed by Student's *t* test; (*) $p < 0.0001$. (C and D) c21/basal cells were incubated in RPMI medium containing GSH, ADP-chelated $FeCl_3$ in the presence or absence of transpeptidation acceptor glycyl-glycine and purified GGT. a) Control; b) control with glycyl-glycine added; c) control with purified GGT added; d) control with glycyl-glycine and GGT added. Each value represents the mean ± S.D. from three independent experiments. Data were analyzed by one-way ANOVA with Newman–Keuls multiple comparisons test. (*) $p < 0.05$ compared with "a"; (**) $p < 0.001$ compared with "b"; (***) $p < 0.001$ compared with "a"; (n.s.) not statistically different from "a".

SBC concomitantly inhibited GSH hydrolysis and DNA damage in GGT-stimulated c21/GGT cells.

3.5. Mechanisms of GGT-mediated DNA damage

The mechanism by which GGT promotes DNA damage was further investigated in experiments performed in the presence of iron chelator DFO. As shown in Fig. 8, DFO completely prevented DNA damage, both in GGT-stimulated cells and in controls incubated in the absence of transpeptidation acceptor glycyl-glycine (Fig. 8A), confirming the participation of extracellular iron in the GGT-dependent damage. GSH consumption was unaffected by DFO, indicating that enzyme activity itself was not disturbed by DFO addition (Fig. 8B).

A marked decrease in DNA damage was also observed when cells were incubated in the presence of superoxide dismutase (SOD) and catalase (CAT), while GGT-mediated GSH hydrolysis remained unaffected (Fig. 9A and B). The same effects were observed when using the chain breaking antioxidants α-tocopherol (Fig. 9C and D), BHT or Trolox C (Fig. 9E and F).

4. Discussion

Early reports described the increase of GGT activity in cells and tissues exposed to carcinogenic treatments, a phenomenon often followed later on by increased cell proliferation and neoplastic growth. In recent years several studies have described alterations of GGT levels in a number of human tumors, and a new perspective has been forwarded proposing GGT as an "active" factor in development of a more aggressive and resistant phenotype of cancer cells [1]. In particular, besides the proposed role of GGT in the 'resistance phenotype' of pre-neoplastic and neoplastic cells (in connection with promotion of cysteine recovery and GSH synthesis), the pro-oxidant effects produced by GGT activity have gained increasing attention. It is likely that a fine equilibrium exists between antioxidant *vs.* pro-oxidant functions of GGT, and that the latter aspect of the enzyme activity may prevail under selected conditions, e.g. in GGT-overexpressing cells and in the presence of redox catalysts, such as metal ions.

Pro-oxidant effects of GGT are mediated through the formation of metabolite cysteinyl-glycine, capable of promoting redox-cycling reactions involving iron and other transition metal ions. Such reactions can eventually lead to the production of reactive oxygen species (superoxide anion radical, hydrogen peroxide) [5]. This phenomenon can represent the source of a low but persistent oxidative stress [3], likely contributing to the "persistent oxidative stress" repeatedly described in genomic instability and cancer [8,9]. The effects of GGT pro-oxidant activity were shown to include lipid and protein oxidation [7,22]. In addition, incubation mixtures containing purified GGT and transition metal ions were shown to be mutagenic in *Salmonella typhimurium* strains [30,31]. The occurrence of persistent oxidative stress in GGT-rich melanoma cells was confirmed in previous studies, showing a higher GSSG/GSH ratio [23] as well as a two-fold higher catalase expression in c21/GGT cells as compared to c21/basal [32].

Against this background, our data indicate that GGT overexpression in melanoma cells is *per se* associated with increased background levels of DNA damage (Fig. 1A) and oxidized bases (Fig. 1B). The effect is magnified when ADP-chelated iron is exogenously added, confirming that the mechanism implicated includes metal ion redox-cycling reactions (Fig. 4A). Basing on this finding, genotoxicity could be thus included among the effects of the more pro-oxidant environment generated by GGT-overexpressing cells.

On the other hand, it has been suggested that the genome of cancer cells could be more prone to oxidative damage due to the higher rate of metabolism associated with increased cellular prolifera-

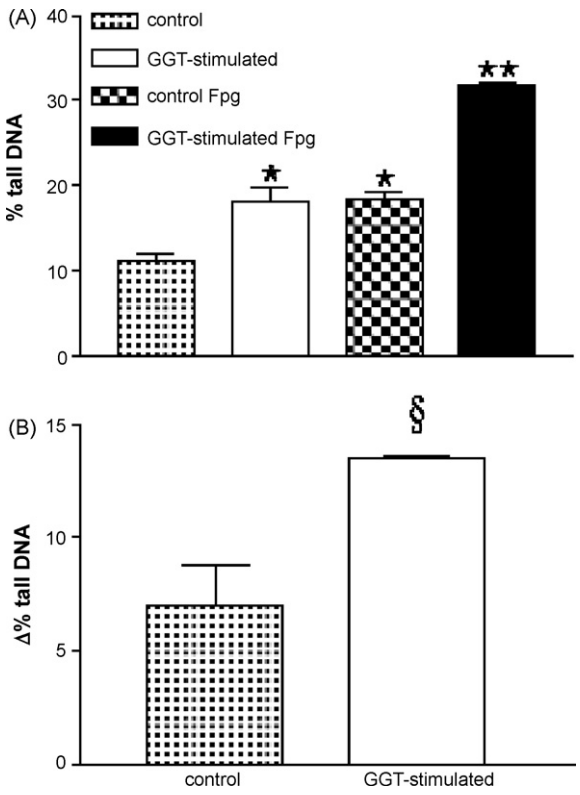

Fig. 5. Effect of GGT stimulation on bases oxidation in c21/GGT cells. c21/GGT cells were incubated in RPMI medium containing GSH, ADP-chelated $FeCl_3$ in the presence ("GGT-stimulated") or absence ("control") of transpeptidation acceptor glycyl-glycine. Data in Fig. 4B were obtained by subtracting the level of DNA damage observed for no treated samples from Fpg treated ones. Each value represents the mean ± S.D. from three independent experiments. Data were analyzed by one-way ANOVA with Newman–Keuls multiple comparisons test. (*) $p < 0.001$ compared with "control"; (**) $p < 0.001$ compared with "GGT-stimulated" and "control Fpg"; (§) $p < 0.01$.

tion [14]. Several studies have documented a relationship between cell proliferative status and induction of DNA damage and repair capabilities, and differences of these parameters were observed in cells as they proceed along G1, S and G2 phases of the cell cycle [33,34]. Moreover, some authors have proposed that apoptosis-associated DNA fragmentation could also be detected by comet assay, and identified the apoptosis-type DNA fragmentation in the so-called 'hedgehog comets' [35,36]. It has however been observed

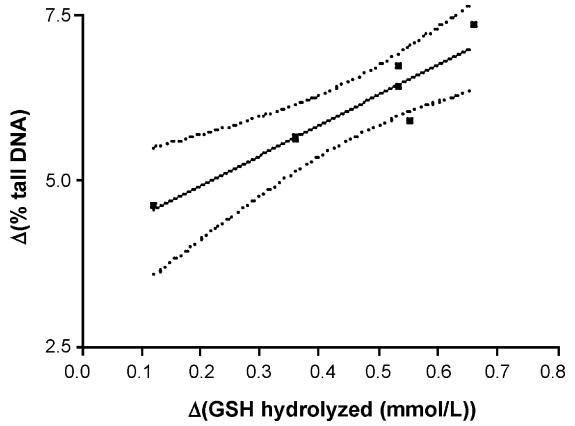

Fig. 6. Direct relationship between GSH hydrolysis and DNA damage in c21/GGT clone. Data were obtained from different experiments performed on c21/GGT clone by subtracting GSH hydrolyzed and percentage of tail DNA values in "GGT-stimulated" samples from "control" ones. $R^2 = 0.867$.

that 'hedgehog comets' are regularly produced by agents – e.g. H_2O_2 – inducing DNA lesions that can be readily repaired, and therefore such comets probably do not represent a reliable indicator of apoptosis [37].

On the basis of the considerations above, we have investigated whether the higher DNA damage basally observed in GGT-overexpressing cells could be explained by differences in cell cycle distribution or by a higher apoptotic rate. No differences were observed between the two clones with respect to cell cycle distribution or apoptotic rate (Fig. 2A and B). Moreover, when the GGT-specific inhibitor ABBA was added to cell culture media, the difference in basal DNA damage was completely abolished (Fig. 1C), while no effect was produced on cell cycle distribution or apoptotic rate (Fig. 2A and B).

It is known that a source of endogenous ROS within the cell is represented by oxidative metabolism, and the question then arises, which can be the predominant mechanism operating in production of the observed DNA damage, i.e. whether the effect is promoted i) directly, through a GGT-mediated production of DNA-damaging reactive species, or ii) indirectly, through the upregulation by GGT activity of cellular processes involved in oxidative metabolism.

With respect to the "indirect" hypothesis, previous studies have shown that GGT expression may represent a growth advantage, insofar as GGT pro-oxidant effects can modulate the redox-sensitive transcription factors NF-kB and AP-1, involved in cell proliferation [7]. It is in fact well assessed that tumors obtained from c21/GGT cells transplanted in nude mice indeed grow faster than those derived from c21/basal cells [23]. A relationship was also documented between exposure to low grade oxidative stress and increased expression of GLUT1 glucose transporter, through the activation of AP-1 [38]. As shown in Fig. 3A, a higher basal glucose transport is indeed present in c21/GGT cells as compared to c21/basal—an effect that was significantly decreased after 24 h pre-incubation with the specific GGT-inhibitor ABBA (Fig. 3B). GGT-rich cells appear thus to enjoy a higher glucose availability as compared to GGT-poor cells, a phenomenon likely leading to a higher metabolic rate and higher oxidative DNA damage.

On the other hand, potentially damaging reactive species are also directly produced by GGT pro-oxidant reactions ("direct hypothesis"). As mentioned above, these are able not only to modulate redox-sensitive signal transduction, but also to produce direct (metal ion-mediated) oxidative damage to biological molecules such as lipids and proteins. In agreement with previous data, the present results demonstrate that conditions of stimulated GGT activity are also accompanied by increased DNA damage (Fig. 4A), and that such damage is indeed oxidative in nature (Fig. 5A and B). ABBA or serine–boric acid complex blocked GSH consumption and the corresponding DNA damage at the same time (Fig. 7). Moreover, when purified GGT was exogenously added to c21/basal cells, GSH hydrolysis and increased DNA damage were concomitantly observed (Fig. 4C and D). Also, a strong correlation was observed between net levels of GSH hydrolysis and DNA damage, further confirming a strict relationship existing between the two (Fig. 6).

Metal ions redox cycling with production of reactive oxygen species is a critical step in the phenomena described. GGT-dependent DNA damage was in fact completely inhibited in the presence of the extracellular iron chelator DFO (Fig. 8A), as well as in the presence of superoxide dismutase and catalase (Fig. 9A). Previous reports described lipid peroxidation (LPO) among the pro-oxidant effects produced by GGT activity, both in acellular [39] and cellular systems [22,40,41]. Accordingly, increased production of malondialdehyde (MDA) was described as a consequence of GGT-dependent lipid peroxidation of isolated LDL lipoproteins [42]. It is well established that LPO end-products (lipid peroxides, aldehydes, carbonyls) can damage DNA, either by reacting directly with bases,

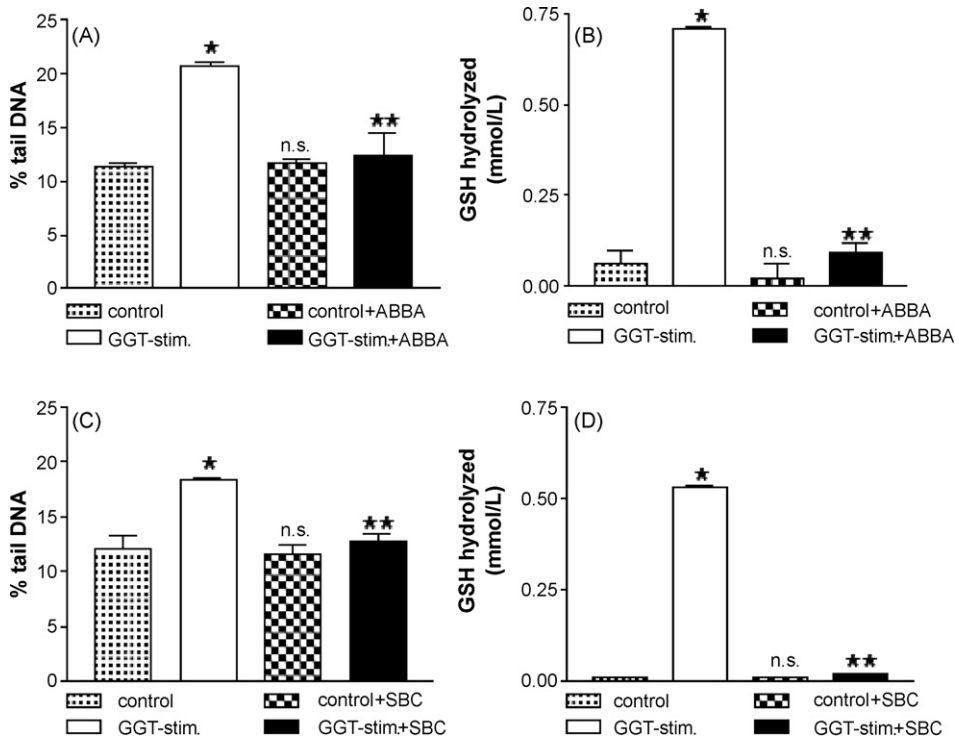

Fig. 7. Effects of GGT inhibitors ABBA (A and B) and SBC (C and D) on GGT-induced DNA damage and GSH hydrolysis in c21/GGT clone. c21/GGT cells were incubated in RPMI medium containing GSH, ADP-chelated $FeCl_3$ in the presence ("GGT-stimulated") or absence ("control") of transpeptidation acceptor glycyl-glycine. Where indicated GGT competitive inhibitors ABBA or SBC were added to incubation mixtures. Each value represents the mean ± S.D. from three independent experiments. Data were analyzed by one-way ANOVA with Newman–Keuls multiple comparisons test. (*) $p < 0.001$ compared with "control"; (**) $p < 0.001$ compared with "GGT-stimulated"; (n.s.) not statistically different from "control".

or by generating more reactive bifunctional intermediates forming exocyclic DNA adducts, DNA nicking and base substitutions [43]. Lipophilic antioxidants such as α-tocopherol or butylated hydroxytoluene were shown to exert strong protection against some types of such DNA damage [44]. Our results document a strong inhibition of GGT-dependent/iron-catalyzed DNA damage by α-tocopherol, butylated hydroxytoluene (a synthetic analogue of vitamin E) and Trolox C (a water-soluble vitamin E analogue) (Fig. 9C and E). It is therefore conceivable that part of the observed GGT-dependent DNA damage may be mediated through the initiation of a lipid peroxidation process, as previously observed in hepatoblastoma cells [22]; future studies will help elucidate this point.

Thus, continuous direct production of DNA damage through a GGT-dependent promotion of iron-dependent oxidative processes can represent a challenge to DNA-repair systems in GGT-expressing cancer cells, a condition likely leading to genomic instability and increased mutation risk. Modulating effects of pro-oxidants have also been reported with regards to DNA repair systems, such as, e.g. 8-oxoguanine DNA glycosylase (OGG1). An oxidative-mediated downregulation of DNA repair would indirectly exacerbate genomic instability; further studies are however needed on these aspects [45]. Fig. 10 depicts a proposed mechanism of DNA damage, induced by GGT pro-oxidant reactions either directly, through the production of reactive species, or/and as a consequence of an increased oxidative metabolism. GGT pro-oxidant activity requires extracellular GSH, which in basal culture conditions can be supplied by continuous GSH efflux from the same cells. Redox active metal ions are also required, and traces of transition metals are indeed known to be present in routine culture media as contaminants. Importantly, we have previously demonstrated that

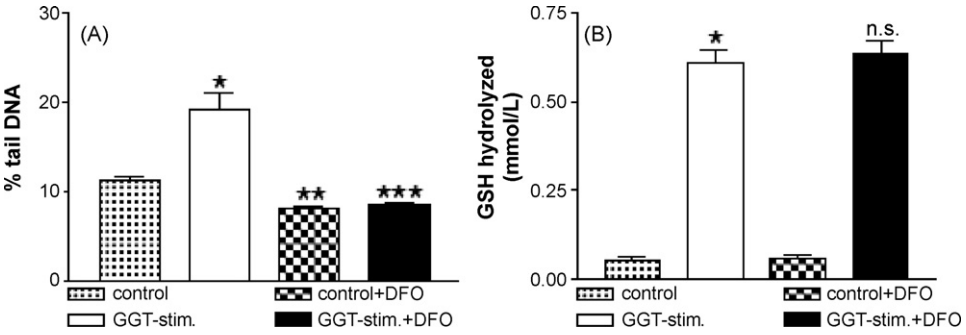

Fig. 8. Effects of iron-chelator DFO on GGT-induced DNA damage (A) and GSH hydrolysis (B) in c21/GGT clone. c21/GGT cells were incubated in RPMI medium containing GSH, ADP-chelated $FeCl_3$ in the presence ("GGT-stimulated") or absence ("control") of transpeptidation acceptor glycyl-glycine. Where indicated the iron chelator DFO was added to incubation mixtures. Each value represents the mean ± S.D. from three independent experiments. Data were analyzed by one-way ANOVA with Newman–Keuls multiple comparisons test. (*) $p < 0.001$, (**) $p < 0.01$ compared with "control"; (***) $p < 0.001$ compared with "GGT-stimulated"; (n.s.) not statistically different from "GGT-stimulated".

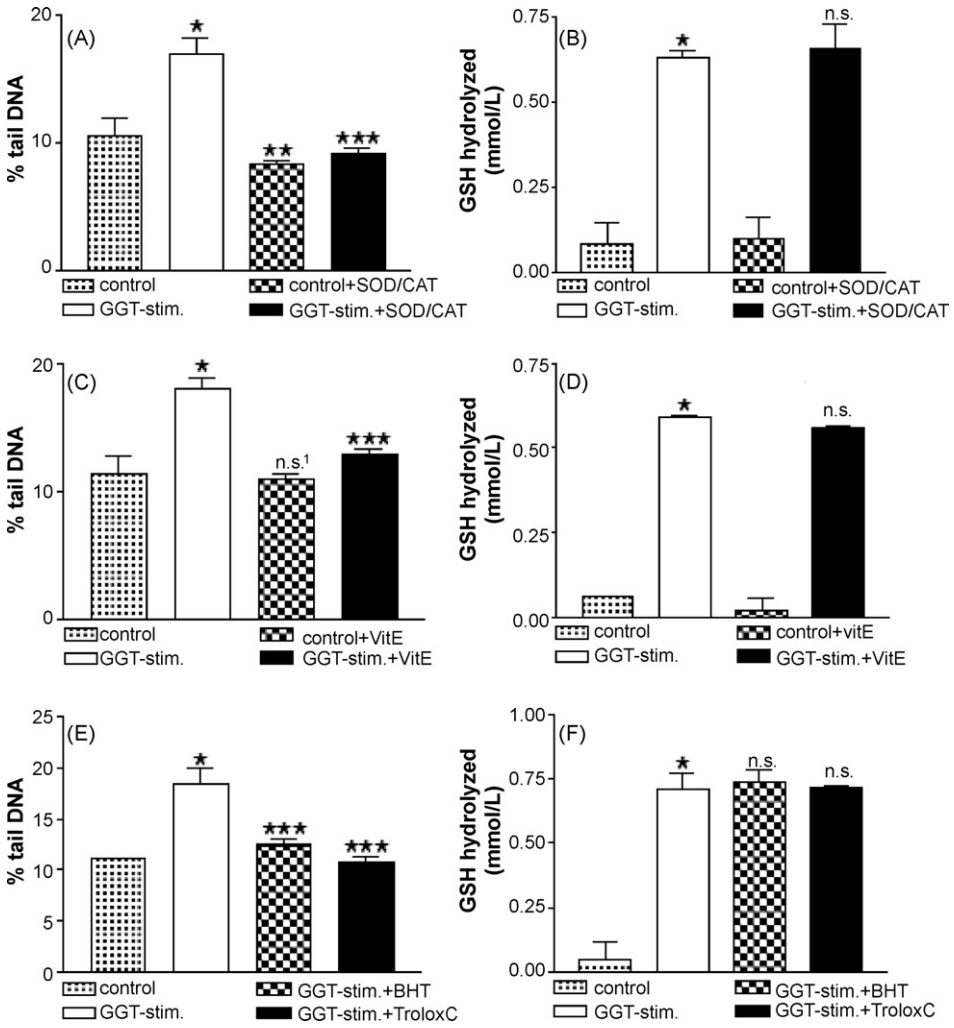

Fig. 9. Effects of antioxidants on GGT-induced DNA damage. c21/GGT cells were incubated in RPMI medium containing GSH, ADP-chelated $FeCl_3$ in the presence ("GGT-stimulated") or absence ("control") of transpeptidation acceptor glycyl-glycine. Where indicated superoxide dismutase (SOD) and catalase (CAT), α-tocopherol (VitE), butylated hydroxytoluene (BHT) or Trolox C were added to incubation mixtures. Each value represents the mean ± S.D. from three independent experiments. (*) $p < 0.001$, (**) $p < 0.05$ compared with "control"; (***) $p < 0.001$ compared with "GGT-stimulated"; (n.s.) not statistically different from "GGT-stimulated"; (n.s.[1]) not statistically different from "control".

GGT-dependent redox-cycling reactions could be efficiently supported *in vivo* by physiological iron sources, such as transferrin and ferritin [46]. On the other hand, it has been documented that tumoral stroma is an environment relatively rich in redox active iron [47].

In conclusion, GGT-dependent DNA oxidative damage adds to previously described redox effects of this enzyme, such as direct oxidative damage to lipids and proteins, modulation of intracellular antioxidants, such as GSH [23] and catalase [32], and regulation of redox-sensitive molecular targets relevant to cell proliferation ability and cancer growth, like NF-kB and AP-1 [3,7] and TNFR-1 [10]. The kidney represents an organ where initiation/progression of neoplastic transformation mediated through GGT-dependent redox processes may represent an important mechanism. The kidney is in fact very rich in GGT activity, expressed in tubular epithelial cells and participating in resorption of GSH from preurine, and it was repeatedly shown that treatment of animals with chelated iron results in lipid peroxidation and induction of kidney carcinomas [39,48]. Moreover, it is known that oxidative conditions occurring during inflammation can induce the expression of GGT [49], and it has been recently shown that the pro-inflammatory cytokine TNF-alpha – implicated at several levels in cancer progression – induces GGT expression *via* NF-kappaB-dependent pathways [50]. It can thus be envisaged that GGT-dependent genomic instabil-

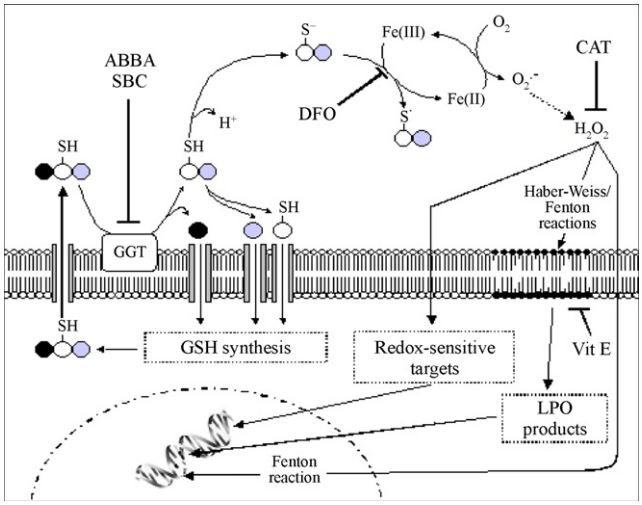

Fig. 10. *Pro-oxidant activity* of GGT and DNA damage. GGT pro-oxidant reactions could promote DNA damage through two distinct mechanisms: i) directly, through the production of directly damaging reactive species, or ii) indirectly, by modulating cellular processes involved in oxidative metabolism (e.g. NF-kB and AP-1 pathways). The inhibitors/antioxidants employed and the steps leading to DNA damage blocked by their action are shown. Further details are given in the text.

ity might contribute to the pathogenesis of inflammation-related neoplasia.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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