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Membrane gamma-glutamyl transpeptidase activity of melanoma cells: effects on cellular H_2O_2 production, cell surface protein thiol oxidation and NF-κB activation status

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SUMMARY

The metabolism of glutathione by membrane-bound gamma-glutamyl transpeptidase (GGT) has been recently recognized as a basal source of hydrogen peroxide in the extracellular space. Significant levels of GGT activity are expressed by malignant tumours, and in melanoma cell lines they were found to correlate with the malignant behaviour. As hydrogen peroxide and other oxidants can affect signal transduction pathways at several levels, the present study was aimed to verify: (i) the occurrence of GGT-dependent production of hydrogen peroxide in melanoma cells; (ii) the effects of GGT-dependent prooxidant reactions on known redox-sensitive cellular targets, i.e. protein thiols, the nuclear transcription factor NF-κB and p53. Two melanoma Me665/2 cell clones, exhibiting traces of (clone 2/21) or high (clone 2/60) GGT activity, were studied. The occurrence of GGT-dependent production of hydrogen peroxide was apparent in 2/60 cells, in which it was accompanied by lower levels of cell surface protein thiols. In 2/60 cells, GGT expression was also associated with higher levels of NF-κB activation, as compared to GGT-poor 2/21 cell clone. Indeed, stimulation or inhibition of GGT activity in 2/60 cells resulted in progressive activation or inactivation of NF-κB, respectively. An analysis of the *p53* gene product indicated lack of protein expression in 2/60 cells, whereas a mutant protein was highly expressed in 2/21 cells. Taken together, these results indicate that the expression of GGT activity can provide melanoma cells with an additional source of hydrogen peroxide, and that such prooxidant reactions are capable to modify protein thiols at the cell surface level. In addition, GGT expression results in an up-regulation of the transcription factor NF-κB, which could explain the higher metastatic behaviour reported for GGT-rich melanoma cells as compared to their GGT-poor counterparts.

Key words: Gamma-glutamyl transpeptidase, Melanoma, Hydrogen peroxide, Protein thiol, NF-κB, p53

INTRODUCTION

Experimental evidence accumulated during the last decade has consistently documented the involvement of oxidation-reduction (redox) reactions in the regulation of a number of key molecular mechanisms, linked to important cellular functions (reviewed by Lander, 1997; Nakamura et al., 1997; Flohé et al., 1997; Sen, 1998). It appears by now clear that low, 'physiological' levels of oxidant agents, and reactive oxygen species in particular, can exert 'positive' roles within the cells, and hence the term 'oxidant-mediated regulation' has been recently proposed as a more accurate alternative to 'oxidant stress' (Cotgreave and Gerdes, 1998). Oxidant agents are capable to induce post-translational modifications in cysteine residues of proteins, often resulting in changes of their functional state. In particular, several recent studies have provided convincing evidence linking these processes with the regulation of the equilibrium between cell proliferation and cell death (Powis et al., 1995; Cotgreave and Gerdes, 1998; Burdon, 1995). For example, low, non-toxic levels of the reactive oxygen species superoxide and hydrogen peroxide have been repeatedly shown to exert a stimulatory effect on cell proliferation (Burdon, 1995), and it has been recently reported by our and other laboratories that pretreatment of cells with a mild oxidative stress can result in their protection against apoptogenic stimuli (Maellaro et al., 1996).

A basal production of low levels of reactive oxygen species exists in normal cells, originating e.g. from mitochondria, cytochromes P450 and b5, xantine oxidase, NADPH-oxidase systems (Halliwell and Gutteridge, 1989). To these known cellular sources, studies from our and other laboratories have recently added gamma-glutamyl transpeptidase (GGT), the

plasma membrane-bound ectoactivity that – in a number of cell types – is in charge of metabolizing extracellular reduced glutathione (GSH). It has been in fact documented that GGT activity can give rise to redox reactions, leading to the production of reactive oxygen species and lipid peroxidation (Stark et al., 1993; Paolicchi et al., 1997; Drozdz et al., 1998; Dominici et al., 1999; Del Bello et al., 1999). In particular, we have recently shown that GGT-dependent prooxidant reactions are involved in inhibition of proliferation in ovarian cancer cells (Perego et al., 1997), and that the low levels of hydrogen peroxide originating as a by-product during GGT activity are capable to prevent apoptosis and maintain proliferation of histiocytic lymphoma cells (Del Bello et al., 1999).

The expression of GGT has been regarded as a marker of neoplastic progression in several experimental models, such as rodent skin and liver chemical carcinogenesis (Warren et al., 1993). Significant levels of GGT have been reported in a number of human malignant neoplasms, e.g. ovary (Paolicchi et al., 1996), colon (Murata et al., 1997), lung (Blair et al., 1997), liver (Tsutsumi et al., 1996), sarcoma (Hochwald et al., 1997), leukemias (Täger et al., 1995); in many instances, GGT levels detectable in metastases are higher than in the corresponding primitive tumours. In a series of 60 different human tumor cell lines, GGT was found to be significantly expressed in 70% of cases (Tew et al., 1996).

GGT is often expressed at high levels in melanomas as well (Murray et al., 1982), and it has been observed that tumour tissue can be a source for GGT circulating in serum (Melezinek et al., 1998). Interestingly, it has also been reported that in different clones of Me665/2 melanoma cells the degree of GGT expression is correlated with the invasive potential (Supino et al., 1992). Against this background, the present study was aimed to verify whether a higher GGT expression in selected melanoma cell clones could result in oxidative alterations of cellular molecular targets. To this purpose, we chose to analyse and compare two melanoma clones, Me665/2/21 and Me665/2/60, presenting with largely different GGT activities.

MATERIALS AND METHODS

Cell cultures

Two clones derived from the melanoma Me665/2 cell line were studied. Cells were cultured in RPMI 1640 medium (Sigma, St Louis, MO), supplemented with 10% heat-inactivated fetal calf serum (FCS)(Sigma), 2 mM L-glutamine, 2.4 mg/l amphotericin B and 50 mg/l gentamycin, at 37°C, under 95% air: 5% CO_2, in a humidified athmosphere. Cells were routinely harvested and reseeded before reaching confluence. For proliferation experiments, after treatments cells were detached by trypsin and counted using a Bürker chamber. In some experiments, catalase (Boehringer Mannheim, FRG; 50 μg/ml, dialyzed before use) was also added to the culture medium.

Determination of cellular GGT activity

GGT activity was assayed according to Grisk et al. (1993), using γ-glutamyl-*p*-nitroanilide (GPNA; Sigma) as donor substrate and glycyl-glycine (Sigma) as glutamate acceptor for the transpeptidation reaction. In a typical experiment 1×10^6 cells were incubated for 30 minutes at 37°C in 0.2 ml of 100 mM Tris-HCl, pH 8.0, in the presence of 2.5 mM γ-glutamyl-*p*-nitroanilide, with or without 20 mM glycyl-glycine. The reaction was stopped by adding 1.8 ml of 1 M sodium acetate (pH 4.0), and *p*-nitroaniline release in the incubation medium was determined by spectrophotometry at 405 nm (ε=11,300). Each experimental value was obtained by subtracting the value of the sample minus glycyl-glycine from the value of the sample plus glycyl-glycine. 1 Unit of GGT activity was defined as 1 μmol of substrate transformed/ml/minute.

Determination of GGT protein by immunoblot

An antiserum directed against a synthetic polypeptide corresponding to the C-terminal 20 amino acid residues of human GGT heavy chain (CDTTHPISYYKPEFYTPDDGG) was obtained after immunization of a rabbit, as described (Hanigan and Frierson, 1996). The antiserum was processed for enrichment of the immunoglobulin fraction, and antibodies were affinity-purified on Affi-Gel® (Bio-Rad) to which the antigenic peptide had been adsorbed. Specificity of the antibodies was tested by verifying that the reaction was not produced (i) when samples were treated with pre-immune serum, and (ii) when antibodies were pre-absorbed with the same synthetic peptide used for immunization.

For determination of GGT expression, melanoma cell monolayers were suspended in lysis buffer (10 mM Tris-HCl, pH 7.5, containing 10 mM NaCl, 1 mM $MgCl_2$ and the following protease inhibitors: leupeptin, 1 μg/ml; pepstatin, 1 μg/ml, and phenylmethylsulfonyl fluoride (PMSF; 50 μg/ml)), and exposed to 5 freezing-thawing cycles at −80°C. Samples were then homogenized with a Dounce homogenizer (20 strokes) and centrifuged at 400 ***g*** (10 minutes, 4°C) to remove cell nuclei. Supernatants were further centrifugated at 100,000 ***g*** (60 minutes, 4°C), and cell membranes (=pellet), and cytosol (=supernatant) were separately recovered. Aliquots of the lysates, containing equivalent amounts of protein, were separated by 8% SDS-PAGE, and gels were blotted onto nitrocellulose membranes (Sartorius, Göttingen, FRG). Protein transfer was verified by reversible staining of nitrocellulose filters with Fast Green FCF (Sigma) dissolved (1 mg/ml) in 1% (v/v) glacial acetic acid. Blots were incubated overnight (4°C) with anti-GGT purified immunoglobulins, corresponding to a 1:1000 dilution of the original antiserum in PBS containing 0.1% (v/v) Tween-20. Visualization of protein bands was obtained using a biotinylated anti-rabbit Ig antibody (Dako, Milan, Italy) and peroxidase-conjugated streptavidin, using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Italy).

Hydrogen peroxide production assay

H_2O_2 production was measured according to the method of Mohanty et al. (1997), by monitoring the horseradish peroxidase (HRP)-catalyzed oxidation of the probe *N*-acetyl-3,7-dihydroxyphenoxazine (A6550; Molecular probes, Eugene, OR, USA), which becomes highly fluorescent only after oxidation by H_2O_2. Cells suspended in RPMI 1640 medium were seeded in 6-well plates (5×10^5 cells/well) and allowed to adhere for at least 12 hours prior to the experiments. All samples were then washed with PBS, and RPMI 1640 medium was replaced by Krebs-Ringer phosphate buffer (2 ml/well), pH 7.4, containing 145 mM NaCl, 5.7 mM Na phosphate, 4.9 mM KCl, 0.5 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 1 g/l glucose, to which 2 mM glycyl-glycine and 5 μM GSH were also added in order to supply cells with GSH and amino acids in concentrations comparable to those present in RPMI 1640 medium. Stimulation of GGT was performed by adding the acceptor dipeptide glycyl-glycine (10 mM) and the substrate GSH (100 μM). Where indicated, GGT inhibition was obtained by preincubating cells with 500 μM of the non competitive inhibitor acivicin (Sigma) for 60 minutes at 37°C in the same medium. In additional experiments the competitive GGT inhibitor serine/boric acid complex (SBC; 10/10 mM) and superoxide dismutase (SOD; Boehringer Mannheim, FRG; 50 μg/ml) were added to samples just prior to the assay for H_2O_2 release. In all samples, A6550 and HRP (Sigma) were present at final concentration of 50 μM and 1 U/ml, respectively. After 60 minutes of incubation at 37°C, aliquots of the incubation medium were withdrawn and fluorescence was measured in a Perkin-Elmer fluorimeter (sensitivity 1; slits 4 nm;

excitation/emission wavelengths: 590/645 nm). Calibration curves obtained with H_2O_2 additions in the concentration range 0.1-5 μM, showing a linear fluorimetric response, were used for calculations. H_2O_2 release was normalized by cell counting, performed in all wells after detachment of cells by trypsin.

Determination of cellular protein thiols

Total cellular protein thiols were determined spectrophotometrically after reaction with 5,5-dithiobis-2-nitrobenzoic acid, essentially as previously described (Pompella et al., 1991). Determination of cell surface protein thiols was accomplished by ELISA assay. Cells were seeded in 96-well microtiter plates (5×10^4 cells / well) and incubated for 24 hours at 37°C. The experimental treatments (GGT stimulation and inhibition, catalase, etc.) were performed in the same plates, at 37°C for 45 minutes. At the end of incubations, cells were washed twice with Tris-buffered saline, and immediately exposed to polymerized maleimide-activated peroxidase (Sigma) at 37°C for 10 minutes. After washing, the peroxidase substrate o-phenylene diamine dichloride (Sigma Fast™ tablet sets) was added and 15 minutes later the development of color was determined in an Bio-Rad Benchmark™ ELISA microplate reader at 405 nm. Cytochemical visualization of cell surface protein thiols by laser scanning confocal fluorescence microscopy was obtained by reacting unfixed cell monolayers with the thiol-specific probe 3-(*N*-maleimidylpropionyl) biocytin (MPB; Molecular probes, USA), as previously described (Pompella et al., 1996).

NF-κB activation status

Activation status of NF-κB was evaluated by determining the presence of activated NF-κB in cell nuclei by western blot, using mouse G96-337 monoclonal antibody (Pharmingen, USA). G96-337 recognizes an independent epitope at the C terminus of the NF-κB subunit p65, which is translocated to cell nucleus following the activation of NF-κB. Nuclear extracts of melanoma cells were prepared as described (Andrews and Faller, 1991); 20 μg of nuclear protein were fractionated by SDS-PAGE and transferred to nitrocellulose membrane. Anti-p65 antibody was diluted 1:2500 (0.2 μg/ml), and a peroxidase-conjugated anti-mouse Ig was used as secondary antibody. Blots were developed using an ECL revelation kit (Boehringer Mannheim, FRG). Densitometric analysis of developed blots was carried out with a Bio-Rad GS-690 imaging densitometer apparatus, using the Molecular Analyst® software (Bio-Rad). Total extinction (E_{tot}) values were obtained by semi-automatic integration of positive bands.

Other determinations

GSH and GSSG were determined by HPLC after derivatization with dinitrofluorobenzene, as described (Reed et al., 1980).

p53 gene product studies were carried out as previously described (Perego et al., 1996). Cells were harvested 24 hours after GGT stimulation or inhibition (0.15 mM acivicin). Cells were then lysed, and after separation of samples by SDS-PAGE, p53 was determined by immunoblot using specific monoclonal antibodies (DO-7, Dako, Denmark) and enhanced chemiluminescence. The allelic status of p53 was determined by direct sequencing (Perego et al., 1996).

Statistical significance of data was assessed by ANOVA with Newman-Keuls test for multiple comparisons. Results are reported as means ± standard error (s.e.m.).

RESULTS

The two Me665/2 melanoma sub-clones chosen for the present study presented with largely different GGT activities, as biochemically assessed by the rate of hydrolysis of GPNA by intact cell monolayers (0.2±0.02 and 84.7±5.7 mU/mg protein, in subclones 2/21 and 2/60, respectively). In agreement with the biochemical data, the immunoblot determination of GGT protein in cell lysates showed a striking difference in the degree of expression of GGT heavy chain. While being undetectable in the cytosol fractions of both clones, GGT heavy chain was expressed in the membrane fractions, with a striking prevalence in 2/60 cells (Fig. 1).

It has been previously shown that GGT activity can give rise to the production of significant amounts of hydrogen peroxide in the extracellular space (Dominici et al., 1999; Del Bello et al., 1999). Hydrogen peroxide production by melanoma cells was therefore assayed, both in basal conditions and in conditions of modulated GGT activity (Fig. 2). As can be seen, basal H_2O_2 production was higher in GGT-rich Me665/2/60 cells, and such a difference was further increased in conditions of stimulated GGT activity (i.e. in the presence of significant concentrations of both substrates, GSH and glycyl-glycine). The GGT-dependence of H_2O_2 production by 2/60 cells was confirmed by the fact that the production of H_2O_2 was reverted to basal levels when GGT activity was almost completely supppressed with two independently acting inhibitors (acivicin: 99-100% inhibition; SBC: 93% inhibition). Interestingly, the addition of superoxide dismutase to the incubation mixture resulted in a further significant gain in H_2O_2 production by 2/60 cells, suggesting that superoxide is the reactive oxygen species being produced during GGT activity. This observation is in agreement with previously published data (Dominici et al., 1999), indicating that GGT-dependent prooxidant effects are mediated through interactions with redox-active iron; the latter are known to primarily result in the formation of superoxide.

Subsequent experiments were carried out to investigate the oxidizing effects of H_2O_2 deriving from GGT activity on cellular protein thiols. Protein thiol levels were selectively determined in proteins of the cell surface, insofar as this cellular pool of protein thiols was previously shown to be preferentially exposed to the extracellular GGT-dependent prooxidant processes (Dominici et al., 1999). In fact, a clear GGT-dependence of surface protein thiol levels was observed in 2/60 cells (Fig. 3). Stimulation of GGT activity resulted in a significant loss of cell surface protein thiols, which was prevented in cells pretreated with the irreversible GGT

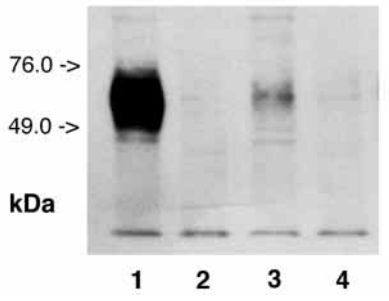

Fig. 1. Western blot of GGT protein heavy chain in membrane and cytosol fractions of Me665/2/21 and 2/60 melanoma cells. Purified polyclonal antibodies against a 20-amino acid peptide belonging to GGT heavy chain were employed. Blots were developed by enhanced chemiluminescence. Lanes 1 and 2: clone 2/60, membrane and cytosol protein fractions, respectively; lanes 3 and 4: clone 2/21, membrane and cytosol protein fractions, respectively.

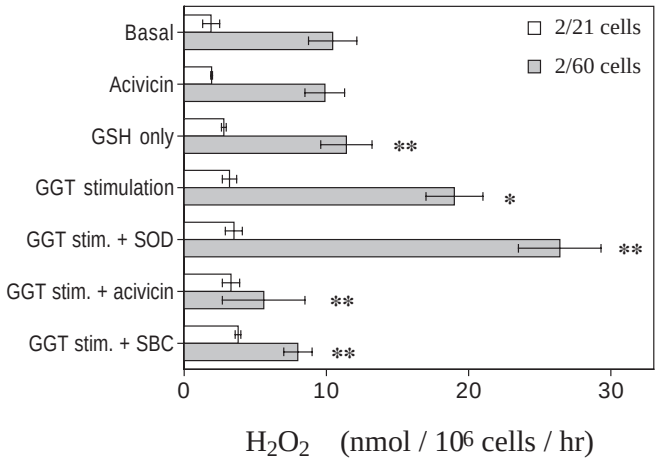

Fig. 2. GGT-dependent and -independent H_2O_2 production in Me665/2 melanoma cells. H_2O_2 production was determined fluorimetrically, by the HRP/A6550 system. 'Basal' values were determined in Krebs-Ringer buffer to which 2 mM glycyl-glycine and 5 μM GSH were also added (see Materials and Methods for details). For 'GSH alone' determinations, a further 95 μM GSH was added (final concn: 100 μM). Further control experiments were performed in Krebs-Ringer buffer with no additions, and it was verified that the sole addition of 10 mM glycyl-glycine, had no effect on H_2O_2 production (data not shown). GGT stimulation conditions were achieved by exposing cells to GSH and glycyl-glycine, 0.1 and 10 mM, respectively. SOD, acivicin and SBC were added where indicated at 50 μg/ml, 500 μM and 10/10 mM (final concentrations), respectively. With the indicated concentrations of inhibitors, GGT residual activity was >1% (acivicin) and >5% (SBC). Results are means ± s.e.m. of 3-6 experiments. Statistical significance (P<0.05) was assessed by one-way ANOVA with Newman-Keuls test for multiple comparisons. (*) Significantly different from basal value; (**) significantly different from 'GGT stimulation' value.

inhibitor, acivicin. This observation was confirmed by laser-scanning confocal fluorescence microscopy, as shown in Fig. 4. Thiol loss was also prevented when the incubations were carried out in the presence of catalase, thus confirming the role of H_2O_2 (Fig. 3). No significant variation was inducible by stimulation nor inhibition of GGT activity in surface protein thiols of GGT-poor 2/21 cells (not shown).

Interestingly, as can be seen in Fig. 3, the opposite effect, i.e. a significant *gain* in protein thiols of the cell surface was observed following the inhibition of *basal* GGT activity, indicating that the prooxidant species originated from the latter may continuously affect the redox status of cell surface proteins in these cells, even in the absence of any exogenous stimulation of GGT.

Whole cellular protein thiols were not affected by GGT stimulation, nor by GGT inhibition in neither subclone (not shown). Cellular levels of glutathione were also compared in the two cell clones, and were found to be significantly lower in 2/60 cells as compared to 2/21 cells (21.3±1.2 and 33.4±2.5 nmol/mg protein, respectively), while GSSG was comparable in the two clones.

Previous studies documented a role for GGT-derived H_2O_2 in the maintainance of cell proliferation rate of U937 lymphoma cells (Del Bello et al., 1999). Thus, the possibility was investigated that extracellular H_2O_2 produced by GGT might represent an additional stimulus for proliferation of Me665 melanoma cells. Indeed, the proliferation of GGT-rich 2/60 cells was markedly inhibited when catalase was added to the incubation medium, while GGT-poor 2/21 cells were totally unaffected (data not shown).

Thus, the expression of high levels of GGT subjects 2/60 cells to a continuous flow of reactive oxygen species. As it is known that prooxidants can modulate the redox status of critical molecular targets implicated in the signal transduction chain, additional experiments were dedicated to verify the possibility that a continuous production of H_2O_2 by GGT-rich 2/60 cells might interfere with the activation status of NF-κB, one of the best studied redox-sensitive transcription factors. Levels of free NF-κB protein were determined by immunoblot in nuclear extracts of the two cell clones studied. Constitutively higher levels of p65 protein were detected in nuclear extracts

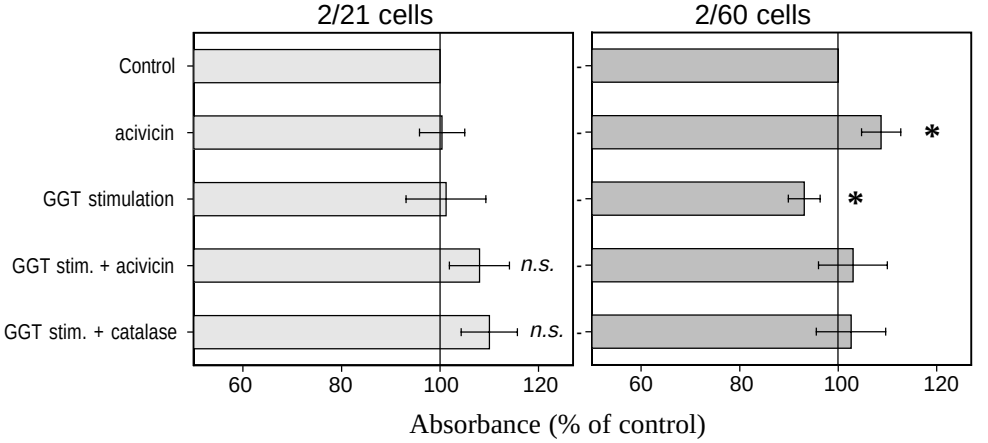

Fig. 3. GGT-dependent modulation of cell surface protein thiols. Protein thiols were determined by ELISA assay on cell monolayers adhering to multiwell microplates, after reaction with polymerized maleimide-activated peroxidase. GGT stimulation conditions were achieved by exposing cells to GSH and glycyl-glycine, 0.1 and 1 mM, respectively. Where indicated, incubations also included acivicin (150 μM, 60 minutes pretreatment, resulting in ≅85% GGT inhibition), or catalase (50 μg/ml). Results are means ± s.e.m. of at least 3 separate experiments. Statistical significance (P<0.05) was assessed by one-way ANOVA with Newman-Keuls test for multiple comparisons. (*) Significantly different from control; (n.s.) not significantly different from control.

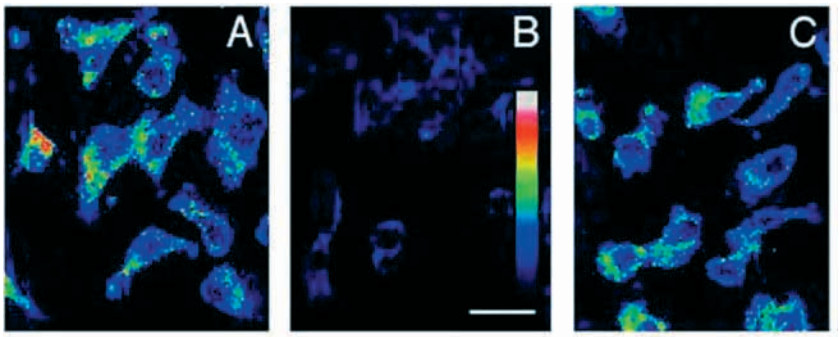

Fig. 4. GGT-dependent loss of cell surface protein thiols in melanoma Me665/2/60 cells. (A) Control 2/60 cells; (B) effects of GGT stimulation after addition of substrates GSH and glycyl-glycine (0.1 and 1 mM, respectively; 45 minutes): cell surface protein thiols are markedly decreased; (C) same incubation conditions as in B, but in the presence of the GGT inhibitor serine/boric acid complex (10/10 mM; GGT residual activity <7%): loss of protein thiols is prevented. Confocal imaging of fluorescence intensities is displayed in false colours (see colour scale bar in B). Unfixed cell monolayers were reacted with the cell-impermeable thiol-specific probe 3-(*N*-maleimidylpropionyl) biocytin (MPB), followed by fluoresceinated extravidin. Laser-scanning fluorescence microscopy was performed with a Bio-Rad MRC-500 confocal imaging system, equipped with an argon ion laser. Bar, 20 μm.

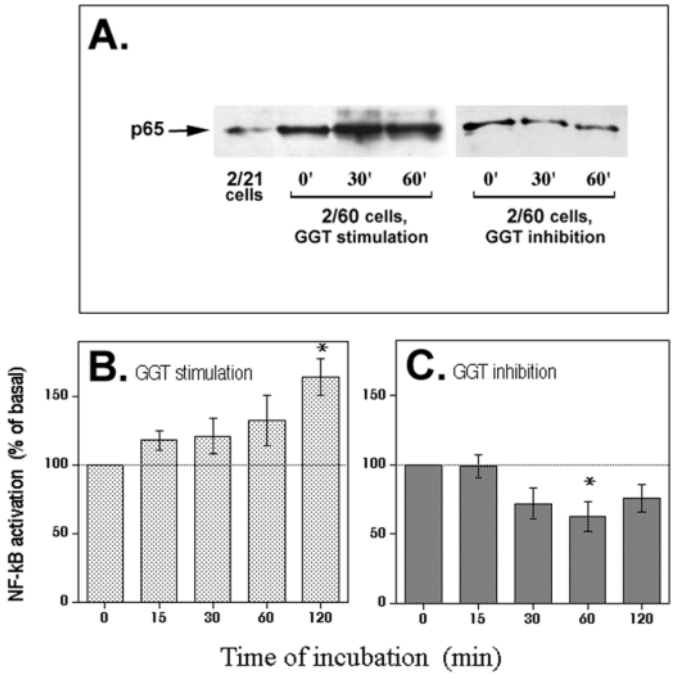

Fig. 5. GGT-dependent activation of NF-κB (nuclear translocation of p65 protein) in Me665/2 cells. For GGT stimulation, cells were exposed for the indicated times to GGT substrates, GSH (0.2 mM) and gamma-glutamyl acceptor glycyl-glycine (2 mM). For GGT inhibition, incubations were carried out in the presence of the specific competitive inhibitor, SBC (10/10 mM). (A) Western blots of proteins of nuclear extracts, performed using anti-p65 monoclonal antibodies and developed by an enhanced chemiluminescence system. Two typical experiments representative of 3-5 are shown. (B,C) Semi-automatic densitometric integration of p65 bands. Values shown are means ± s.e.m. of 3-5 separate experiments. (*) Significantly different (P<0.05) from 0-time value.

of 2/60 cells, as compared to GGT-poor 2/21 cells (Fig. 5), indicating a higher degree of NFkB activation. Moreover, in 2/60 cells nuclear translocation of p65 was increased in conditions of stimulated GGT activity, and progressively decreased in cells exposed to the competitive GGT inhibitor SBC.

Since p53 has been proposed to be involved in cellular response to oxidative stress, we also examined whether GGT stimulation or inhibition was capable of modulating *p53* expression. Although RT-PCR analysis indicated the expression of p53 in both clones, western blot analysis showed no protein expression in the 2/60 clone (not shown). The reasons of this discrepancy are not known. A marked *p53* expression was found in 2/21 cells. When GGT activity was inhibited by acivicin or stimulated with GSH and glycil-glycine, no modulation of *p53* expression was found (not shown). Sequencing analysis indicated that the *p53* gene product in the 2/21 clone was mutated at exon 7, codon 236: TAC (Tyr)→CAC (His). Thus, both clones lack a functional *p53*, suggesting that *p53* is not a critical determinant of oxidative stress response in this cellular context.

DISCUSSION

Data reported in the present study document that GGT present on plasma membrane of melanoma Me665/2/60 cells gives rise to production of significant amounts of hydrogen peroxide, as a by-product of the metabolism of extracellular GSH. This phenomenon was characterized in previous work from our laboratory, in which it was shown that GGT of U937 histiocytic lymphoma cells can produce H_2O_2 and other free radical species, leading to significant alterations of the cell proliferation capacity (Dominici et al., 1999; Del Bello et al., 1999). The observation that comparable H_2O_2 amounts can originate from GGT of a different tumor cell line, Me665/2/60 cells, supports thus the possibility that such a prooxidant function of GGT activity may represent a general feature of this enzyme. In this perspective, it is conceivable that GGT-mediated prooxidant reactions could be a general feature of those human malignant neoplasms in which high levels of GGT activity have been documented to occur.

Stimulation of GGT activity was used in the present study as a means of maximizing GGT-dependent prooxidant reactions, as opposed to conditions of GGT inhibition by acivicin or SBC. To this aim, our experimental conditions in vitro included relatively high concentrations of substrates (GSH, glycyl-glycine), chosen basing on the described K_m values (Huseby and Strömme, 1974). The question indeed arises whether conditions can occur in vivo for a GGT activity to continuously produce H_2O_2 in significant concentrations – although conceivably lower than those reported in the present study. As far as the role of γ-glutamyl acceptor, which was sustained by glycyl-glycine in our experimental conditions, this can be played in vivo by a number of different amino acids and small peptides, including GSH itself and its metabolites

(Tate and Meister, 1985). Concerning the availability of extracellular GSH, it has been documented that a continuous GSH efflux – through specific membrane transporters – takes place from mammalian cells (Sze et al., 1993), and is capable of mediating the efflux of as many as 0.02 to 0.07 nmol GSH/10^6 cells/minute. Such efflux of GSH is present at significant levels in melanoma Me665 cells as well (A. Paolicchi et al., unpublished observations). In principle, these transport systems could maintain extracellular GSH much above the micromolar range, possibly generating concentrations in the range of those we have used in the present and previously published studies (Paolicchi et al., 1999). It has been observed that the continuous efflux of GSH – in association with its concomitant GGT-mediated metabolism – actually give rise to a continuous *'GSH cycling'* across the plasma membrane (Forman et al., 1997). GGT activity can in fact be considered as the main activity for plasma GSH concentrations being kept so low in vivo*:* genetic deficiency (Schulman et al., 1975) or in vivo inhibition of GGT (Griffith and Meister, 1979) are accompanied by a substantial increase in circulating levels of GSH, and with the urinary excretion of *millimolar* amounts of GSH. Basing on these considerations, it is thus likely that GGT-dependent H_2O_2 production and other prooxidant reactions can take place spontaneously in in vivo conditions as well. The fact that GGT-rich 2/60 cells presented with significantly lower intracellular levels of GSH is in favour of this interpretation. In addition, GGT inhibition resulted in a shift of cell surface protein thiols towards a more reduced state (cf. Fig. 3), which seems to indicate that in 2/60 cells GGT is responsible of a continuous, basal oxidant stress.

Lower but anyway sizable H_2O_2 production was also observed in the GGT-poor 2/21 cells, and H_2O_2 production was present in GGT-rich 2/60 cells after specific inhibition of GGT (cf. Fig. 2), indicating the existence of GGT-independent sources of H_2O_2. Actually, production of reactive oxygen species is a well known phenomenon in tumor cells (Toyokuni et al., 1995; Szatrowski and Nathan, 1991), including melanoma (Bittinger et al., 1998), in which it has been attributed to prooxidant reactions taking place during melanogenesis (Nappi and Vass, 1996). However, the implication of other mechanisms such as mitochondrial uncoupling or NADPH-oxidase systems (Toyokuni et al., 1995), or thiol oxidase activity (Lash and Jones, 1986) cannot be excluded.

Prooxidants have long been known to exert cytotoxic effects, through interactions with critical oxidizable macromolecular targets. On the other hand, there is strong evidence that – at significantly lower concentrations – prooxidants can have a physiological role as well, by modulating the activation status of several proteins involved throughout the signal transduction pathways, including growth factor receptors, protein kinases and transcription factors (Lander, 1997; Suzuki et al., 1997; Monteiro and Stern, 1996). Several studies have addressed the effects of such modulations on the cellular balance between proliferation and susceptibility to apoptosis. In particular, H_2O_2 has been repeatedly shown to stimulate proliferation of different cell types, when exogenously added in low μM concentrations to the culture media (Nose et al., 1991; Gallagher et al., 1993; Burdon, 1995; Herbert et al., 1996). Low levels of H_2O_2 and other prooxidants have also been shown to induce resistance to several apoptogenic stimuli (Maellaro et al., 1996; Genaro et al., 1995; Clément and Stamenkovic, 1996). Against this background, it should be emphasized that the GGT-dependent production of H_2O_2 actually represents an *endogenous*, as yet unrecognized source, capable under some circumstances of supplying GGT-rich cells with additional amounts of H_2O_2. It is by now established that the latter can act as sort of a 'life signal' for the sustainment of cell proliferation and possibly protection from apoptosis, as previously reported by us in U937 histiocytic lymphoma cells (Del Bello et al., 1999). The observation that proliferation of 2/60 cells was inhibited when extracellular H_2O_2 was removed with catalase could suggest that endogenously produced H_2O_2 acts as a proliferative stimulus also in the GGT-rich Me665/2/60 clone. H_2O_2 could in this way contribute to growth factor independence of melanoma cells (Rodeck, 1993), playing the role of an additional, autocrine 'growth factor'.

Concerning the biochemical mechanisms underlying the prooxidant effects of GGT activity, our results suggest that these could be mediated through modulation of the thiol redox status of critical macromolecules. Indeed, GGT activity was observed to result in oxidation of cell surface protein thiols (Figs 3 and 4). Such observation is not surprising, for oxidation of thiols by H_2O_2 is well established (Radi et al., 1991; Seres et al., 1996), and GGT-dependent generation of H_2O_2 and free radicals takes place extracellularly (Dominici et al., 1999), its active site being oriented towards the outer portion of the plasma membrane. Importantly, the sole inhibition of the enzyme in 2/60 cells was sufficient to induce an increase in the amount of reduced protein thiols detectable at the cell surface (cf. Fig. 3), indicating that GGT-dependent prooxidant action may be continuously operative in maintaining protein thiols in a partially oxidized status. This observation is of considerable potential importance, insofar as it has been documented that oxidative alterations in critical cysteine residues in receptor proteins at the cell surface can modify the ligand binding affinity and the activation status of important growth factor receptors, such as EGFR (Clark and Konstantopoulos, 1993; Huang et al., 1996), b-FGFR (Herbert et al., 1996) and insulin receptor (Schmid et al., 1998). Such processes could interfere with the cellular responsivity to exogenous proliferative signals, thus providing one possible explanation for the observed inhibitory effects of catalase on the proliferation of 2/60 cells.

An alternative mechanism for the observed GGT-dependent oxidation of protein thiols should be also mentioned, i.e. the so called 'GSH-oxidase' activity of GGT. It was shown in fact that variable amounts of oxidized glutathione (GSSG) are produced during GGT enzymatic activity (Griffith and Tate, 1980); and formation of protein mixed disulfides mediated by GSSG ('protein-S-glutathiolation') has been described in several experimental models (Thomas et al., 1994). However, this aspect was not investigated in the present study.

GGT expression is known to be a marker of neoplastic progression in several experimental models, such as rodent skin and liver chemical carcinogenesis (Warren et al., 1993). In melanomas, GGT is often expressed in significant levels (Murray et al., 1982), and in particular, in different clones of Me665/2 melanoma cells the degree of GGT expression was found to correlate with the metastatic potential, as estimated from invasiveness and migration experiments in vitro. As far as the cell lines selected for the present study, they actually

represent the two opposite ends in the spectrum of GGT expression exhibited by six Me665 cell clones, 2/21 and 2/60 cells presenting with the lowest and the highest degree, respectively, of GGT activity, invasiveness and migration (Supino et al., 1992).

The coexistence of high GGT levels and high invasion/migration ability in 2/60 cells raises the possibility that the described GGT-mediated prooxidant reactions might concur to determine the higher malignancy of 2/60 cells as compared to GGT-poor 2/21 cells. GGT-mediated extracellular H_2O_2 production might e.g. participate in the endothelial damage, which is regarded as a necessary step in the establishment of metastasis (Bittinger et al., 1998). On the other hand, other important effects could well involve critical targets in the intracellular signal transduction cascade. Among several possible redox-sensitive targets, GGT-derived H_2O_2 could modulate the activation status of the transcription factor NF-κB (Flohé et al., 1997). Endogenous activation of NF-κB was recently shown to occur in melanoma cells due to post-translational modification of the inhibitory element IκB (Shattuck-Brandt and Richmond, 1997), an effect known to result from oxidative processes. The present study actually documents that in 2/60 cells a higher constitutive activation of NF-κB exists, and that this is likely the result of a constitutive prooxidant status induced in these cells by their high GGT activity. This interpretation is strengthened by the observation that stimulation or inhibition of GGT activity in 2/60 cells indeed resulted in stimulation or inhibition of nuclear translocation of p65, respectively (Fig. 5). Activation of NF-κB could play a major role in determining the reported higher malignancy of 2/60 cells as compared to their GGT-poor counterparts. Several studies point in fact to the particular relevance of NF-κB activation in the malignant behaviour of cancer cells, due to its involvement in the expression of several gene products participating to cancer invasion. Indeed, antisense inhibition of NF-κB has been shown to inhibit tumorigenicity in nude mice injected with tumor-derived cell lines (Higgins et al., 1993).

In conclusion, the present study provides evidence for a role of GGT activity in the production of hydrogen peroxide by melanoma cells, and for the involvement of the latter in the regulation of critical protein thiols of the cell surface and other intracellular targets, such as the transcritpion factor NF-κB. These observations might open a novel therapeutic window, basing on the possibility of modifying cellular GGT activity by a variety of chemical and molecular means.

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