

Membrane gamma-glutamyl transpeptidase activity of melanoma cells: effects on cellular H₂O₂ production, cell surface protein thiol oxidation and NF-κB activation status

Emilia Maellaro¹, Silvia Dominici¹, Barbara Del Bello¹, Marta A. Valentini¹, Lisa Pieri¹, Paola Perego², Rosanna Supino³, Franco Zunino², Evelina Lorenzini³, Aldo Paolicchi³, Mario Comporti¹ and Alfonso Pompella^{1,*}

¹Department of Pathophysiology & Experimental Medicine, University of Siena, Italy

²Istituto Nazionale Tumori, Milan, Italy

³Department of Experimental Pathology, University of Pisa, Italy

*Author for correspondence (e-mail: apompella@unisi.it)

Accepted 18 May; published on WWW 10 July 2000

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; Drozd 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% CO₂, in a humidified atmosphere. 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 1×10⁶ 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 MgCl₂ 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

H₂O₂ 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 H₂O₂. Cells suspended in RPMI 1640 medium were seeded in 6-well plates (5×10⁵ 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 CaCl₂, 1.2 mM MgSO₄ 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 H₂O₂ 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 H₂O₂ additions in the concentration range 0.1–5 µM, showing a linear fluorimetric response, were used for calculations. H₂O₂ 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 (5×10⁴ 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 H₂O₂ 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 H₂O₂ production by 2/60 cells was confirmed by the fact that the production of H₂O₂ was reverted to basal levels when GGT activity was almost completely suppressed 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 H₂O₂ 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 H₂O₂ 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

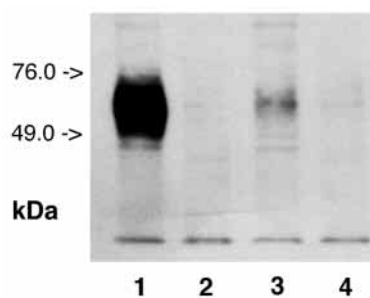


Fig. 1. Western blot of GGT protein heavy chain in membrane and cytosol fractions of Me665/2/21 and 2/60 melanoma cells. Purified polyclonal antibodies against a 20-amino acid peptide belonging to GGT heavy chain were employed. Blots were developed by enhanced chemiluminescence. Lanes 1 and 2: clone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

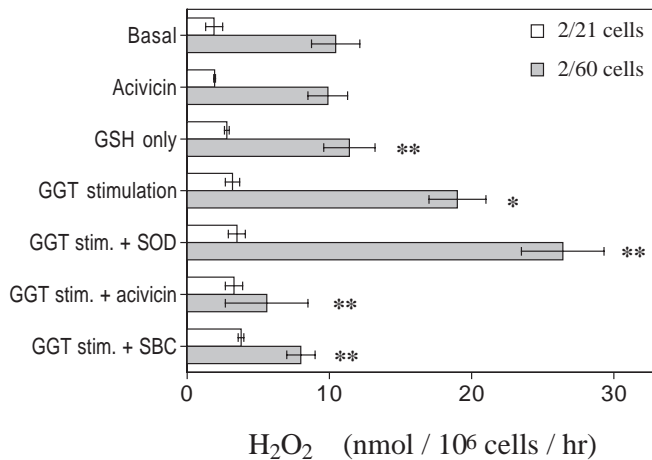


Fig. 2. GGT-dependent and -independent H₂O₂ production in Me665/2 melanoma cells. H₂O₂ production was determined fluorimetrically, by the HRP/A6550 system. 'Basal' values were determined in Krebs-Ringer buffer to which 2 mM glycyl-glycine and 5 μ M GSH were also added (see Materials and Methods for details). For 'GSH alone' determinations, a further 95 μ M GSH was added (final concn: 100 μ M). 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 H₂O₂ 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 μ g/ml, 500 μ M 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 \pm 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 H₂O₂ (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 \pm 1.2 and 33.4 \pm 2.5 nmol/mg protein, respectively), while GSSG was comparable in the two clones.

Previous studies documented a role for GGT-derived H₂O₂ in the maintenance of cell proliferation rate of U937 lymphoma cells (Del Bello et al., 1999). Thus, the possibility was investigated that extracellular H₂O₂ 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 H₂O₂ by GGT-rich 2/60 cells might interfere with the activation status of NF- κ B, one of the best studied redox-sensitive transcription factors. Levels of free NF- κ B 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

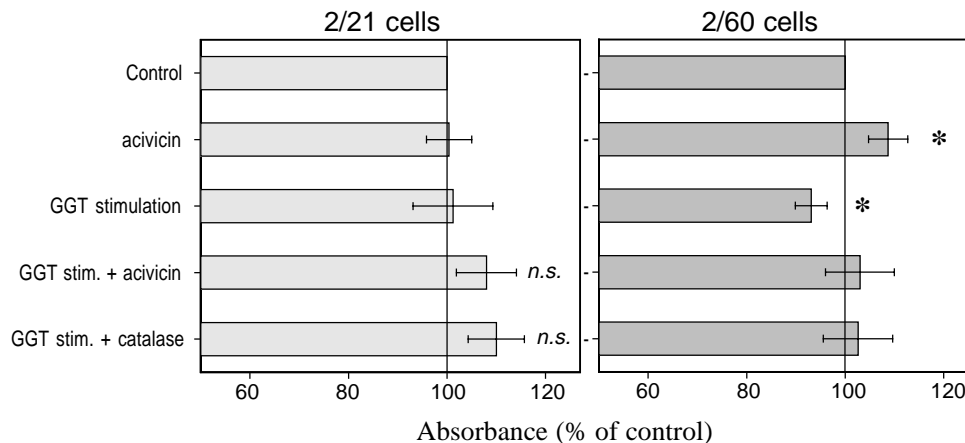


Fig. 3. GGT-dependent modulation of cell surface protein thiols. Protein thiols were determined by ELISA assay on cell monolayers adhering to multiwell microplates, after reaction with polymerized maleimide-activated peroxidase. GGT stimulation conditions were achieved by exposing cells to GSH and glycyl-glycine, 0.1 and 1 mM, respectively. Where indicated, incubations also included acivicin (150 μ M, 60 minutes pretreatment, resulting in \approx 85% GGT inhibition), or catalase (50 μ g/ml). Results are means \pm 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.

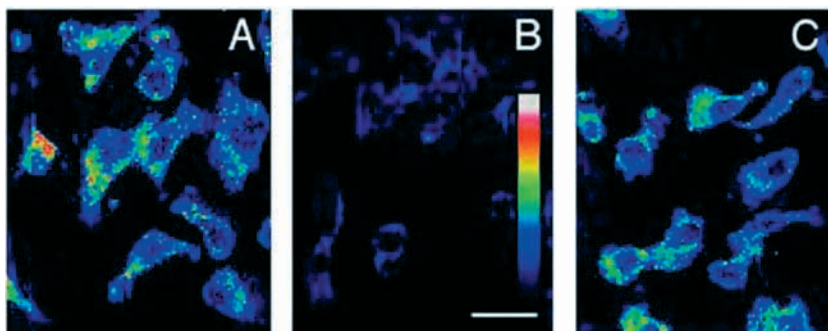


Fig. 4. GGT-dependent loss of cell surface protein thiols in melanoma Me665/2/60 cells. (A) Control 2/60 cells; (B) effects of GGT stimulation after addition of substrates GSH and glycyl-glycine (0.1 and 1 mM, respectively; 45 minutes): cell surface protein thiols are markedly decreased; (C) same incubation 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 μ m.

of 2/60 cells, as compared to GGT-poor 2/21 cells (Fig. 5), indicating a higher degree of NF κ B 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 glycyl-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) \rightarrow 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.

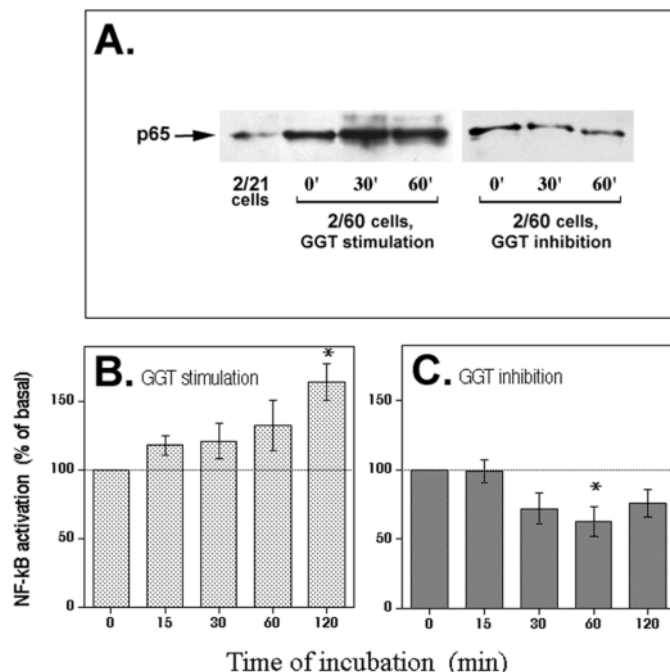


Fig. 5. GGT-dependent activation of NF- κ B (nuclear translocation of p65 protein) in Me665/2 cells. For GGT stimulation, cells were exposed for the indicated times to GGT substrates, GSH (0.2 mM) and gamma-glutamyl acceptor glycyl-glycine (2 mM). For GGT inhibition, incubations were carried out in the 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 \pm s.e.m. of 3-5 separate experiments. (*) Significantly different ($P < 0.05$) from 0-time value.

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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 responsiveness 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 H₂O₂ 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 H₂O₂ could modulate the activation status of the transcription factor NF- κ B (Flohé et al., 1997). Endogenous activation of NF- κ B was recently shown to occur in melanoma cells due to post-translational modification of the inhibitory element I κ B (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- κ B 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- κ B 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- κ B 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- κ B 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 transcription factor NF- κ B. 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.

The authors are indebted to Dr G. Parmiani (Istituto Nazionale Tumori, Milan) for making Me665/2 cells available for the present study. The support of the Associazione Italiana Ricerca sul Cancro (A.I.R.C., Italy) and the Association for International Cancer Research (A.I.C.R., UK) is gratefully acknowledged. Additional funds were derived from the Italian Ministries for Public Health and for University (MURST-Cofinanziamento 1998). The authors thank Dr L. Schiaffonati (University of Brescia, Italy) for useful suggestions.

REFERENCES

Andrews, N. C. and Faller, D. V. (1991). A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucl. Acids Res.* **19**, 2499-2504.

- Bittinger, F., González-García, J. L., Klein, C. L., Brochhausen, C., Offner, F. and Kirkpatrick, C. J. (1998). Production of superoxide by human malignant melanoma cells. *Melanoma Res.* **8**, 381-387.
- Blair, S. L., Heerdt, P., Sachar, S., Abolhoda, A., Hochwald, S., Cheng, H. and Burt, M. (1997). Glutathione metabolism in patients with non-small cell lung cancers. *Cancer Res.* **57**, 152-155.
- Burdon, R. H. (1995). Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Rad. Biol. Med.* **18**, 775-794.
- Clark, S. and Konstantopoulos, N. (1993). Sulfhydryl agents modulate insulin- and epidermal growth factor (EGF)-receptor kinase via reaction with intracellular receptor domains: differential effects on basal versus activated receptors. *Biochem. J.* **292**, 217-223.
- Clément, M.-V. and Stamenkovic, I. (1996). Superoxide anion is a natural inhibitor of Fas-mediated cell death. *EMBO J.* **15**, 216-225.
- Cotgreave, I. A. and Gerdes, R. G. (1998). Recent trends in glutathione biochemistry – Glutathione-protein interactions: a molecular link between oxidative stress and cell proliferation? *Biochem. Biophys. Res. Commun.* **242**, 1-9.
- Del Bello, B., Paolicchi, A., Comperti, M., Pompella, A. and Maellaro, E. (1999). Hydrogen peroxide produced during gamma-glutamyl transpeptidase activity is involved in prevention of apoptosis and maintenance of cell proliferation in U937 cells. *FASEB J.* **13**, 69-79.
- Dominici, S., Valentini, M., Maellaro, E., Del Bello, B., Paolicchi, A., Lorenzini, E., Tongiani, R., Comperti, M. and Pompella, A. (1999). Redox modulation of cell surface protein thiols in U937 lymphoma cells: the role of gamma-glutamyl transpeptidase-dependent H₂O₂ production and S-thiolation. *Free Rad. Biol. Med.* **27**, 623-635.
- Drozd, R., Parmentier, C., Hachad, H., Leroy, P., Siest, G. and Wellman, M. (1998). gamma-Glutamyltransferase dependent generation of reactive oxygen species from aglutathione/transferrin system. *Free Rad. Biol. Med.* **25**, 786-92.
- Flohé, L., Brigelius-Flohé, R., Saliou, C., Traber, M. G. and Packer, L. (1997). Redox regulation of NF-kappa B activation. *Free Rad. Biol. Med.* **22**, 1115-1126.
- Forman, H. J., Liu, R.-M. and Tian, L. (1997). Glutathione cycling in oxidative stress. *Lung Biol. Health Dis.* **105**, 99-121.
- Gallagher, D. L., Betteridge, L. J., Patel, M. K. and Schachter, M. (1993). Effect of oxidants on vascular smooth muscle proliferation. *Biochem. Soc. Trans.* **21**, 98S.
- Genaro, A. M., Hortelano, S., Alvarez, A., Martínez-A., C. and Boscà, L. (1995). Splenic B lymphocyte programmed cell death is prevented by nitric oxide release through mechanisms involving sustained Bcl-2 levels. *J. Clin. Invest.* **95**, 1884-1890.
- Griffith, O. W. and Meister A. (1979). Translocation of intracellular glutathione to membrane bound gamma-glutamyl transpeptidase as a discrete step in the gamma-glutamyl cycle: glutathionuria after inhibition of the transpeptidase. *Proc. Nat. Acad. Sci. USA* **76**, 268-279.
- Griffith, O. W. and Tate, S. S. (1980). The apparent glutathione-oxidase activity of γ -glutamyl transpeptidase. *J. Biol. Chem.* **255**, 5011-5014.
- Grisk, O., Küster, U. and Ansörge, S. (1993). The activity of gamma-glutamyl transpeptidase (γ -GT) in populations of mononuclear cells from human peripheral blood. *Biol. Chem. Hoppe-Seyler* **374**, 287-290.
- Halliwell, B. and Gutteridge, J. M. C. (1989). *Free Radicals in Biology and Medicine* (2nd edn). Clarendon Press, Oxford.
- Hanigan, M. H. and Frierson, H. F. Jr (1996). Immunohistochemical detection of gamma-glutamyl transpeptidase in normal human tissue. *J. Histochem. Cytochem.* **44**, 1101-1108.
- Herbert, J. M., Bono, F. and Savi, P. (1996). The mitogenic effect of H₂O₂ for vascular smooth muscle cells is mediated by an increase of the affinity of basic fibroblast growth factor for its receptor. *FEBS Lett.* **395**, 43-47.
- Higgins, K. A., Perez, J. R., Coleman, T. A., Dorshkind, K., McComas, W. A., Sarmiento, U. M., Rosen, C. A. and Narayanan, R. (1993). Antisense inhibition of the p65 subunit of NF- κ B blocks tumorigenicity and causes tumor regression. *Proc. Nat. Acad. Sci. USA* **90**, 9901-9905.
- Hochwald, S. N., Harrison, L. E., Rose, D. M., Anderson, M. and Burt, M. E. (1997). Elevation of glutathione and related enzyme activities in high-grade and metastatic extremity soft tissue sarcoma. *Ann. Surg. Oncol.* **4**, 303-309.
- Huang, R. P., Wu, J. X., Fan, Y. and Adamson, E. D. (1996). UV activates growth factor receptors via reactive oxygen intermediates. *J. Cell Biol.* **133**, 211-220.
- Huseby, N. E. and Strømme, J. H. (1974). Practical points regarding routine determination of gamma-glutamyl transferase (γ -GT) in serum with a kinetic method at 37°C. *Scand. J. Clin. Lab. Invest.* **34**, 357-361.

- Lander, H. M. (1997). An essential role for free radicals and derived species in signal transduction. *FASEB J.* **11**, 118-124.
- Lash, L. H. and Jones, D. P. (1986). Purification and properties of the membranal thiol oxidase from porcine kidney. *Arch. Biochem. Biophys.* **247**, 120-130.
- Maellaro, E., Del Bello, B. and Comporti, M. (1996). Protection by ascorbate against apoptosis of thymocytes: implications of ascorbate-induced nonlethal oxidative stress and poly(ADP-ribosylation). *Exp. Cell Res.* **226**, 105-113.
- Melezinek, I., Borovansky, J., Elleder, M. and Bubnova, E. (1998). Tumour tissue is a source of gamma-glutamyl transpeptidase sialoform in the sera of melanoma-bearing mice. *Melanoma Res.* **8**, 39-45.
- Mohanty, J. G., Jaffe, J. S., Shulman, E. S. and Raible, D. G. (1997). A highly sensitive fluorescent micro-assay of H₂O₂ release from activated human leukocytes using a dihydroxyphenoxazine derivative. *J. Immunol. Meth.* **202**, 133-141.
- Monteiro, H. P. and Stern, A. (1996). Redox modulation of tyrosine phosphorylation-dependent signal transduction pathways. *Free Rad. Biol. Med.* **21**, 323-333.
- Murata, J., Ricciardi-Castagnoli, P., Dessous L'Eglise Mange, P., Martin, F. and Juillerat-Jeanneret, L. (1997). Microglial cells induce cytotoxic effects toward colon carcinoma cells: measurement of tumor cytotoxicity with a gamma-glutamyl transpeptidase assay. *Int. J. Cancer* **70**, 69-174.
- Murray, J. L., Lerner, M. P. and Nordquist, R. E. (1982). Elevated gamma-glutamyl transpeptidase levels in malignant melanoma. *Cancer* **49**, 1439-1443.
- Nakamura, H., Nakamura, K. and Yodoi, J. (1997). Redox regulation of cellular activation. *Annu. Rev. Immunol.* **15**, 351-369.
- Nappi, A. J. and Vass, E. (1996). Hydrogen peroxide generation associated with the oxidations of eumelanin precursors 5, 6-dihydroxyindole and 5, 6-dihydroxyindole-2-carboxylic acid. *Melanoma Res.* **6**, 341-349.
- Nose, K., Shibamura, M., Kikuchi, K., Kageyama, H., Sakiyama, S. and Kuroki, T. (1991). Transcriptional activation of early-response genes by hydrogen peroxide in a mouse osteoblastic cell line. *Eur. J. Biochem.* **201**, 99-106.
- Paolicchi, A., Pompella, A., Tonarelli, P., Gadducci, A., Genazzani, A. R., Zunino, F., Pratesi, G. and Tongiani, R. (1996). Gamma-glutamyl transpeptidase activity in human ovarian carcinoma. *Anticancer Res.* **16**, 3053-3058.
- Paolicchi, A., Tongiani, R., Tonarelli, P., Comporti, M. and Pompella, A. (1997). Gamma-glutamyl transpeptidase-dependent lipid peroxidation in isolated hepatocytes and HepG2 hepatoma cells. *Free Rad. Biol. Med.* **22**, 853-860.
- Paolicchi, A., Minotti, G., Tonarelli, P., Tongiani, R., De Cesare, D., Mezzetti, A., Dominici, S., Comporti, M. and Pompella, A. (1999). Gamma-glutamyl transpeptidase-dependent iron reduction and LDL oxidation – a potential mechanism in atherosclerosis. *J. Invest. Med.* **47**, 151-160.
- Perego, P., Giarola, M., Righetti, S. C., Supino, R., Caserini, C., Delia, D., Pierotti, M. A., Miyashita, T., Reed, J. C. and Zunino, F. (1996). Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. *Cancer Res.* **56**, 556-562.
- Perego, P., Paolicchi, A., Tongiani, R., Pompella, A., Tonarelli, P., Carenini, N., Romanelli, S. and Zunino, F. (1997). The cell-specific anti-proliferative effect of reduced glutathione is mediated by γ -glutamyl transpeptidase-dependent extracellular pro-oxidant reactions. *Int. J. Cancer* **71**, 246-250.
- Pompella, A., Romani, A., Benedetti, A. and Comporti, M. (1991). Loss of membrane protein thiols and lipid peroxidation in allyl alcohol hepatotoxicity. *Biochem. Pharmacol.* **41**, 1255-1259.
- Pompella, A., Cambiaggi, C., Dominici, S., Paolicchi, A., Tongiani, R. and Comporti, M. (1996). Single-cell investigation by laser scanning confocal microscopy of cytochemical alterations resulting from extracellular oxidant challenge. *Histochem. Cell Biol.* **105**, 173-178.
- Powis, G., Briehl, M. and Oblong, J. (1995). Redox signalling and the control of cell growth and death. *Pharmac. Ther.* **68**, 149-173.
- Radi, R., Beckman, J. S., Bush, K. M. and Freeman, B. A. (1991). Peroxynitrite oxidation of sulfhydryls – The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266**, 4244-4250.
- Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W. and Potter, D. W. (1980). High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal. Biochem.* **106**, 55-62.
- Rodeck, U. (1993). Growth factor independence and growth regulatory pathways in human melanoma development. *Cancer Metast. Rev.* **12**, 219-226.
- Schmid, E., El Benna, J., Galter, D., Klein, G. and Dröge, W. (1998). Redox priming of the insulin receptor β -chain associated with altered tyrosine kinase activity and insulin responsiveness in the absence of tyrosine autophosphorylation. *FASEB J.* **12**, 863-870.
- Schulman, J. D., Goodman, S. I., Mace, J. W., Patrick, A. D., Tietze, S. and Butler, E. J. (1975). Glutathionuria: inborn error of metabolism due to tissue deficiency of gamma-glutamyl transpeptidase. *Biochem. Biophys. Res. Commun.* **65**, 68-74.
- Sen, Ch. K. (1998). Redox signaling and the emerging therapeutic potential of thiol antioxidants. *Biochem. Pharmacol.* **55**, 1747-1758.
- Seres, T., Ravichandran, V., Moriguchi, T., Rokutan, K., Thomas, J. A. and Johnston, R. B. (1996). Protein S-thiolation and dethiolation during the respiratory burst in human monocytes – A reversible post-translational modification with potential for buffering the effects of oxidant stress. *J. Immunol.* **156**, 1973-1980.
- Shattuck-Brandt, R. L. and Richmond, A. (1997). Enhanced degradation of I- κ B alpha contributes to endogenous activation of NF- κ B in Hs294T melanoma cells. *Cancer Res.* **57**, 3032-3039.
- Stark, A.-A., Zeiger, E. and Pagano, D. A. (1993). Glutathione metabolism by γ -glutamyl transpeptidase leads to lipid peroxidation: characterization of the system and relevance to hepatocarcinogenesis. *Carcinogenesis* **14**, 183-189.
- Supino, R., Mapelli, E., Sanfilippo, O. and Silvestro, L. (1992). Biological and enzymatic features of human melanoma clones with different invasive potential. *Melanoma Res.* **2**, 377-384.
- Suzuki, Y. J., Forman, H. J. and Sevanian, A. (1997). Oxidants as stimulators of signal transduction. *Free Rad. Biol. Med.* **22**, 269-285.
- Szatrowski, T. P. and Nathan, C. F. (1991). Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* **51**, 794-798.
- Sze, G., Kaplowitz, N., Ookhtens, M. and Lu, S. C. (1993). Bidirectional membrane transport of intact glutathione in HepG2 cells. *Am. J. Physiol.* **28**, G1128-G1134.
- Täger, M., Ittenson, A., Franke, A., Frey, A., Gassen, H. G. and Ansoerge, S. (1995). γ -Glutamyl transpeptidase – cellular expression in populations of normal human mononuclear cells and patients suffering from leukemias. *Ann. Hematol.* **70**, 237-242.
- Tate, S. S. and Meister, A. (1985). γ -Glutamyl transpeptidase from kidney. *Meth. Enzymol.* **113**, 400-419.
- Tew, K. D., Monks, A., Barone, L., Rosser, D., Akerman, G., Montali, J. A., Wheatmley, J. B. and Schmidt, D. E. Jr (1996). Glutathione-associated enzymes in the human cell lines of the National Cancer Institute Drug Screening Program. *Mol. Pharmacol.* **50**, 149-159.
- Thomas, J. A., Chai, Y.-C. and Jung, C.-H. (1994). Protein S-thiolation and dethiolation. *Meth. Enzymol.* **233**, 385-395.
- Toyokuni, S., Okamoto, K., Yodoi, J. and Hiai, H. (1995). Persistent oxidative stress in cancer. *FEBS Lett.* **358**, 1-3.
- Tsutsumi, M., Sakamuro, D., Takada, A., Zang, S. C., Furukawa, T. and Taniguchi, N. (1996). Detection of a unique gamma-glutamyl transpeptidase messenger RNA species closely related to the development of hepatocellular carcinoma in humans: a new candidate for early diagnosis of hepatocellular carcinoma. *Hepatology* **23**, 1093-1097.
- Warren, B. S., Naylor, M. F., Winberg, L. D., Yoshimi, N., Volpe, J. P., Gimenez-Conti, I. and Slaga, T. J. (1993). Induction and inhibition of tumor progression. *Proc. Soc. Exp. Biol. Med.* **202**, 9-15.