Endogenous production of reactive oxygen species by the NADPH oxidase complexes is a determinant of γ-glutamyltransferase expression

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ABSTRACT

γ-Glutamyltransferase (GGT) plays a significant role in antioxidant defence and participates in the metabolism of glutathione (GSH). The enzyme is upregulated after acute oxidative stress and during pro-oxidant periods, but the underlying regulatory mechanisms are not well known. In the present investigation we studied whether the endogenous reactive oxygen species (ROS) level was a determinant for GGT expression. A substantial amount of ROS is produced through the NADPH oxidase (NOX) system, and knockdown of p22phox, a subunit of NOX1-4, resulted not only in reduced ROS levels but also in reduced GGT expression in human endometrial carcinoma cells. Phorbol-12-myristate-13-acetate (PMA) is an activator of NOX, and we found that PMA treatment of human colon carcinoma cells both increased cellular ROS levels and subsequently upregulated GGT expression. On the other hand, the NOX inhibitor apocynin reduced ROS levels as well as GGT expression. The GGT mRNA subtype A was increased after PMA induced NOX activation. These results demonstrate that ROS generated from NOX enzymes are a significant determinant for GGT expression and activity.

KEY WORDS:

NADPH oxidase, reactive oxygen species, γ-glutamyltransferase, glutathione.

INTRODUCTION

Glutathione (GSH) has an important role in maintaining intracellular redox balance and acts as an antioxidant, protecting cells against oxidative stress, particularly by detoxifying peroxides and free radicals (for recent reviews, see [1, 2]). GSH is the most abundant non-protein thiol in cells, present at levels as high as 0.5-10 mM [3]. The synthesis of this tripeptide (γ-glutamyl-cysteinyl-glycine) is an intracellular process that is regulated through the activity of γ-glutamyl-cysteine ligase, which catalyses the first step in the GSH synthesis. A significant rate limiting factor for GSH biosynthesis is the availability of cysteine [4, 5].

The enzyme γ-glutamyltransferase (GGT) is a transmembrane protein with the enzymatic domain located extracellularly [6, 7]. GGT initiates the breakdown of extracellular GSH by hydrolysing the γ-glutamyl-cysteinyl bond whereas the remainder, the cysteinyl-glycine moiety, is subsequently hydrolysed by peptidases. The action of GGT and the uptake of the resulting amino acids will provide cells with the monomers needed for intracellular GSH biosynthesis. As most cells are unable to take up extracellular GSH, GGT plays an important role in GSH salvage and homeostasis, particularly at low cyst(e)ine concentrations [8, 9]. The intracellular level of GSH is significantly reduced when cells are grown in cystine-depleted medium, which leads to oxidative stress and apoptosis [2, 5, 10] . When supplementing the medium with GSH the intracellular GSH is maintained due to the activity of GGT. Thus, GGT acts as a glutathionase and protects the cells from GSH depletion and oxidation-induced cell death [8-11].

GGT is expressed at high levels in several types of cancer cells [12] and has been used as a marker of experimental carcinogenesis [13]. In studies on metastatic growth of melanoma cells in mouse liver, cells with higher levels of GGT showed increased invasive growth [9]. GGT expression is induced by oxidative stress and by several anticancer drugs, and the enzyme has been suggested to have a protective role against apoptosis [11, 14-16]. We have earlier reported that GGT expression is upregulated after acute oxidative stress through Ras and several downstream signalling pathways [17, 18]. Such increased expression of the enzyme might be necessary to reach sufficient cysteine/GSH levels and thus to counteract the acute high level of reactive oxygen species (ROS).

Human GGT is encoded by a multigene family of at least seven genes or pseudogenes, of which one gene (GGT1) encodes for the active enzyme detectable in human tissues [19, 20]. The transcription is controlled by several tandemly organised promoters. Five mRNA subtypes have been described which exhibit different 5'-UTRs but identical coding regions [21]. A differential regulation of these subtypes was reported [22], and one of these (type C) is activated by NF-κB and Sp1 during TNF-α induced GGT expression [23].

ROS, such as H_2O_2, are recognized as signalling molecules, with significant effects on tumor progression [24-26]. At low concentrations, ROS act as intracellular second messengers affecting proliferation, differentiation and cell death. Endogenous ROS production by the NADPH oxidase (NOX) systems is tightly regulated. NOX is a multicomponent family of oxidases, consisting of seven members that can be classified

into three distinct groups: NOX1-4, NOX5 and DUOX1-2 [27, 28]. The main phagocytic NOX complex is composed of the well-conserved catalytic domain gp91phox (also known as NOX2) and the smaller subunit p22phox that are embedded in the plasma membrane, and four cytosolic regulators, p47phox, p40phox, p67phox and Rac1. Upon activation, the cytosolic components associate with the membrane-bound NOX2-p22phox complex, and superoxide is produced. NOX1, 3 and 4 are homologous to gp91phox/NOX2 and all require p22phox for their activity [27-30]. Several NOX activators are known, including the protein kinase C agonist phorbol myristate acetate (PMA) [31].

As oxidative stress is reported to affect expression and activation of GGT, we hypothesized that increased endogenous production of ROS through the NOX system could have similar effects. In the present study we therefore investigated the role of NOX generated ROS on the regulation of GGT expression.

MATERIALS AND METHODS

Cell lines and culture conditions

HT-29 is a human colon carcinoma cell line undifferentiated at normal cell growth conditions and was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). The Ishikawa cell line is a well differentiated human endometrial adenocarcinoma and was obtained from Sigma-Aldrich, St.Louis, MO, USA. Both cell lines were cultured in RPMI-1640 medium containing L-glutamine (Sigma-Aldrich, St.Louis, MO, USA) with 10% fetal calf serum (BIOCHROM AG, Berlin, Germany) in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were replated by trypsinization each 4-6th day. Trypsin-EDTA was purchased from LONZA, Belgium.

Antibodies

A primary antibody against GGT was generated in rabbits using a synthetic polypeptide corresponding to the C-terminal human GGT heavy chain (DDTTHPISYYKPEFYTPDDGG-OH) as antigen (Biotrend Chemikalien GmbH, Cologne, Germany). Specific antibodies were affinity purified using the same peptide coupled to CNBr-activated Sepharose 4B (GE Healthcare, Fairfield, CT, USA) and eluted with 100 mM glycine pH 2.5. Tris-HCl (1 M, pH 8.0) was added to the antibody solution until a pH of 7.4 was reached. The rabbit anti-p22phox antibody FL-195 (SC:20781) was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). HRP-linked anti-rabbit secondary antibody (#A0545) and the HRP-conjugated anti-β-actin antibody (#A3854) were obtained from Sigma-Aldrich

(Sigma-Aldrich, St. Louis, MO, USA). The anti-biotin antibody (#7727) was purchased from Cell Signaling Technology (Cell Signaling Technology, Danvers MA, USA).

RNA extraction

Total RNA was extracted from cell lines using RNeasy Plus Mini kit from Qiagen (Qiagen, Hilden, Germany). On-column DNase treatment of the RNA samples was performed using RNase-Free DNase (Qiagen, Hilden, Germany) according to the product manual. Quantity and purity of the extracted RNA was determined using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Reverse transcription PCR

The mRNA expression levels in the cell lines were quantified by reverse transcription quantitative PCR (RT-qPCR) performed on a Stratagene Mx3000P instrument (Stratagene, La Jolla, CA, USA) and the mRNA subtypes A-C in HT-29 cells were semiquantitatively estimated using RT-PCR on Gene Amp PCR System 9700 (version 2.25, Applied Biosystems, Life Technologies, USA). Reverse transcription of total RNA was performed using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) with 1 µg of RNA per 20 µl cDNA reaction. For real-time estimations, cDNA corresponding to 9 ng RNA was amplified for 40 cycles in a 25 µl PCR mix (RT^2 SYBR® Green/ROX PCR Master Mix (SA Biosciences, Frederick, MD, USA)) containing a final concentration of 240 nM (for p22phox, GAPDH and ACT-B) and 400 nM (for GGT1) of each primer. Cycling conditions: 95 ˚C for 10 min, 40 cycles at 95 ˚C for 30 sec, and 55 ˚C for 1 min, 72 ˚C for 30 sec, 1 cycle at 95 ˚C for 1 min. All primers are listed in Table 1. Primer specificities and absence of primer dimers were determined by SYBR green melting curve analysis. Duplicate reverse transcriptase

reactions were performed for each RNA sample, and duplicate PCR analyses were performed on each cDNA sample. The absence of genomic DNA was confirmed by performing a no reverse transcriptase (NoRT) control for every RNA sample, and absence of contaminations was assessed by including a no template control (NTC) in every run. The ΔΔCt method [32] was used to determine the relative amount of target gene, normalizing against the average expression of the two reference genes *GAPDH* and *ACT-B.*

For estimation of GGT mRNA subtypes, cDNA corresponding to 9 ng RNA was amplified in 25 µl PCR mix (JumpStart REDTaq ReadyMix, Sigma-Aldrich, St.Louis, MO, USA), with the following cycling conditions: 92 ^{o}C for 2 min, 35 cycles at 92 ^{o}C for 30 sec, 56 ^{o}C for 30 sec, and 72 ^{o}C for 30 sec, followed by 72 ^{o}C for 10 min. As an internal control, amplification of GADPH was performed using the same cycling conditions for 22 cycles. Primer sequences have been described earlier [33, 34]. PCR products were visualized by ethidium bromide staining after electrophoresis. The sizes of the PCR products are: GGT subtype A (fetal liver) – 308bp, subtype B (HepG2) – 300bp, and subtype C (placenta) – 386 bp; GADPH – 222 bp [33, 34].

Western blot

Cell lysates of Ishikawa and HT-29 cells (10^4 cells/µl) were prepared using NuPAGE LDS Sample buffer (Invitrogen, Carlsbad, CA, USA), and subjected to reduced SDS-PAGE (NuPAGE Novex 4-12% Bis-Tris gels, Invitrogen, Carlsbad, CA). A biotinylated protein ladder (Cell Signaling Technology, Danvers, MA, USA) was used as a molecular weight marker. Samples were transferred onto PVDF membranes (Millipore, Billerica, MA), blocked for 1 hour in 5 % skim milk in TBS-T (150 mM

NaCl, 0.25% Tween-20, 20 mM Tris-HCl, pH 7.4) at room temperature for 1 hour, and incubated overnight at 4˚C with primary antibodies against GGT (1:200 dilution) or p22phox (FL-195) (1:250 dilution). Membranes were subsequently washed in TBS-T, and incubated with HRP-linked anti-rabbit secondary antibody (1:5000 dilution) and anti-biotin antibody (1:500 dilution). As a loading control, blots were probed with HRP-conjugated anti-β-actin antibody (1:40000 dilution). The Western Blotting Luminol Reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used for detection by a Fujifilm LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Enzyme activity measurements

For GGT activity measurements, the cells were harvested by trypsinization and solubilized at a concentration of 2 x 10^6 cells/200 µl in phosphate buffered saline (PBS) with 1% Triton X-100. The supernatant was collected after a brief centrifugation (5 min at 5000 x g) and GGT activity measurement was performed at 37^{o}C using a commercial kit (ABX Pentra GGT-CP, Horiba Group, California, USA) with L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine as substrates. The activity was expressed as units pr gram protein in cell lysates, U/g. Protein concentrations were measured using the BioRad DC protein assay kit (BioRad Laboratories, Oslo, Norway).

ROS measurements

Intracellular ROS was determined using the CM-H_2DCFDA probe (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and flow cytometry. When oxidised, the probe is detected by fluorescence with excitation at 485 nm and emission at 515 nm. Viable cells (0.5x10^6) were seeded in 6 well cell culture plates with 2 ml of complete growth medium and incubated overnight at 37°C, 5% CO_2. The next day, the cells were washed

2 times with Hanks Balanced Salt Solution with $CaCl_2$ and $MgCl_2$ (HBSS)(GIBCO, Invitrogen, Carlsbad, CA) and incubated for 30 min with 5 µM CM-H_2DCFDA in HBSS for HT-29 cells and 2.5 µM CM-H_2DCFDA in HBSS for Ishikawa cells. Subsequently the cells were trypsinized, resuspended in 0.5 ml HBSS and immediately analysed by flow cytometry (FACSAria, BD Biosciences). Ten thousand events were collected and analyzed by BD FACSDiva Version 5.0.2. The numbers presented are the median values of the fluorescent intensity.

PMA treatment

HT-29 cells were incubated for 24 h in growth medium supplemented with 100 nM PMA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich). The cystine concentration was kept low (50 µM) for 24 h prior to, during, and following the PMA treatment for GGT enzyme activity measurements. Control cells were treated with the same volume of DMSO as the PMA treated cells.

Apocynin treatment

HT-29 and Ishikawa cells were incubated for 4 days in growth medium supplemented with 0.5 mM apocynin (Calbiochem, Merck, Darmstadt, Germany) dissolved in DMSO. Fresh apocynin containing medium was added to the cells each day. Control cells were treated with the same volume of DMSO as the apocynin treated cells.

Knockdown of p22phox

The HIV-based lentiviral vector shRNA system from Open Biosystems (Thermo Fisher Scientific Inc, Waltham, MA, USA) was used for knockdown of p22phox in Ishikawa cells. The cells were transfected with the empty vector control pLKO.1 and two

different shRNA vectors for p22phox (named p22phox-78 and p22phox-81) using Lipofectamine LTX with Plus reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Single cell derived clones were isolated and expanded in complete growth medium supplemented with 0.6 µg/ml puromycin.

Validated p22-phox siRNA (h) (SC-36149) and Control siRNA-A (SC-37007), purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA), were transfected by Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA 92008, USA) into HT-29 cells according to the manufactures instructions, using 80 nM siRNA pr $0.15x10^6$ cells. The levels of p22-phox protein in control and siRNA treated cells were monitored 72 h after transfection by Western blotting using anti-p22-phox (FL-195) antibody (SC-20781, Santa Cruz) to verify the efficiency of reduction in p22-phox expression.

Statistical Analysis

Data are presented as mean±SEM. Statistical data were obtained by comparison of mean values using Student's *t*-test. Differences with $p < 0.05$ were considered significant.

RESULTS

Knockdown of p22phox expression reduced ROS production in Ishikawa cells.

The p22phox subunit has been found to be necessary for the activity of NOX1, 2, 3 and 4, while NOX5 is independent of p22phox [35]. Previous studies have shown that Ishikawa cells express NOX2, NOX4 and NOX5 (Ravuri, unpublished results). In order to lower the endogenous ROS levels generated by NOX2 and NOX4 in these cells, the p22phox subunit was knocked down by transfection of two different shRNA constructs targeting the p22phox mRNA. After transient transfection with the two shRNA plasmids (p22phox-78 and p22phox-81, see Methods), the p22phox level was significantly reduced when compared to cells transfected with the empty vector control (pLKO.1). This was shown both at the mRNA and protein levels demonstrating that both shRNA constructs were functional in reducing p22phox levels (Fig. 1).

Several stably transfected clones of the Ishikawa cell line that were isolated showed a clear reduction in p22phox mRNA and protein levels (Fig. 2A and B). Two clones were selected for further studies; one from each of the two shRNA constructs (p22phox-78(9) and p22phox-81(5)). To analyze the intracellular ROS production, the cells were loaded with the cell permeable ROS indicator CM-H_2DCFDA and analysed by flow cytometry. As shown in Fig. 2C, both p22phox knockdown clones showed reduced ROS levels compared to the control clone.

Downregulation of p22phox levels reduces the expression of GGT.

To investigate whether reduction in endogenous ROS would affect GGT, we analysed the GGT mRNA, protein and enzyme activity in the p22phox knockdown clones. As

shown in Fig. 3A-C, the GGT mRNA, protein and enzyme activity levels were reduced in the p22phox-78(9) and GGT activity and protein in p22phox-81(5) clones. The GGT mRNA expression in the 81(5) clones showed variable and not significant changes. We also found a reduction in GGT mRNA levels in transiently transfected cells (data not shown), demonstrating that the effect on GGT expression is detectable as soon as two days after p22phox downregulation. Taken together, this demonstrates that a reduction in ROS generated by NOX enzymes is a determinant for GGT expression and activity.

Activation of NOX with phorbol ester increases ROS production in HT-29 cells.

Treatment with the PKC activator phorbol myristate acetate (PMA) has previously been shown to activate NOX enzymes and subsequently ROS production [31]. To confirm that the increase in ROS levels after PMA stimulation was a result of NOX activity in the human colorectal adenocarcinoma cell line HT-29, two experiments were performed. First, the ROS level was measured in cells treated with PMA and the NOX inhibitor apocynin for 24 h. The results showed an increase in ROS after PMA treatment (Fig. 4A), and a slight decrease after apocynin treatment. Following the combined PMA and apocynin treatment, however, the ROS level was significantly reduced when compared to cells treated only with PMA (Fig. 4A). In the second experiment, knockdown of p22phox by siRNA resulted in a clear reduction of the p22phox protein level after 72 h when compared to cells transfected with the control siRNA (Fig. 4B). When these cells were then treated with PMA for 24 h significantly less ROS was measured in the siRNA p22phox transfected cells compared to the control cells (Fig. 4C). These experiments demonstrate that NOX enzymes are producing ROS as a result of PMA stimulation in HT-29 cells.

Activation of NOX with phorbol ester upregulates GGT and in particular the expression of GGT mRNA subtype A.

It has been reported that PMA treatment can upregulate GGT expression in HeLa cells [36]. We confirmed these findings using HT-29 cells and obtained a significant increase in GGT expression using 100 nM PMA for 24 h (Fig. 5A-C). Similar results were obtained with 500 nM PMA (data not shown). Thus, stimulation of HT-29 cells with PMA increases both intracellular ROS levels and GGT expression.

It has been suggested that three subtypes of GGT mRNA (A-C) account for the major part of GGT mRNA [22]. To see which of the GGT mRNA subtypes were regulated by PMA induced NOX activity, we analysed the mRNA subtype composition 24 h after PMA treatment. The theoretical size of the amplification products using subtype specific primers are 300 bp (subtype A), 308 bp (subtype B) and 386 bp (subtype C) [33]. As shown in Figure 5D, subtype A was increased in PMA stimulated cells compared to untreated cells, whereas subtypes B and C were slightly but not consistently altered.

Inhibiting NOX activity with apocynin reduces both ROS production and GGT expression in HT-29 cells.

To confirm that cellular GGT is affected by a reduction in NOX activity and a subsequent reduction in endogenous ROS production, HT-29 cells were incubated with the NOX inhibitor apocynin for 4 days. This treatment downregulated the expression of GGT, as seen both by reduced mRNA levels, protein levels, and enzyme activity (Fig. 6A-C). Furthermore, using flow cytometry we demonstrated a reduction in ROS levels after apocynin treatment in both HT-29 cells (Fig. 6D) and the endometrial adenocarcinoma cell line Ishikawa (data not shown).

DISCUSSION

GGT participates in maintaining GSH and cysteine homeostasis, and a significant role for the enzyme in antioxidant defence and inflammatory processes has been emphasized [37]. GGT-deficient mice revealed a disrupted GSH homeostasis, with cysteine deficiency and reduced tissue GSH, whereas serum and urine GSH were elevated [38]. These animals also showed signs of oxidative stress related injuries in the lungs [39]. Numerous cell experiments have shown that GGT is upregulated after acute oxidative stress using either exogenous oxidants such as H_2O_2 or menadione (a redox cycling quinone) or substances that initiate an intracellular generation of oxidants, such as androgens, TNF-α or PMA [18, 36, 40-42].

Endogenous ROS in cancer cells is largely produced by the NOX system and the mitochondria [28, 43]. The highly regulated NOX enzymes generate ROS not only for bactericidal activity, but also in response to signalling agents such as growth factors and cytokines [44, 45]. The phorbol ester PMA is a documented activator of NOX in various cells [31, 46]. Furthermore, PMA has been reported to upregulate GGT in HeLa cells [36]. In the present study we confirmed that PMA not only activated the NOX system and produced higher ROS levels in HT-29 cells, but also that this ROS production upregulated the expression of GGT. The reported necessity for low cystine concentrations in these experiments [36] may be linked to the reduced GSH levels demonstrated after PMA incubations. A need for higher cysteine/cystine uptake in these cells may then initiate an upregulation of GGT. In a previous study using rat colon carcinoma cells, we found a 50% reduction in intracellular GSH after 24 h of incubation in cystine depleted medium [10]. Intracellular GSH was normalized by adding GSH to

the medium, and we demonstrated that this was due to GGT-dependent cyst(e)ine uptake. In the present study using HT-29 cells the need for low cystine was not a critical factor for the increased production of ROS and the increased GGT mRNA expression. In fact, PMA incubations in normal medium also showed GGT upregulation (data not shown).

In this study, we saw a significant downregulation of intracellular ROS levels using shRNA against p22phox in Ishikawa cells Furthermore, reduction of p22phox resulted in reduced GGT expression, both after transient transfection (data not shown) and in several clones obtained after stable transfection. Correspondingly, when inactivating NOX in HT-29 cells by apocynin treatment for 3-4 days, we detected a significant reduction in ROS levels as well as in GGT expression. Thus, there is a clear link between NOX activity and GGT levels in both Ishikawa and HT-29 cells. Although the p22phox mRNA level was more reduced in the p22phox-81 clones compared to the p22phox-78 clones (Figure 2A), the p22phox-81(5) clone showed somewhat higher ROS and GGT levels than the p22phox-78(9) clone (Figure 2C and Figure 3A-C). This discrepancy may be explained by an increased level of NOX5 in the p22phox-81(5) clone compared to the p22phox-78(9) clone (data not shown). Thus, the total ROS in the p22phox-81(5) clone might be higher due to ROS production from several NOX members including NOX5.

Cancer cells are characterized by a higher endogenous ROS production when compared to normal, untransformed cells, and this is generated by increased NOX activity or release from mitochondria [16, 47, 48]. Such increased ROS levels appear to play an active role during increased malignancy, and during adaptation to oxidative stress,

chemoresistance and cell survival. Others have shown that downregulation of NOX activity and subsequent ROS signalling may result in reduced cellular invasion, angiogenesis and cell growth [29, 49]. In prostate cancer cells, elevated levels of ROS were reported to be responsible for malignant phenotypes and critical for migration and invasiveness [50]. Increased androgen levels in prostate cells were reported to elevate oxidative stress and alter the GSH and GGT levels in a prooxidant shift [16]. Several studies have demonstrated connections between Ras pathways and NOX activity (for recent reviews, see [28, 51]). Oncogenic Ras upregulates NOX1 expression and data implies that increased NOX1-derived ROS is necessary for oncogenic Ras transformation [52, 53]. NOX1 is overexpressed in human colon cancers, and has been shown to strongly correlate with activating mutations in K-Ras [26]. We have previously found that increased Ras activity upregulates GGT in HT-29 cells, and that several MAPK pathways were activated [17, 18]. The involvement of NOX activity in these processes has not been investigated.

Human GGT is regulated through several mRNA subtypes characterized by different 5'UTRs but with the same coding region. It has been suggested that these 5'UTRs play an important role in regulation of GGT translation [54]. In an investigation on four different cell lines, it was shown that different treatments resulted in different modulations of the three subtypes. Subtype A was increased significantly in HepG2 cells after PMA treatment, although the total GGT mRNA level was not significantly increased [22]. Additional studies must be performed to establish the response elements involved in the NOX mediated regulation of GGT. The additional bands after PCR reported by Daubeuf et al [22], and explained by the presence of an unspliced intron in

the sequence of the three 5'UTRs [55], were also detected in the present study but not shown in Figure 4.

In this study we present data showing that variations in the endogenous level of ROS, generated by NOX, are a significant determinant for basal GGT expression. Higher levels of oxidative stress are correlated with cancer malignancy and poor prognosis [16, 49, 52, 56] which indicates that cancer cells are able to compensate and benefit from such increased oxidative stress situations. GGT may act as one of several adaptive responses to protect the cells against severe oxidative stress and apoptosis and, as has been suggested for melanoma therapy [9], the GGT enzyme could thus be a target for cancer treatment.

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LIST OF ABBREVIATIONS

NOX; NADPH oxidase, ROS; reactive oxygen species, GGT; γ-glutamyltransferase, GSH; glutathione, PMA; phorbol-12-myristate-13-acetate, CM-H_2DCFDA; 5-(and-6)-chloromethyl-2`,7`-dichlorodihydrofluorescein diacetate, acetyl ester.

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LEGEND TO FIGURES.

Figure 1. Knockdown of p22phox levels in Ishikawa cells after transient transfection with shRNA constructs.

Ishikawa cells were transiently transfected with empty vector (pLKO.1) or the shRNA constructs p22phox-78 or p22phox-81, respectively. The knockdown effect was determined 48 h post transfection. (A) The p22phox mRNA level was analysed using the ΔΔCt method as described in Methods, and the expression was normalized to the housekeeping genes *hACTB* and *hGAPDH*. The results show the relative p22phox mRNA levels to that in empty vector transfected cells and are mean values of three independent experiments. The asterix * indicates a significant difference ($p < 0.05$) in mean value compared to the level in control cells. (B) Total cell lysates were subjected to Western blotting using antibodies for p22phox and β-actin, respectively. The data are representative of three independent experiments.

Figure 2. Knockdown of p22phox levels in Ishikawa cells after stable transfection with shRNA.

Ishikawa cells were stably transfected with empty vector (pLKO.1) or the shRNA constructs p22phox-78 and p22phox-81, respectively. Individual clones were selected and analysed for expression of p22phox. (A) The p22phox mRNA level was analysed using the ΔΔCt method as described in Methods, and the expression was normalized to the housekeeping genes *hACTB* and *hGAPDH*. (B) Total cell lysates were subjected to Western blotting using antibodies for p22phox and β-actin, respectively. (C) The intracellular ROS levels were determined by incubating the cells with 2.5 μM CM-H_2DCFDA in HBSS for 30 minutes followed by flow cytometric analysis. Ten

thousand events were collected and the median values are presented. The results are representative of three independent experiments.

Figure 3. Decreased expression of GGT in p22phox knockdown Ishikawa cells.

The effect of p22phox knockdown on the level of GGT were analysed in the p22phox-78(9) and p22phox-81(5) clones. (A) The relative GGT mRNA level was estimated using the ΔΔCt method as described in Methods, and normalized to the housekeeping genes *hACTB and hGAPDH.* The results are mean values (+SEM) of three independent experiments shown relatively to that in control cells. (B) Total cell lysates were subjected to Western blotting using antibodies for p22phox and β-actin, respectively. Similar results were obtained in two other independent experiments. (C) GGT enzyme activity was measured kinetically as described in Methods. Data are means (+SEM) of at least four independent experiments. The asterix * indicates a significant difference ($p < 0.05$) in mean value compared to the level in control cells.

Figure 4. Increase in ROS production in HT-29 cells due to PMA activation of NOX.

HT-29 cells were exposed to 100 nM PMA for 24 h and the intracellular ROS level was determined by incubating the cells with 5 μM CM-H_2DCFDA in HBSS for 30 minutes followed by flow cytometric analysis. Ten thousand events were collected and the median values are presented. (A) ROS levels were measured after 24 h treatments with PMA (100 nM), apocynin (0.5 mM) and PMA + apocynin. The data are from 3 independent experiments and expressed relatively to that in control cells. (B) Cells were transfected with p22phox siRNA and control siRNA-A, harvested after 72 h and total cell lysates were subjected to Western blot analysis using antibody for p22phox.

(C) Cells transfected with control siRNA or siRNA p22phox, respectively, were treated for 24 h with 100 nM PMA before measurements of ROS levels. The data shown are median values (+SEM) of 3 independent experiments expressed relatively to that in control cells. The asterix * indicates a significant difference ($p < 0.05$) in mean value compared to the levels in control cells, whereas ** indicates a significant difference compared to the PMA treated cells.

Figure 5. Increased expression of GGT in HT-29 cells after PMA stimulation.

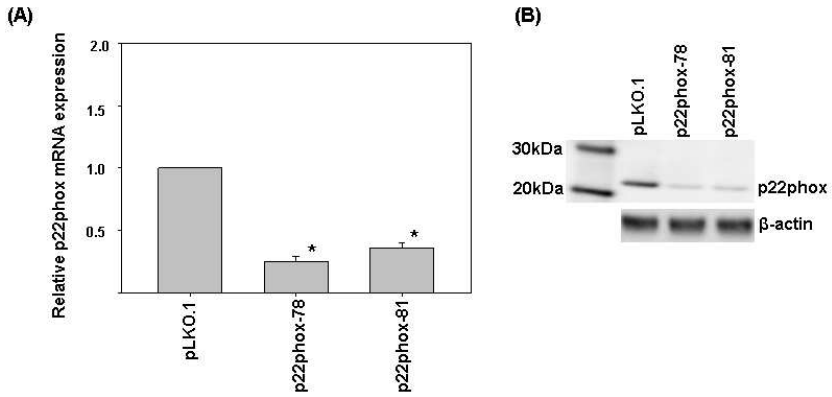
HT-29 cells were exposed to 100 nM PMA for 24 h followed by 4 days of culturing. (A) The relative GGT mRNA expression was estimated using the ΔΔCt method as described in Methods, normalized to the housekeeping genes *hACTB and hGAPDH*. The results are median values (+SEM) of 3 independent experiments expressed relatively to that in control cells. (B) Total cell lysates were subjected to Western blotting using antibodies for GGT and β-actin, respectively. The results are representative of three independent experiments. (C) GGT enzyme activity was measured kinetically as described in Methods. Data are means (+SEM) of at least four independent experiments. (D) Total RNA from HT-29 cells incubated with 100 nM PMA for 24 h were isolated. Using subtype specific primers, semi-quantitative levels of each GGT mRNA subtype were estimated. Amplification of GADPH was included as control. The estimated band sizes are shown. The results shown are representative of three independent experiments. The asterix * indicates a significant difference ($p < 0.05$) in mean value compared to the levels in control cells.

Figure 6. Decreased GGT expression and ROS production after inhibition of NOX by apocynin in HT-29 cells.

HT-29 cells were incubated with 0.5 mM apocynin for 4 days. (A) The relative GGT mRNA expression was estimated using the ΔΔCt method as described in Methods, normalized to the housekeeping genes *hACTB and hGAPDH.* The data shown are median values (+SEM) of 3 independent experiments expressed relatively to that in control cells. (B) Total cell lysates were subjected to Western blotting using antibodies for GGT and β-actin, respectively. The results are representative of three independent experiments. (C) GGT enzyme activity was measured kinetically as described in Methods. Data are means (+SEM) of at least four independent experiments. (D) The intracellular ROS levels were determined by incubating the cells with 5 μM CM-H_2DCFDA in HBSS for 30 minutes followed by flow cytometric analysis. Ten thousand events were collected and the median values are presented relatively to that in control cells. The results are representative of three independent experiments. The asterix * indicates a significant difference ($p < 0.05$) in mean value compared to the level in control cells.

Table 1: Primer sets used in RT-qPCR analysis

Gene symbol	Assay Name	Cat. No	Amplicon Length(bp)	Gene Bank Accession Number
GGT1	γ-glutamyltransferase 1	PPH02093E	119	NM_005265.2
CYBA	Hs_CYBA_1_SG (p22PHOX)	QT00082481	106	NM_000101
ACTB	Hs_ACTB_1_SG	QT00095431	146	NM_001101
GAPDH	Hs_GAPDH_2_SG	QT01192646	119	NM_002046



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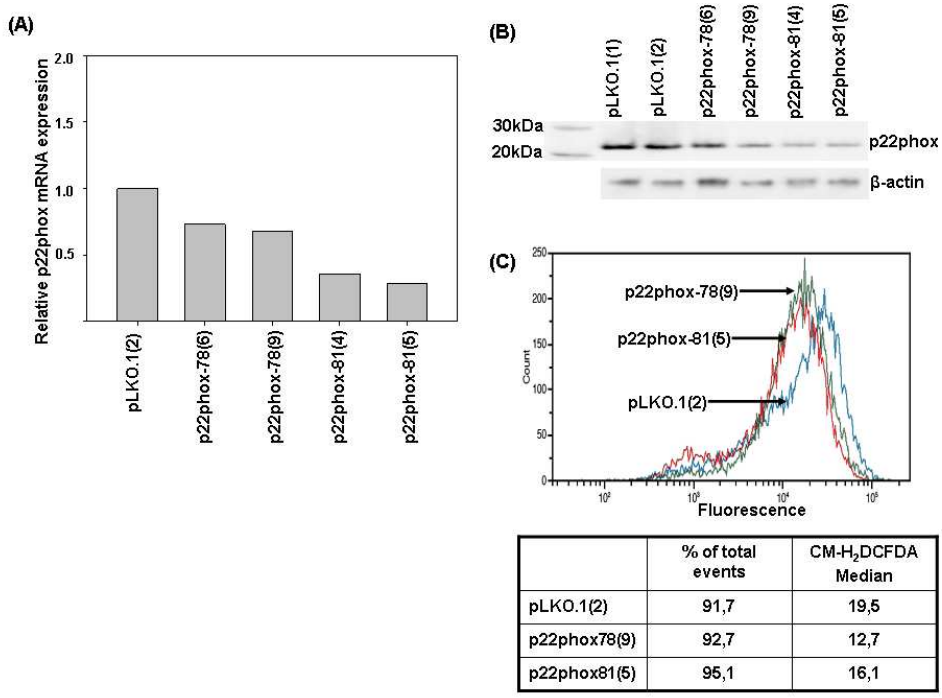
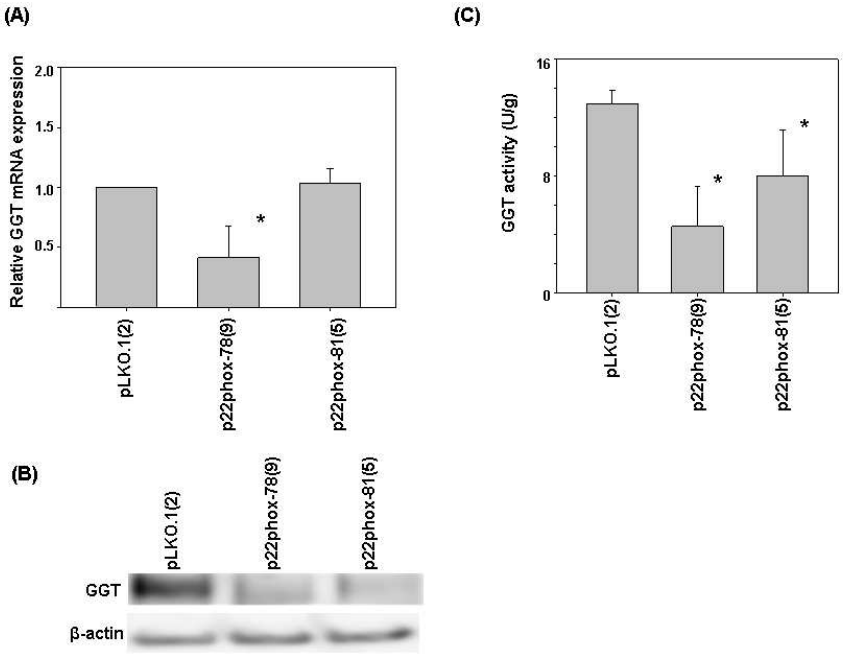


	% of total events	CM-H_2DCFDA Median
pLKO.1(2)	91,7	19,5
p22phox78(9)	92,7	12,7
p22phox81(5)	95,1	16,1

254x190mm (96 x 96 DPI)



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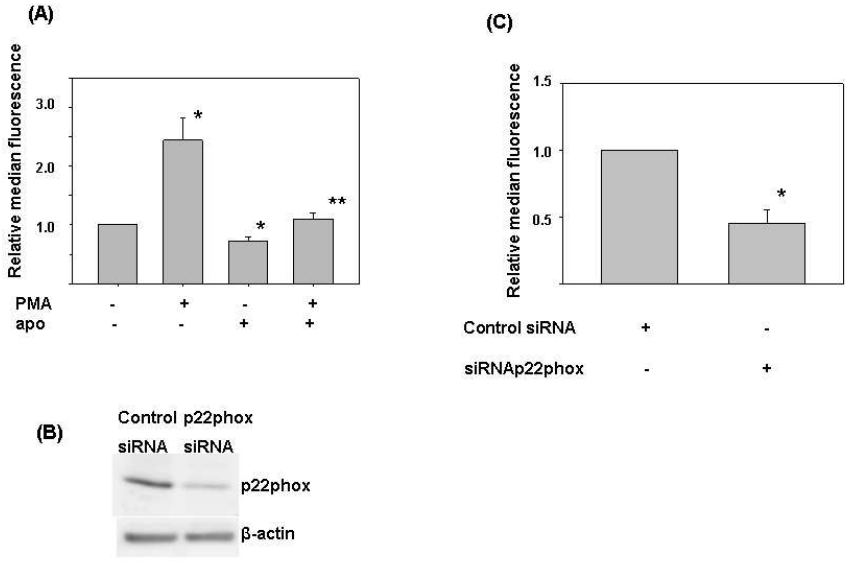

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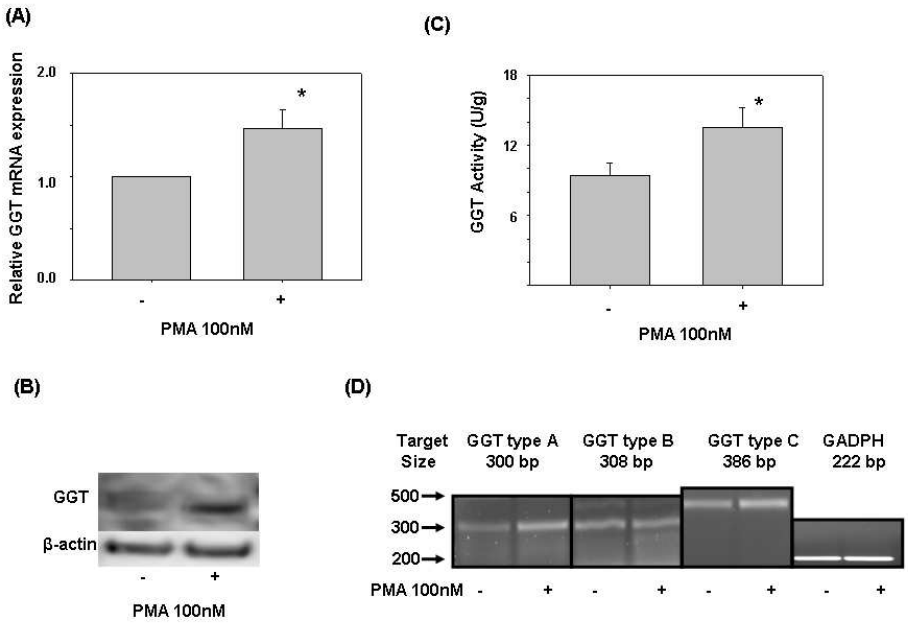

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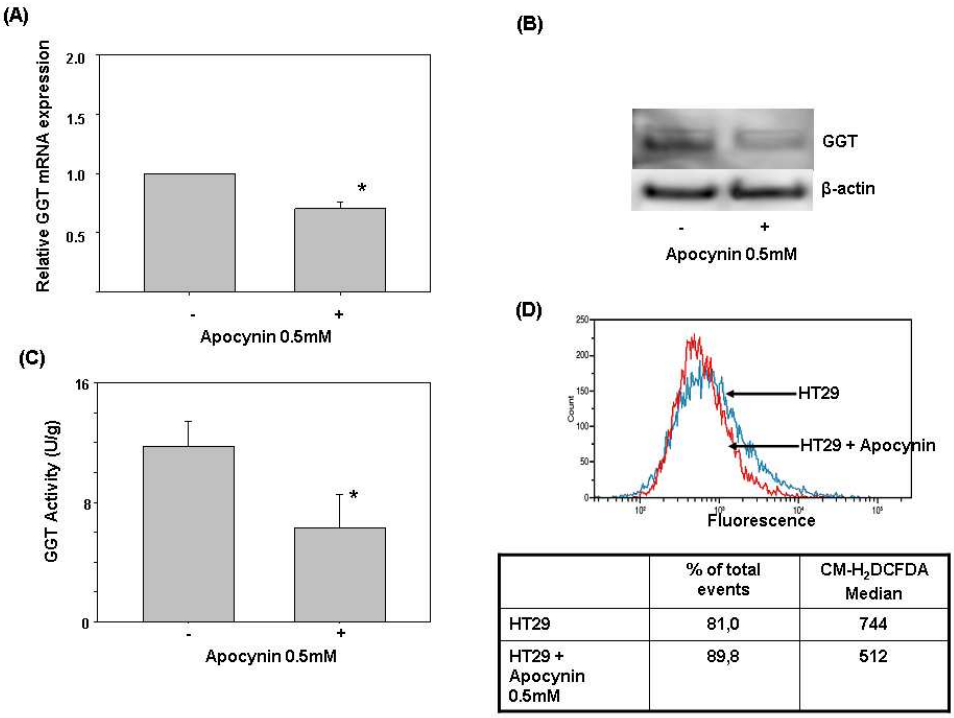

	% of total events	CM-H_2DCFDA Median
HT29	81,0	744
HT29 + Apocynin 0.5mM	89,8	512

254x190mm (96 x 96 DPI)