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Hydrogen peroxide mediates doxorubicin-induced transglutaminase 2 expression in PC-14 human lung cancer cell line

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Abbreviations: TGase, transglutaminase; MDR, multidrug resistance; DCF, 2,7-dichlorofluorescein; NAC, N-acetylcysteine; GSH, glutathione; ROS, reactive oxygen species

Abstract

Increased expression of Transglutaminases 2 (TGase 2, TGase C) was observed in PC-14 human lung cancer cells in association with doxorubicin resistance and the reduction of the enzyme expression was correlated with the increasing cytotoxicity of the drug (Han and Park, 1999). Hydrogen peroxide was suggested to be a key mediator for doxorubicin-induced DNA fragmentation leading to apoptosis. A possible role of hydrogen peroxide as a putative mediator of TGase 2 expression in the doxorubicin sensitive PC-14 cells was examined. TGase 2 expression was increased in PC-14 cells treated with doxorubicin in a dose-dependent manner resulting in the concomitant increase of reactive oxygen species. The rise of TGase 2 expression by doxorubicin treatment was inhibited by N-acetylcysteine or glutathione treatment, while direct addition of hydrogen peroxide to PC-14 cells induced TGase 2 expression. These results suggest that generation of hydrogen peroxide induced by doxorubicin treatment is one of the key factors in an enhancement of TGase 2 expression in PC-14 cells.

Keywords: Transglutaminase 2, Hydrogen peroxide, Doxorubicin, PC-14 cells

Introduction

Doxorubicin is one of the most widely used chemotherapeutic agents. However, the cross-resistance to unexposed drugs which share no obvious structural resemblance with doxorubicin has been a big problem in the doxorubicin-based chemotherapy. This multidrug resistance (MDR) phenomenon frequently occurred in using alkaloid com-pounds and antibiotics including anthracyclines such as doxorubicin and daunomycin, vinca alkaloids, actinomycin D, etoposide or paclitaxel (Beck, 1987). The MDR pheno-type has been partially explained in connection with a variety of biological factors; overexpression of 170 kDa P-glycoprotein or 190 kDa multidrug resistance-associated protein (Beck, 1987; Marquartdt *et al*., 1990; Cole *et al*., 1992), quantitative or qualitative alterations of target molecule, topoisomerase II (Ross *et al*., 1984; Harker *et al*., 1991; Sullivan *et al*., 1993), increased drug detoxifying systems, such as glutathione, γ-glutamylcysteine synthetase or γ-glutamyl transpeptidase (Hamilton *et al*., 1985; Godwin *et al*., 1992), decreased susceptibility to oxygen free radicals, which is associated with increased superoxide dismutase, glutathione peroxidase or anionic glutathione transferase (Batist *et al*., 1986; Mimnaugh *et al*., 1989), the increased activity of protein kinase C (Fine *et al*., 1988; Lee *et al*., 1992), or overexpression of c-Fos (Scanlon *et al*., 1991; Scanlon *et al*., 1994). Recently, overexpression of TGase 2 was suggested to be related with the resistance to doxorubicin (Mehta, 1994; Han and Park, 1999).

Transglutaminases (EC 2.3.2.13; TGases) are Ca^{2+}-dependent enzymes that carry out acyl-transfer reactions between γ-carboxamide groups of peptide-bound glutamine residues and ε-amino groups of peptide-bound lysine residues or primary amino groups of polyamines, resulting in the isopeptide bond formation (Folk, 1980; Folk and Chung, 1985; Davies *et al*., 1988). Among the several isozymes, TGase 2 is a ubiquitous enzyme implicated in diverse cellular functions including differentiation (Birckbichler *et al*., 1976; Scott *et al*., 1982), inhibition of cell growth (Cai *et al*., 1991; Mian *et al*., 1995; Katoh *et al*., 1996), cell adhesion (Byrd and Licht, 1987; Gentile *et al*., 1992), receptor-mediated endocytosis (Davies *et al*., 1980; Julian *et al*., 1983), secretion (Bungay *et al*., 1984; Fesus *et al*., 1985; Bungay *et al*., 1986), apoptosis (Fesus *et al*., 1987; Piacentini *et al*., 1991) and receptor-mediated intracellular signaling (Lee *et al*., 1989; Nakaoka *et al*., 1994; Feng *et al*., 1996).

In addition, increased TGase 2 expression was observed in doxorubicin-resistant MCF-7 human breast cancer cells or PC-14 human lung cancer cells (Mehta, 1994; Han and Park, 1999). But the precise mechanism by which doxorubicin induced TGase 2 expression was not clearly understood. Doxorubicin has been known to generate

reactive oxygen species (ROS) due to one electron transfer from its quinone redox cycling, which modify membrane lipids, DNA and proteins (Slater, 1984). Recently, it was reported that topoisomerase II modified by doxorubicin was bound to DNA covalently and thereby DNA breaks were ensued (Ross *et al*., 1984). Hydrogen peroxide was suggested to be a key mediator for doxorubicin-induced DNA fragmentation leading to apoptosis (Simizu *et al*., 1998). Association of two doxorubicin-induced cellular events may provide possible mechanism of regulation of TGase 2 expression in PC-14 cells. In this study, a possibility of hydrogen peroxide generated in the redox cycling of doxorubicin as a putative mediator of TGase 2 expression in the doxorubicin sensitive PC-14 cells was examined.

Materials and methods

Materials

RPMI 1640 media, fetal bovine serum, penicillin G sodium/streptomycin sulfate were purchased from GIBCO BRL (Geithersburg, MD). [1,4-^{14}C]putrescine and enhanced chemiluminescence (ECL) kit were purchased from Amersham (Little Chalfont, UK). 2,7-Dichlorofluorescein-diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR) and all other chemicals used in this study were obtained from Sigma (St. Louis, MO).

Cell culture

PC-14 cells were provided by Dr. Young Sook Son (Korea Cancer Center Hospital, Seoul, Korea) and maintained in RPMI 1640 media containing 10% fetal bovine serum, 100 units/ml penicillin G sodium and 100 mg/ml strepto-mycin sulfate. Cells were grown at 37˚C in a humidified air with 5% CO_2.

TGase activity assay

TGase activity assay was carried out according to the methods described (Folk and Chung 1985). Cells (5×10^6) were harvested and homogenized in TBS (100 mM Tris-HCl, pH 7.4 and 1.5 M NaCl) containing 1 mM EDTA, 5 mM benzamidine HCl, 1 mM PMSF, 0.5 μg/ml leupeptin and 2 μg/ml aprotinin. The 100 mg homogenate protein and 0.25 μCi [1,4-^{14}C]putrescine (S.A. 10^8 mCi/mmol) were added into the 400 μl substrate solution (0.1 M Tris-acetate, 0.5 mM EDTA, 10 mM $CaCl_2$, 0.5% Triton X-100, 5 mM dithiothreitol and 1% N, N-dimethylcasein) and incubated at 37˚C for 1 h. Subsequently, 7.5% ice-cold trichloroacetic acid was added to the reaction mixture to stop the reaction and proteins were precipitated overnight at 4˚C, followed by a filtration with a Whatman GF/A filter. The radioactivities of the precipitates were counted by a liquid scintillation counter and the enzyme activity was calculated by the [^{14}C]putrescine incorporation rate into dimethylcasein.

Western blot analysis

Cells ($5\times 10^{6)}$ were harvested and homogenized in TBS (100 mM Tris-HCl, pH 7.4 and 1.5 M NaCl) containing 1 mM EDTA, 5 mM benzamidine HCl, 1 mM PMSF, 0.5 μg/ml leupeptin and 2 μg/ml aprotinin. The protein concentration was determined by the Bradford method (Bradford, 1976) and the protein at 20 μg concentration was subjected to 10% SDS-PAGE. The gel was blotted onto nitrocellulose membrane for 1.5 h at 1 Ampere and the membrane was incubated with a TGase 2 antibody (polyclonal, Lee *et al*., 1995) and then secondary antibody conjugated with horseradish peroxidase. The chemiluminescence was performed with a ECL kit according to the manufacturer's instruction.

Measurement of ROS production

Reactive oxygen species (ROS) production in cells was assessed using 2,7-dichlorofluorescein (DCF) according to the method described (Duranteau *et al*., 1998; Simizu *et al*., 1998) with slight modifications. Cells (2×10^6) were treated with diacetate form of the dye (DCFH-DA) at a final concentration of 5 μM. The esterified form of DCFH-DA can permeate cell membranes and then be deacetylated by intracellular esterases. The resulting compound, dichlorofluorescin (DCFH), is reactive with ROS, mainly H_2O_2 or hydroxyl radical, to give a oxidized fluorescent compound, dichlorofluorescein (DCF). Fluore-scence was measured using an excitation wavelength of 488 nm and emitter wavelength of 525 nm with 1% attenuation of the excitation light in a luminescence spectrometer (Hewlett-Packard).

Results

TGase 2 expression by doxorubicin treatment

Induction of TGase 2 expression in PC-14 cells treated with doxorubicin was examined at two different concentrations; 0.2 μg/ml or 2 μg/ml. The enzyme activity has remained at the basal levels in the cells treated with 0.2 μg/ml concentration of doxorubicin upto 88 h and began to increase. The cells treated with 2 μg/ml concentration of doxorubicin responded by three-fold elevation of TGase acitivity in a biphasic mode peaking at 10 and 64 h and returned to a basal level at 40 and 94 h (Figure 1A). The elevation of TGase activity was accomanied with a concomitant increase of TGase 2 protein. Fig 1B shows elevated TGase 2 protein levels at 16 and 64 h and lower levels at 0 and 40 h suggesting that specific activity of TGase 2 remain unchanged during the experimental period. These results indicate that doxorubcin induced a *de novo* synthesis of TGase 2.

Generation of ROS by doxorubicin treatment

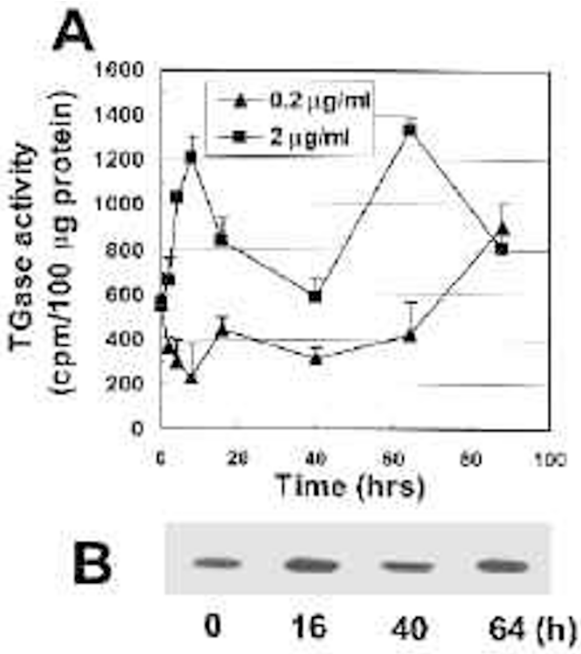

Figure 1. Changes of TGase 2 expression level by the treatment of doxorubicin in PC-14 cells. (A) Cells were treated with doxorubicin in a concentration of 0.2 μg/ml (▲) or 2 μg/ml (■) as a function of time. Then TGase enzyme activity was measured according to the method described in Materials and Methods. Data represent the mean values ± standard deviations from 3 experiments. (B) Representative immunoblot of TGase 2 protein from cells treated with 2 μg/ml doxorubicin for indicated time.

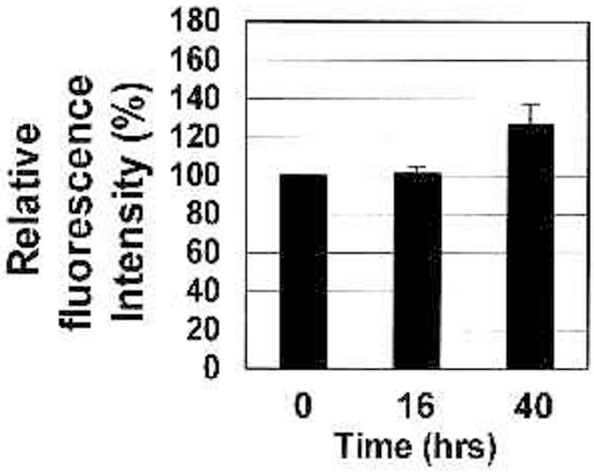

Figure 2. Measurement of ROS generation by doxorubicin treatment in PC-14 cells. Cells were treated with 2 μg/ml doxorubicin as a function of time and the amount of intracellular ROS was measured by the method described in 'Materials and Methods'. Data represent the mean values ± standard deviations from 4 experiments.

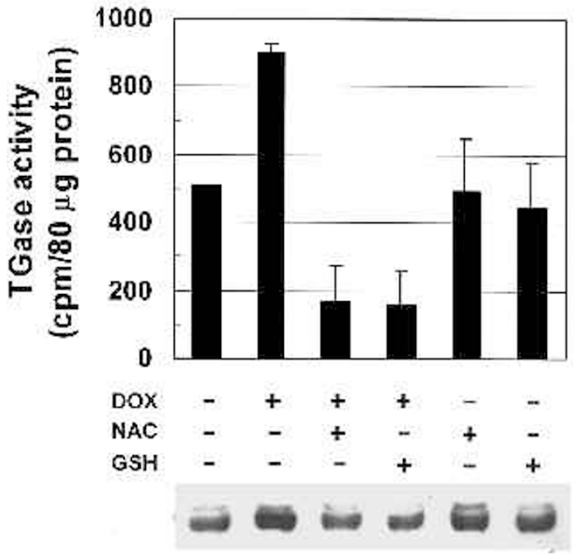

Figure 3. Effect of NAC or GSH pretreatment on doxorubicin-induced TGase 2 expression in PC-14 cells. Cells were pretreated with 10 μM NAC or 10 nM GSH for 1 hour and then followed by a treatment with doxorubicin in 2 μg/ml concentration for 64 h. TGase enzyme activity was measured according to the method described in 'Materials and Methods' (*upper pannel*). Data represent the mean values ± standard deviations from 3 experiments. And the amount of TGase 2 protein was compared by Western blot analysis (*lower pannel*).

Generation of reactive oxygen species (ROS) in PC-14 cells treated with doxorubicin were examined using dichlorofluorescei-diacetate (DCFH-DA) probe. A small but significant increment of relative fluorescence intensity was increased in the cells treated with 2 μg/ml concentration of doxorubicin for 40 h (Figure 2). This result demonstrates that ROS are generated by doxorubicin within PC-14 cells.

Effects of NAC or GSH pretreatment on doxorubicin-induced TGase 2 expression

In an effort to investigate a possible inter-relationship of TGase 2 expression and hydrogen peroxide generation in the doxorubicin treated PC-14 cells, the hydrogen peroxide neutralizing agents, N-acetylcysteine (NAC) or glutathione (GSH) were used for conditioning of the cells prior to doxorubicin treatment. As shown in Figure 3, the pretreatment of the cells with 10 μM NAC or 10 nM GSH for one hour resulted in an inhibition of elevation of TGase 2 expression observed with doxorubicin treatment but rather a significant lowering of TGase enzyme activity without changes in the protein amount. A treatment with 10 μM NAC or 10 nM GSH for 1 h had little effect on the enzyme activity in itself (Figure 3). These data suggest that hydrogen peroxide could probably be related to an increase of TGase 2 expression by doxorubicin in PC-14 cells.

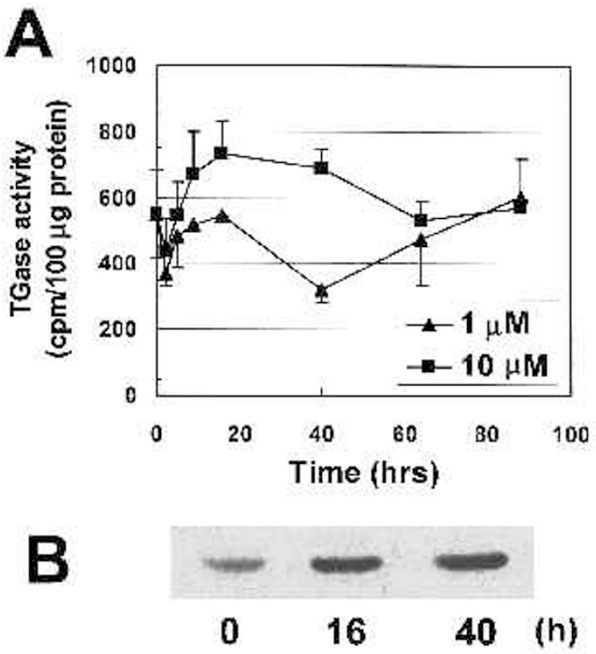

Figure 4. Changes of TGase 2 expression level by H_2O_2 treatment in PC-14 cells. (A) Cells were treated with H_2O_2 in a concentration of 1 μM (s) or 10 μM (n) for various times. Then TGase enzyme activity was measured according to the method described in 'Materials and Methods'. Data represent the mean values ± standard deviations from 3 experiments. (B) Representative immunoblot of TGase 2 protein from cells treated with 10 μM H_2O_2 for indicated time, respectively.

Increase of TGase 2 expression by hydrogen peroxide treatment

A poosible affect of hydrogen peroxide exposure of PC-14 cells on the expression of TGase 2 were examined. As shown in Figure 4, TGase 2 enzyme activity and protein levels were increased with 10 μM hydrogen peroxide treatment within 24 h. All these data suggest that hydrogen peroxide plays a key role in an increase of TGase 2 expression in doxorubicin-treated PC-14 cells.

Discussion

We have previously reported that TGase 2 expression was increased with the acquisition of resistance to doxorubicin or vincristine, and this increase was one of the mechanism for the acquisition of resistance to these drugs in PC-14 cells (Han and Park, 1999). In addition, the findings that TGase 2-reduced clones showed increased sensitivity to cisplatin as well as to doxorubicin or vincristine suggested the possibility that TGase 2 might play a role in the acquisition of drug resistance through a general cellular protective mechanism other than MDR-related system (Han and Park, 1999).

In addition, it was recently reported that hydrogen peroxide functioned as a common mediator for apoptosis induced by doxorubicin, vinblastine, campothecin and inostamycin in Ms-1 human small cell lung cancer cells (Simizu *et al*., 1998). It was also reported that hydrogen peroxide production was a critical event for cisplatin-induced cytotoxicity in S_3 renal terminal tubular cells (Tsutsumishita *et al*., 1998). These findings strongly suggest that hydrogen peroxidemay also function as an important mediator for the doxorubicin- or vincristine-induced increase of TGase 2 expression, which might be responsible for cellular defense activity against a variety of cytotoxic agents in PC-14 cells.

In the present study, hydrogen peroxide generated by doxorubicin was shown to mediate an increase of TGase 2 expression in doxorubicin-treated PC-14 cells. According to our data, TGase 2 expression was increased by 2 μg/ml doxorubicin in a biphasic manner, which had two peakes at 8 h and 64 h (Figure 1). It is not clear why the two peak of TGase 2 during the 4 days of exposure to doxorubicin. There appeared to be a thresh fold level of the enzyme synthesis. Whether there exists any phenotypic factors regulating TGase 2 in the doxorubicin treated cells are not known at present.

TGase 2 is a sulfhydryl enzyme, of which a cysteine residue in the active site is essential for its activity (Folk, 1980). Therefore, the enzyme is very vulnerable to oxidizing agents including ROS. Actually, the enzyme itself is inactivated by hydrogen peroxide (Jung *et al*., 1992). Therefore, doxorubicin treatment might inactivate the enzyme activity, but the cellular response to the drug was opposite to the previous view, which suggests the strong possibility of the enzyme-inducing function by the drug. Actually, we have reported that the development of drug resistance to doxorubicin was accompanied by induction of TGase 2 in PC-14 human lung cancer cell line (Han and Park, 1999).

The induction of TGase 2 by doxorubicin treatment could be mediated by hydrogen peroxide, since doxorubicin treatment generated ROS in the cells (Figure 2), and hydrogen peroxide induced the TGase 2 expression (Figure 4). Moreover, we have shown that a long term treatment with hydrogen peroxide of the human primary fibroblast induced the cellular senescence-like changes concomitantly with the induction of TGase 2 expression (Park *et al*., 1999). The ROS generation including hydrogen peroxide by doxorubicin has been well documented (Doroshow, 1983; Muindi *et al*., 1984). In addition, the role of hydrogen peroxide or other ROS in the doxorubicin-induced TGase 2 expression was confirmed by the effect of NAC and GSH treatment (Figure 2). Both agents are well known as the radical scavengers, blocking the activity of hydrogen peroxide.

It is not clear yet how hydrogen peroxide can induce TGase 2 expression. It should be studied whether

hydrogen peroxide or other ROS can directly activate the trans-criptional mode of the enzyme or they modulate the activities of transcriptional factors or inhibitors. It has been already reported that ROS could modulate the activity of NF-kappaB, which affected a variety of gene expression (Pinkus *et al*., 1996; Schmidt *et al*., 1996). But it is not clearly shown that NF-kappaB or other ROS sensitive transcriptional factors are involved in genetic regulation of TGase 2 (Lu *et al*., 1995).

From these results, it could be concluded that the doxorubicin-induced TGase 2 expression might be mediated by hydrogen peroxide generated by doxorubicin treatment

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