Changes in GSH-antioxidant system induced by daunorubicin in human normal and diabetic fibroblasts

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We investigated the effect of daunorubicin on glutathione content and activity of GSH-related enzymes in cultured normal and diabetic human fibroblasts. Cells were incubated with $4\,\mu$M daunorubicin (DNR) for 2 h followed by culture in drug-free medium for up to 72 h. Treatment of diabetic cells with the drug caused a time-dependent depletion of intracellular GSH and a decrease of the GSH to total glutathione ratio. GSH depletion was accompanied by apoptotic changes in morphology of the nucleus. Analysis of GSH-related enzymes showed a significant increase of the activities of Se-dependent and Se-independent peroxidases and glutathione S-transferase. In contrast, glutathione reductase activity was reduced by 50%. Significant differences between normal and diabetic cells exposed to DNR were observed in the level of GST and Se-dependent glutathione peroxidase activities.

These findings indicated that daunorubicin efficiently affects the GSH antioxidant defense system both in normal and diabetic fibroblasts leading to disturbances in glutathione content as well as in the activity of GSH-related enzymes.

The anthracycline antibiotic daunorubicin is still one of the most frequently used antitumor agents in clinical oncology. Anthracycline cytotoxicity is generally believed to be the result of intercalation-induced DNA damage and stabilization of the cleavable complex formed between DNA and topoisomerase II (Meyers *et al.*, 1988; Gewirtz, 1999). Moreover, daunorubicin metabolizes to form reactive oxygen species (ROS), superoxide anion radical, hydroxyl free radical and hydrogen peroxide, which interact with cell membranes,

proteins and DNA (Powis, 1989; O'Brien, 1991). An increase of intracellular concentration of ROS is tightly regulated by multiple defense mechanisms involving ROS scavenging enzymes and small antioxidant molecules. Among these antioxidant systems acting as antioxidants or scavengers are glutathione (GSH) and GSH-dependent enzymes (Sies, 1999; Cnubben *et al.*, 2001). Glutathione, the main intracellular thiol antioxidant, participates in a number of cellular antitoxic and defensive functions. It restores damaged molecules by hydrogen donation, reduces H_2O_2 and lipid peroxides and plays an important role in drug detoxification (Sies, 1999; Griffith, 1999). Recent studies have demonstrated the role of GSH in regulation of gene expression and apoptosis (Hammond *et al.*, 2001).

GSH constitutes more than 95% of total intracellular glutathione (GSH + GSSG) (Wernerman *et al.*, 1999). GSH depletion has been identified in the pathogenesis of a variety of diseases as well as in drug-treated cells (Samiec *et al.*, 1998; Paranka & Dorr, 1994). Furthermore, a decrease of the GSH/GSSG ratio is considered as an early indicator of oxidative stress.

Although considerable progress has been made, the precise role of the GSH-dependent antioxidant system in protection of cells from drug-induced oxidative damage is still poorly understood. The aim of this study was to obtain further insights into the role of endogenous glutathione in drug-treated cells. Because changes in glutathione content are involved in the pathogenesis of many diseases, including diabetes, we decided to analyze the levels of GSH and GSH-related enzymes in cultured diabetic human fibroblasts exposed to oxidative stress. Diabetic cell lines were subjected to daunorubicin treatment and changes in the levels of GSH and the enzymes were estimated spectrophotometrically. We also investigated the effect of the drug-induced GSH depletion on the induction of apoptosis in the cells.

MATERIALS AND METHODS

Materials. Daunorubicin was obtained from Laboratoire Roger Bellon (France). Eagle's Minimal Essential Medium was from the Sera and Vaccines Factory (Lublin, Poland), calf serum and lactalbumin hydrolysate were from Gibco BRL (Scotland). Gentamicin was purchased from Biochemie Gesellschaft m.b.H (Vienna, Austria). All other chemicals and solvents were of high analytical grade and were obtained from commercial sources.

Cell culture. C-2 and C-5 are cell lines derived from the skin of diabetic patients type II, and S-126 cell line from the skin of a normal donor. They were obtained from the tissue bank of the Centre of Child Health (Warsaw, Poland). Cells were cultured as monolayers in Eagle's Minimal Essential Medium supplemented with 10% newborn calf serum, 10% lactalbumin hydrolysate and 5 μg/ml gentamicin. Fibroblasts were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For the experiments, cells between 5th and 15th passage were used. Under the culture conditions we have observed no differences in the growth properties of the normal and diabetic cell lines.

Drug treatment. Solutions of daunorubicin in PBS (sodium phosphate buffered saline), stored as small aliquots at –20°C, were thawed immediately prior to use. The cells were incubated with 4 μM daunorubicin at 37°C for 2 h. After incubation, cells were rinsed twice with cold PBS to remove free drug. Then fresh medium was added and the cells were cultured at 37°C for further 6–72 h.

Cytotoxicity assays. Cytotoxicity was measured using the colorimetric MTT assay as described previously (Przybylska *et al.*, 2001). Briefly, fibroblasts were plated at 1×10^4 cells/well per 200 μl in 96-well microtiter plates. DNR was added at a concentration of 1–15 μM and incubated at 37°C for 2 h. The medium was removed, replaced with drug-free medium, and plates were incubated for a fur-

ther 72 h. Then MTT was added and incubation was continued for a further 4 h. After aspiration of the culture medium, the resulting formazan crystals were dissolved in dimethyl sulfoxide and absorbance was immediately read at 570 nm. The fraction of surviving cells was determined by dividing the mean absorbance values of the drug-treated samples by the mean absorbance value of the untreated control. The IC_{50} parameter is the DNR concentration that reduces the absorbance value to 50% of the value in control cells.

Measurement of enzyme activities. Cells plated in dishes were rinsed twice with cold PBS, scraped and lysed in 1% Triton X-100, 0.25 M sucrose and 10 mM Tris/HCl, pH 7.4 and centrifuged at $1000 \times \boldsymbol{g}$ for 5 min at 4°C. The supernatants were used for enzyme activity assays.

Glutathione reductase (GR) activity. Activity of the enzyme was determined as described by Calberg & Mannervik (1975). The reaction mixture consisted of 0.1 M phosphate buffer, pH 7.6, containing 0.5 mM EDTA, 1.0 mM GSSG, 0.1 mM NADPH and 100 μl aliquots of the supernatant in a final volume of 1.0 ml. The consumption of NADPH was monitored spectrophotometrically at 340 nm. The enzyme activity was expressed as nmol of NAPDH oxidized per min per milligram of protein, using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

GSH-peroxidase (GPX) activity. This was also measured at 340 nm by the glutathione reductase-coupled method reported by Rice-Evans *et al.* (1991). The reaction mixture for the measurement of enzyme activity contained the following constituents at the final concentrations indicated: 0.05 M phosphate buffer, pH 7.0, 0.1 mM EDTA, 0.15 mM NADPH, 240 U/ml GSH reductase, 1.0 mM GSH and 100 μl aliquots of the supernatants. In addition, the substrates 0.12 mM *tert*-butyl hydroperoxide, to quantify total (Se-dependent and Se-independent) GSH-peroxidase activity or 0.015 mM hydrogen peroxide for Se-dependent peroxidase activity were added

to the reaction mixture. When H_2O_2 was used as a substrate, 1 mM sodium azide was added to the reaction mixture to inhibit catalase activity. The activity of Se-independent glutathione peroxidase was calculated as the difference between the activity of total and Se-dependent peroxidase. The enzyme activity was expressed as above glutathione reductase.

Glutathione S-transferase (GST) activity. This was assayed by measuring at 340 nm the rate of formation of a GSH conjugate with 1-chloro-2,4-dinitrobenzene (CDNB) (Rice-Evans *et al.*, 1991). The reaction mixture contained 100 μl aliquots of the supernatant, 1.0 mM GSH, 1.0 mM CDNB and 0.1 M phosphate buffer, pH 6.5, in a final volume of 1.0 ml. A milimolar extinction coefficient of 9.6 was used to calculate the enzyme activity. The activity was expressed in nmol/min per mg of protein.

Glutathione measurement. Control cells and cells after incubation with the drug were mixed with 2 M perchloric acid and 4 mM EDTA solution and centrifuged 5 min at 5000 $\times \boldsymbol{g}$. The supernatant was neutralized with 2 M KOH in 0.3 Mops solution, centrifuged and assayed for GSH and total glutathione as described by Akerboom & Sies (1981). GSH content in the supernatant was determined by reaction with *o*-phtaldialdehyde. Total glutathione (the sum of GSH and GSSG) was determined in the supernatant by a kinetic assay in which catalytic amounts of GSH or GSSG and glutathione reductase caused a continuous reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) by NADPH. The rate of DTNB reduction was measured spectophotometrically at 412 nm. GSH and total glutathione were expressed in nmol/mg protein.

Apoptosis. Apoptosis was assessed by fluorescence microscopy. After 2 h exposure to daunorubicin cells were washed with PBS and incubated in drug-free medium for 24, 48 and 72 h. Cells were then stained with Hoechst 33342 (10 μg/ml) for 1 h at 37°C and analyzed for nuclear morphology changes on an Olympus fluorescence microscope.

Protein measurement. Protein content was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Statistical analysis. Statistical significance was assessed by Student's *t*-test and $P < 0.05$ (*) and $P < 0.01$ (**) were considered to be statistically significant. Data are given as mean ± S.D. for five to eight separate experiments.

RESULTS

Glutathione and GSH-dependent enzymes in control fibroblasts

The level of total and reduced glutathione and the activity of GSH-related enzymes in diabetic and normal cultured cell lines are shown in Table 1. There were no significant differences in the levels of glutathione and glutathione reductase (GR) between the examined cells. Significant changes were observed

respectively, in comparison with the normal cell line (S-126).

Cytotoxicity assays

The responses of fibroblasts to DNR treatment were analyzed by drug cytotoxicity, the level of glutathione and GSH-dependent enzymes and by fluorescence microscopy. The cells were exposed to 4 μM daunorubicin for

Table 2. Values of IC$_{50}$ in human fibroblast lines after daunorubicin treatment

Cell line	Origin	Karyotype	IC$_{50}$
Control cells			
S-126	skin	46, XY	4.3 ± 0.1 µM
Diabetic cells			
C-2	skin	46, XY	10.2 ± 0.5 µM
C-5	skin	46, XX	9.7 ± 0.6 µM

Table 1. Levels of glutathione and GSH-dependent enzymes in normal and diabetic fibroblasts.

Mean ± S.D. of 5–8 determinations (*$P < 0.05$).

	Normal fibroblasts	Diabetic fibroblasts	
	S-126	C-2	C-5
Total glutathione (nmol/mg protein)	17.0 ± 2.5	16.9 ± 0.6	16.9 ± 1.0
GSH (nmol/mg protein)	16.3 ± 1.3	15.1 ± 0.1	15.0 ± 1.0
Total glutathione peroxidase (nmol/min/mg protein)	18.0 ± 1.5	24.1 ± 2.0*	26.0 ± 1.9*
Glutathione reductase (nmol/min/mg protein)	6.2 ± 0.8	6.2 ± 0.5	5.3 ± 0.5
Glutathione S-transferase (nmol/min/mg protein)	11.9 ± 1.2	9.0 ± 0.5*	9.5 ± 0.7

in total glutathione peroxidase (GPX$_x$) and glutathione *S*-transferase (GST) activities. In the diabetic cell lines (C-2 and C-5) the activity of GPX$_x$ was enhanced by 34% and 44%, and GST levels were decreased by 32% and 26%,

2 h and then cultured in drug-free medium for up to 72 h. As shown in Table 2 the IC$_{50}$ values for DNR were 10.2 μM and 9.7 μM for the C-2 and C-5 cell lines and 4.3 μM for the normal cells, respectively. The diabetic cells were thus

Table 3. The effect of DNR on GSH and total glutathione contents in diabetic (lines C-2 and C-5) and normal (line S-126) fibroblasts.

Values are mean ± S.D. for eight to ten separate experiments. Values significantly different from the respective controls at time zero are indicated (*P < 0.05 and **P < 0.01).

Time after drug treatment (h)	Total glutathione nmol/mg of protein			GSH nmol/mg of protein			GSH/total glutathione		
	S-126	C-2	C-5	S-126	C-2	C-5	S-126	C-2	C-5
0	17.0 ± 2.5	16.9 ± 0.6	16.9 ± 1.0	16.3 ± 1.3	15.1 ± 0.1	15.0 ± 1.0	1.0	0.9	0.9
6	15.6 ± 2.6	17.3 ± 0.6	17.3 ± 1.1	14.8 ± 1.3	14.2 ± 0.3	14.7 ± 0.4	1.0	0.8	0.9
12	18.7 ± 2.0	18.5 ± 1.0	17.5 ± 0.9	14.1 ± 1.5	13.7 ± 0.7*	14.0 ± 1.1	0.8	0.7	0.8
24	20.1 ± 1.3*	19.1 ± 0.8*	19.4 ± 0.8*	13.2 ± 0.9*	13.3 ± 0.5*	12.3 ± 0.8*	0.7	0.7	0.6
48	22.3 ± 1.0*	18.0 ± 0.6	18.7 ± 1.0	12.0 ± 1.1**	12.1 ± 0.7**	11.5 ± 0.6**	0.5	0.7	0.6
72	18.3 ± 1.3	17.2 ± 1.1	17.7 ± 0.7	11.3 ± 1.0**	11.0 ± 0.5**	10.6 ± 0.9**	0.6	0.6	0.6

2-fold more resistant to the drug than the normal cell line S-126.

Effect of DNR on glutathione content

The changes in intracellular GSH and total glutathione content in drug-treated diabetic and normal cells are presented in Table 3. Treatment of the cells with DNR exerted a time-dependent effect on the level of glutathione.

The concentration of total glutathione in both diabetic cell lines increased slowly with time of culture. The levels of total glutathione were elevated by 13–14% in drug-treated cells at 24–48 h compared to the respective controls at time 0. At the same time a gradual decrease in the level of GSH was observed. The concentration of GSH was lowered by 27–30% after 72 h of drug exposure. Consequently, the ratio of GSH to total glutathione was decreased from 0.9 to 0.6 (mean values for both cell lines).

Treatment of normal fibroblasts (line S-126) with DNR caused a decrease in total glutathione level within the initial 6 h of incubation. Then the content of total glutathione markedly increased (to 118–131% of the control value) in the 24–48 h period. The level of GSH and the GSH/total glutathione ratio were lowered by 30% and 35%, respectively, at 72 h. These results indicate that the same concentration of DNR induced similar changes in GSH content in the diabetic and normal cells.

Effect of DNR on the activity of GSH-associated enzymes

Changes in the activity of GSH-related enzymes were also analyzed in the cells exposed for 2 h to 4 μM DNR. The results are shown in Fig. 1–3. As illustrated in Fig. 1A, B, the drug exposure led to a significant increase in the total as well as Se-dependent and Se-independent glutathione peroxidase activities in the diabetic cell lines. The level of total GPX was elevated by 81% and 106% respectively, in DNR-treated diabetic cell lines C-2 and C-5 at 48 h compared to their respective controls (time 0). At the same time, the Se-dependent and Se-independent glutathione peroxidase activities increased by 84% and 139% (mean maximal increase for the C-2 and C-5 cell lines, respectively, at 48 h). Figure 1C shows alterations in the activity of GPX$_X$ in the normal fibroblasts. In drug-treated cell line S-126 the maximal increase of total and Se-dependent glutathione peroxidase activities was observed at 72 h (respectively to 229% and 303% of the control values). The highest enhance-

ment in the activity of Se-independent glutathione peroxidase was noted at 48 h (241% of control at time 0).

The activity of glutathione reductase in drug-treated cells was non-significantly changed
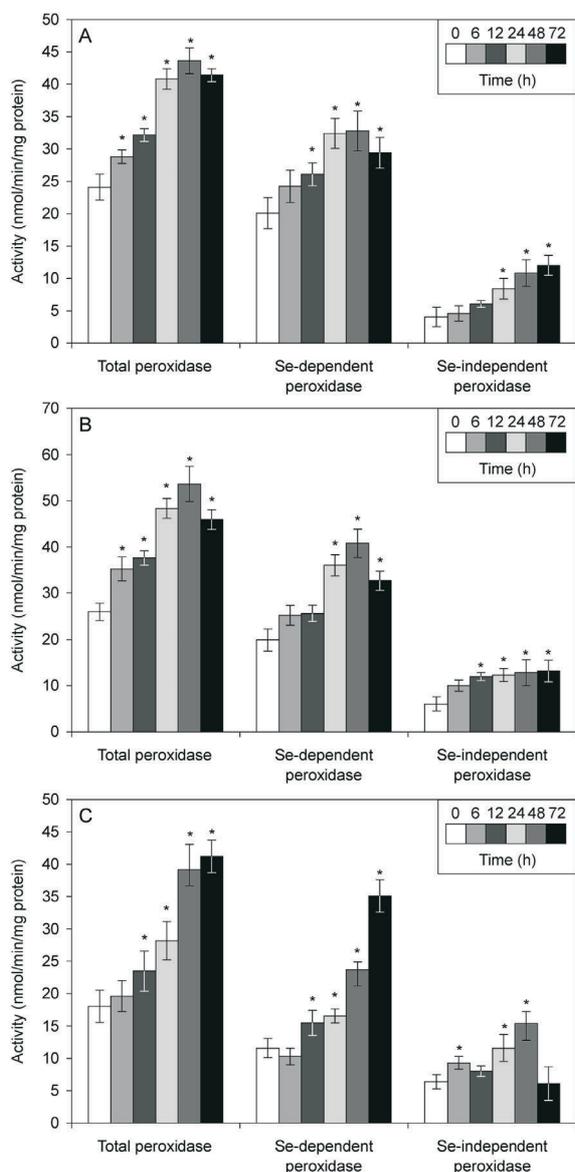

Figure 1. Effect of daunorubicin on total, Se-dependent and Se-independent glutathione peroxidase activity in C-2 (A), C-5 (B) and S-126 (C) fibroblasts.

Cells were incubated with 4 μM DNR for 2 h followed by culture in drug-free medium for up to 72 h. Data are shown as the mean ± S.D. of six experiments. The asterisk (*) indicates a statistically significant difference from control (time zero for respective cells) by Student's *t*-test analysis ($P < 0.05$)
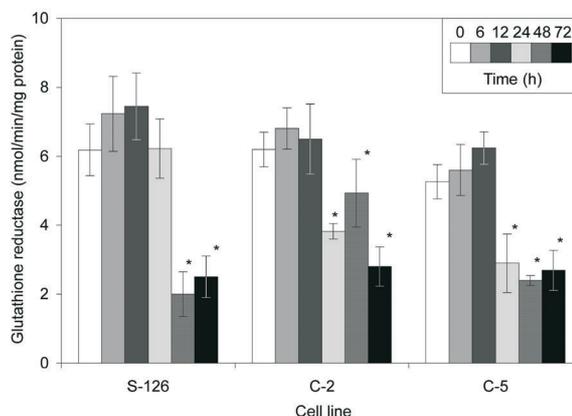
during the first 24 h in the normal and 12 h in the diabetic cells (Fig. 2). During the incubation of the C-2 and C-5 cell lines for 24–72 h the enzyme activity decreased (mean depletion by 52% at 72 h). In the normal S-126 cells

Figure 2. Effect of daunorubicin on glutathione reductase activity in diabetic (C-2 and C-5) and normal (S-126) fibroblasts.

Cells were processed as in the legend to Fig. 1. Data are expressed as the mean ± S.D. of at least six independent determinations. *$P < 0.05$ as compared with control (time zero for respective cells).

DNR also markedly lowered the reductase activity at 48 and 72 h after drug treatment, to 32.4% and 40.4% of control values at time 0, respectively.

As shown in Fig. 3, DNR induced a progressive enhancement of GST activity in the diabetic cell lines (mean increase 70% at 72 h). The GST activity in drug-treated normal cells was increased by 20–30% at 12–24 h and declined at 72 h after drug exposure.

Morphology of drug-treated cells

The morphological changes of the diabetic and normal cells induced by DNR exposure are presented in Fig. 4. The cultured cell lines were stained with Hoechst 33342 and analyzed by fluorescence microscopy. Hoechst 33342 is a dye that complexes with condensed DNA in apoptotic cells. Under the experimental conditions, cells treated with the drug showed the characteristic morphological hallmarks of apoptosis such as cell shrinkage,

perinuclear clumping of condensed chromatin at 24 h and the presence of apoptotic bodies at 72 h.
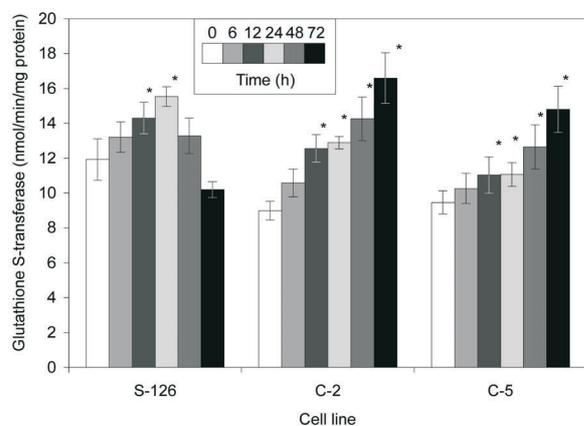

Figure 3. Effect of daunorubicin on glutathione *S*-transferase activity in diabetic (C-2 and C-5) and normal (S-126) fibroblasts.

Cells were processed as in the legend to Fig. 1. Data are expressed as the mean ± S.D. of at least six independent determinations. *$P < 0.05$ as compared with control (time zero for respective cells).

DISCUSSION

In the present study we have examined the role of the GSH-antioxidant system in the protection of cultured diabetic human fibroblasts against oxidative stress. We first compared the level of glutathione and GSH-related enzymes in untreated (control) normal and diabetic cells. These findings indicated a significantly elevated activity of total glutathione peroxidase and decreased activity of glutathione *S*-transferase in diabetic fibroblasts (cell lines C-2 and C-5) in comparison with normal cells (cell line S-126). The levels of GSH and glutathione reductase were similar in both types of cells. Furthermore, the ratio of GSH to total glutathione was also comparable and amounted 0.9 and 1.0 in the diabetic and normal cells, respectively. Many studies have demonstrated similar levels of glutathione in diabetic patients and healthy population (Di Simplico *et al.*, 1995; Srivastava *et al.*, 1989). However, there are also papers noting a lower glutathione content in patients with diabetes mellitus than in control group (Mukherjee *et al.*, 1998; Thornalley *et al.*, 1996).

At present, there is no data concerning the role of glutathione and GSH-dependent enzymes in diabetic patients exposed to anticancer drugs. Using the cultured diabetic cells we showed that daunorubicin induced in these cells considerable changes in the glutathione homeostasis. The drug caused a time-dependent decrease of GSH and glutathione reductase levels and reduction of the ratio of GSH to total glutathione. At the same time a significant increase in the activity of total, Se-dependent as well as Se-independent glutathione peroxidase and glutathione transferase was observed. Our results suggest that a loss of GSH in drug-treated diabetic cells may be due both to greater detoxification activity and lower efficacy in the reduction of GSSG.

Many reports have demonstrated in the last few years that anthracycline drugs decrease GSH content in different cells. A decreased level of GSH was observed in cultured heart cells exposed to doxorubicin treatment (Julicher *et al.*, 1985; Olson *et al.*, 1980; Paranka & Dorr, 1994) and in trisomic human fibroblasts following exposure to DNR (Zatorska & Jóźwiak, 2002). Under oxidative stress conditions, changes in glutathione levels may be associated with a number of processes such as free radical scavenging, reduction of peroxides and detoxification of electrophilic agents to less toxic compounds (Meister, 1995; Palmer & Paulson, 1997; Bladeren, 2000). Based on our results, DNR-treated diabetic cells were unable to maintain stable GSH content as well as the ratio of GSH to total glutathione. Moreover, the reduced GSH content and a concomitant decrease of glutathione reductase activity suggest that the reductive capacity of the cells was insufficient to protect them against GSSG formation. Simultaneously, the drug significantly enhanced the Se-dependent and Se-independent peroxidase (GPX_x) and GST activities. These enzymes play a crucial role in GSH-mediated de-
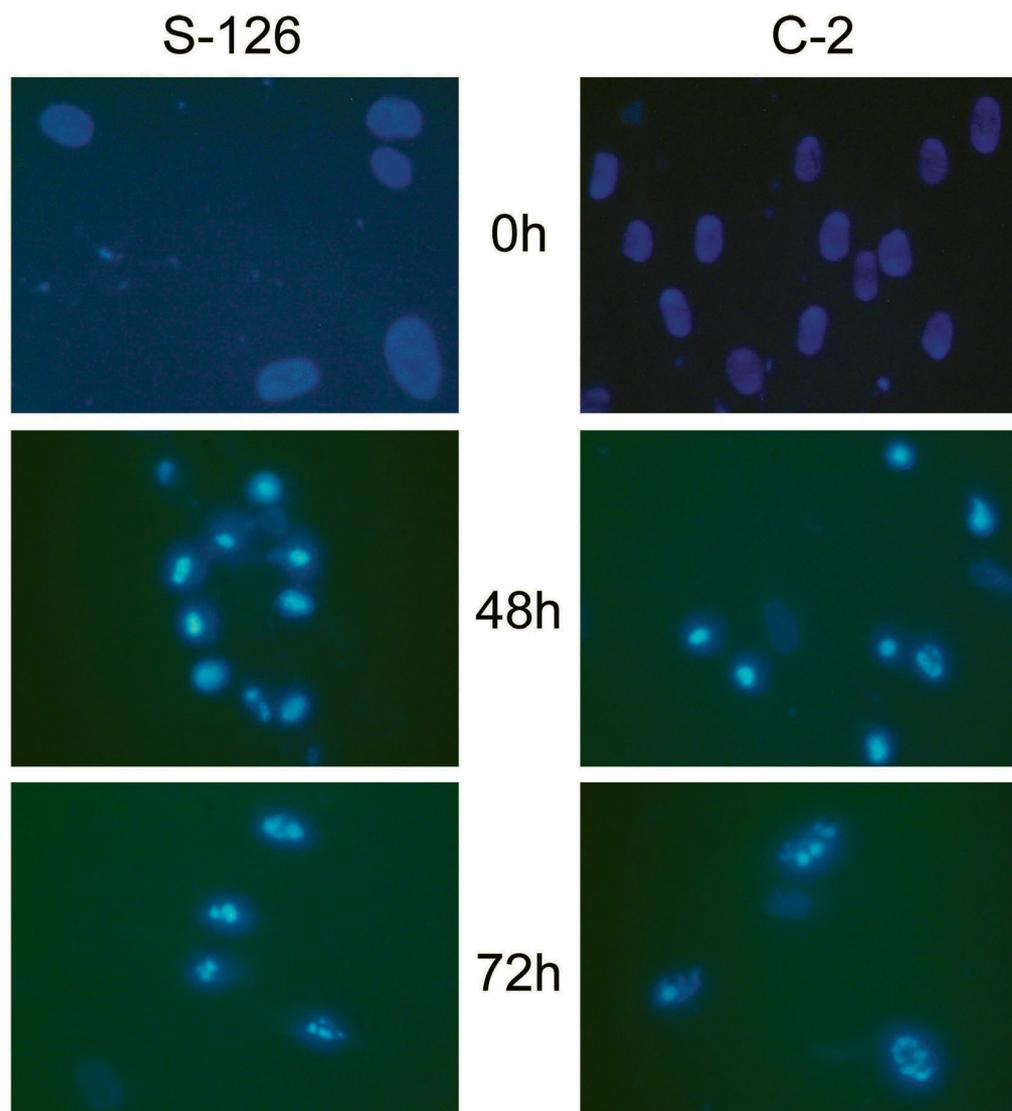
Figure 4. Morphological changes in daunorubicin treated diabetic (C-2) and normal (S-126) fibroblasts.

Cells were treated with 4 μM DNR for 2 h and cultured in drug-free medium for 48 and 72 h. Control (0 h) and drug-treated cells were stained with Hoechst 33342, transferred onto glass slides, representative areas were photographed under an Olympus fluorescence microscope equipped with a NU filter using a 400 $\times$ magnification.

fense mechanisms against ROS and oxidative stress-induced cellular damage (Cnubben *et al.*, 2001; Hayes & McLellan, 1999; Tew, 1994). GPX$_x$ are able to reduce endogenously produced H$_2$O$_2$ and organic hydroperoxides. GSH, as a substrate for GPX reactions also results in GSSG formation. The second enzyme GST exerts its protective functions utilizing GSH by two main mechanisms. The enzyme catalyzes the conjugation of GSH with various electrophilic compounds, including anthracycline antibiotics. GST also catalyzes a pero-xidative reaction with production of GSSG (Hayes & McLellan, 1999; Tew, 1994). The ability of GST to alter the level of intracellular glutathione in response to generation of ROS has been implicated in protection of cells against free-radical inducing agents (Baez *et al.*, 1997; Tew & Ronai, 1999). An increase in GST activity has been reported in cultured heart cells and trisomic fibroblasts following exposure to anthracycline drugs (Julicher *et al.*, 1985; Paranka & Dorr, 1994; Zatorska & Jóźwiak, 2002).

A major mechanism for the up and down regulation of the GSH-related enzymes in drug-treated diabetic cells is based on the ROS-mediated induction of oxidative stress. Recently, we have also examined the level of H_2O_2 in diabetic cells. The level of H_2O_2 in the C-2 cell line was increased by 40% in comparison with normal fibroblasts. In DNR-treated diabetic cells the H_2O_2 content increased at 6 h and slowly declined after 24–72 h of drug exposure to the respective control values at time 0 (unpublished data). These findings imply that ROS-mediated oxidative modifications of GSH-related enzymes may lead to changes in their activity which, in turn, cause a perturbation in the intracellular glutathione level. Further studies are needed to determine the exact regulatory mechanism of GSH-dependent enzymes in cells exposed to anticancer drugs treatment.

The cellular GSH-redox status is also an important factor during apoptosis mediated by endogenous ROS (Tan *et al.*, 1998; Zucker *et al.*, 1997). Reduction of the intracellular GSH content has been shown to induce apoptosis in cultured mouse MxC11 and C3H 10T1/2 fibroblasts (Zucker *et al.*, 1997), human B lymphoma and T cells (Amstrong *et al.*, 2002; Chiba *et al.*, 1996) and cultured neurons (Ahlemeyer & Krieglstein, 2000; Ratan *et al.*, 1994). The findings reported by Armstrong *et al.* (2002) suggest that GSH depletion can act as a potent early activator of apoptotic signaling. Recently, a relation between the extent of apoptosis and intracellular glutathione concentrations has been shown in cultured human neuroblastoma SK-N-SH cells (Shimizu *et al.*, 2002). Our results, obtained by simultaneous microscopy analysis, indicated that DNR treatment induced changes in morphology of the nucleus in diabetic cells. The occurrence of condensed chromatin and apoptotic bodies was accompanied by a decrease of GSH content.

A comparison of the GSH-antioxidant system in diabetic cells and normal fibroblasts exposed to the drug revealed that a given DNR concentration induced similar alterations in the GSH content and the ratio of GSH to total glutathione. Substantial differences between the two types of cells were observed in GST and Se-dependent glutathione peroxidase activities. In drug-treated normal cells, a smaller increase of the GST and a significantly higher enhancement of Se-dependent peroxidase activities were noted than in diabetic fibroblasts. The differences in the response of diabetic and normal fibroblasts may result from different sensitivity of the cells to DNR treatment as well as from different cellular antioxidant capacity.

In summary, our results indicate that daunorubicin induces in cultured normal and diabetic fibroblasts significant changes in the GSH antioxidant system. We suggest that drug-induced oxidative stress in the cells is tightly connected with the disturbances both in the enzymatic and non-enzymatic antioxidant defenses. Under oxidative stress, GSH depletion may play an important role in the apoptotic process of diabetic cells.

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