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The changes of prooxidant and antioxidant enzyme activities in bovine leukemia virus-transformed cells

Their influence on quinone cytotoxicity

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It was found that the activities of prooxidant enzymes (NAD(P)H oxidases and NAD(P)H:cytochrome *c* reductases) in bovine leukemia virus-transformed calf and lamb embryo kidney fibroblasts (lines Mi-18 and FLK) were by 1.25–18 times higher when compared to corresponding nontransformed calf cells. The activity of DT-diaphorase was also increased by about one order of magnitude in transformed cells. The activities of antioxidant enzymes were almost unchanged (superoxide dismutase), decreased by 13% or 53% (catalase) or increased by 25% or 90% (glutathione reductase) in Mi-18 or FLK cells, respectively. These changes of enzyme activity increased the toxicity of simple redox-cycling quinones (duroquinone, naphthazarin) towards transformed cells, but did not affect the toxicity of daunorubicin. The latter was most probably related to the inhibition of plasma membrane NADH dehydrogenase.

Bovine leukemia virus; Cell transformation; Quinone cytotoxicity

1. INTRODUCTION

Anthracycline quinones are important anticancer agents [1–6]. Some mechanisms of their cytotoxicity are related to the interaction with redox enzymes. One of the mechanisms is the redox cycling mediated by various NAD(P)H-oxidizing flavin dehydrogenases, leading to the formation of reactive oxygen species [1,2]. Another mechanism of their action is the inhibition of plasma membrane NADH dehydrogenase [4–6].

It is generally accepted that tumours and virus-transformed cells possess increased prooxidant activities, i.e. decreased activities of antioxidant enzymes superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6) [7–11], thus sometimes making these cells more susceptible to the redox cycling of quinoidal compounds [7,11]. The content of glutathione peroxidase (EC 1.19.1.9) also decreases during transformation but the content of glutathione reductase (EC 1.6.4.2) is highly variable [10,12]. Some data show that the activities of certain $O_2^{\cdot-}$ and H_2O_2 generating enzymes, e.g. plasma membrane NADH oxidase [13] or the peroxisomal fatty acid β-oxidation enzyme system [14] are higher in transformed cells. The increased activities of these or similar redox enzymes, catalyzing the redox cycling of quinonones may also increase their cytotoxicity. On the other hand, cell tranformation may change the activity of plasma membrane NADH dehydrogenase and increase its sensitivity to anthracyclines [4–6]. Thus the cell transformation is accompanied by complex changes of enzyme activity, which complicate the discrimination between the mechanisms of toxicity of anthracyclines or the evaluation of their contribution.

In the present paper, we demonstrate that two lines of bovine leukemia virus (BLV)-transformed fibroblasts possess significantly higher activities of prooxidant enzymes as compared to nontransformed cells, whereas the activities of antioxidant enzymes were almost unchanged. The influence of these enzymatic changes towards the cytotoxicity of quinones, including anthracycline antibiotic daunorubicin was investigated.

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Abbreviations: BLV, bovine leukemia virus; FLK and Mi-18, BLV-transformed lamb and calf embryo kidney fibroblast lines.

2. MATERIALS AND METHODS

The cultures of calf embryo kidney fibroblasts and their BLV-transformed line (Mi-18), and BLV-transformed lamb embryo kidney fibroblasts (line FLK) were grown and maintained in Eagles medium supplemented with 10% fetal bovine serum as described previously [15–17]. For enzymatic analysis, the cells were grown until confluency, detached by trypsinization, twice washed with 0.1 M K-phosphate, pH 7.4, containing 1 mM EDTA, and sonicated on ice in four cycles of 20 s. The homogenate was centrifuged at 14,000 × *g* for 45 min and the resulting supernatant was used for enzymatic analysis. Protein determination was performed according to the method of Bradford [18]. For experiments using whole cells, after trypsinization, the cells were washed twice and finally suspended in Hanks' balanced salt solution (pH 7.4), which was used during experiments. In cytotoxicity

experiments, cells were grown in the presence of various amounts of quinones, trypsinized and counted using a haemocytometer with viability determined by exclusion of Trypan blue. Unattached dead cells were removed prior to trypsinization.

All spectrophotometric measurements were performed using a Hitach-557 spectrophotometer at 25°C. The activity of catalase was determined following the decomposition of 10 mM of H_2O_2 in 0.1 M Tris-HCl, pH 8.0 [19]; the activity of superoxide dismutase was determined using the xanthine oxidase-nitrobluetetrazolium assay at pH 7.8 (0.1 M K-phosphate) [20]. The enzymatic reduction of cytochrome *c* (50 μM) or dichlorophenolindophenol (50 μM) by NAD(P)H (100 μM) was monitored using $\Delta\varepsilon_{550} = 20$ $mM^{-1} \cdot cm^{-1}$ and $\Delta\varepsilon_{600} = 21$ $mM^{-1} \cdot cm^{-1}$, respectively, in the absence or presence of 20 μM of dicumarol. In these assays, Tween 20 (0.01%) and bovine serum albumin (0.25 $mg \cdot ml^{-1}$) were used as activators [21]. NAD(P)H:oxidase and NADPH:glutathione reductase activities were determined using $\Delta\varepsilon_{340} = 6.2$ $mM^{-1} \cdot cm^{-1}$ (concentration of NAD(P)H, 100 μM; concentration of glutathione, 300 μM). These assays were performed in 0.1 M K-phosphate, pH 7.0, containing 1 mM EDTA. The reduction of ferricyanide (0.125 mM) by whole cells was measured using the dual wavelength mode by substracting the change in absorbance at 500 nm from change at 420 nm, using $\Delta\varepsilon_{420} = 1.0$ $mM^{-1} \cdot cm^{-1}$ [6]. Typically, concentrations of ca. 10^6 cells $\cdot ml^{-1}$ were used.

Daunorubicin was purchased from Minmedprom (Russia), naphthazarin from Fluka AG, all other enzymes and reagents from Sigma and Serva.

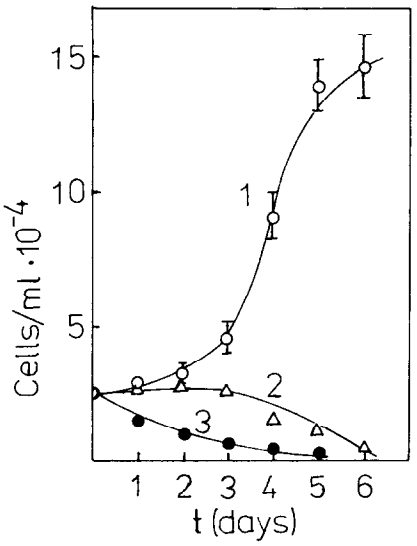

Fig. 1. The effect of daunorubicin on the growth of BLV-transformed fibroblasts (line FLK). Concentratin of daunorubicin, 0 (1), 0.01 μM (2), 1.0 μM (3). Results are means from 3–4 determinations ± S.D.

3. RESULTS

The data in Table I indicate that the activities of certain redox enzymes were markedly changed in BLV-transformed fibroblasts when compared with nontransformed ones. The NADH oxidase and NADPH:-dichlorophenoloindophenol reductase activities were increased about by one order of magnitude, the latter being partially sensitive to dicumarol, a specific inhibitor of DT-diaphorase (EC 1.6.99.2) [21]. Thus, an increase of content of DT-diaphorase in transformed cells could be identified unequivocally. The activities of NAD(P)H:cytochrome *c* reductase and NADPH oxidase increased up to 5.4 times in transformed cells. NAD(P)H oxidase activities were insensitive to rotenone (2 μM) or azide (1 mM). They could be evidently attributed to $O_2^{\cdot-}$-generating NAD(P)H oxidases of the plasma membrane [13,22,23], since according to the used method of lysate preparation, the latter should include a fibroblast membrane fraction as well [22,23]. Besides, the reduction of cytochrome *c* is inhibited by 40% by adding superoxide dismutase (30 $\mu g \cdot ml^{-1}$), thus being partially mediated by superoxide. The activities of antioxidant enzymes, however, were changed less markedly in transformed cells. The activity of superoxide dismutase was almost unchanged, the activity of catalase was decreased by 13% or 53%, and that of glutathione reductase was even increased by 25% or 90% (Table I).

Table I

The activities of prooxidant and antioxidant enzymes in lysates of nontransformed and BLV-transformed fibroblasts

Enzyme activity	Cell line		
	Nontrans-formed	Mi-18	FLK
NADH:oxidase[a]	22 ± 2.4	400 ± 20	359 ± 5
NADPH:oxidase[a]	8 ± 1	10 ± 1	43 ± 6
NADH:cytochrome *c* reductase[a]	42 ± 1	110 ± 7	141 ± 8
NADPH:cytochrome *c* reductase[a]	9.6 ± 1	30 ± 2	43 ± 1
NADPH:dichlorophenol-indophenol reductase[a]			
(− dicumarol)	42 ± 3	453 ± 53	502 ± 44
(+ dicumarol)	22 ± 3	290 ± 43	246 ± 24
Glutathione reductase[a]	28 ± 4	35 ± 5	53 ± 4
Catalase[b]	44 ± 2	38 ± 1	21 ± 1
Superoxide dismutase[c]	14 ± 2	13.5 ± 2	13 ± 2

[a] nmol · mg protein^{-1} · min^{-1};
[b] μmol · mg protein^{-1} · min^{-1};
[c] units · mg protein^{-1}; one unit of enzyme activity is defined as amount of protein needed to inhibit the rate of reduction of nitrobluetetrazolium by 50%.

Results are means from 3-4 determinations ± S.D.

The addition of 10 μM naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) or 100 μM duroquinone (tetramethyl-1,4-benzoquinone) to reaction mixtures increased the corresponding rates of oxidation of NAD(P)H (Table I) and superoxide dismutase-sensitive reduction of cytochrome *c* by 2–2.5 times for all cell lysates investigated. It means, that redox cycling of these quinones leading to the formation of superoxide takes place. On the other hand, 100 μM of daunorubicin did not stimulate these reactions noticeably.

The effect of daunorubicin on the growth of FLK cells is presented in Fig. 1. It is evident that even low

concentrations of this compound (< 1 μM) are highly cytotoxic, especially during long incubation. The same effects were also observed for nontransformed fibroblasts. However, after 24 h growth the toxicity of daunorubicin was similar both to transformed and nontransformed cells, showing an LD_{50} close to 1 μM in all cases (Fig. 2). On the other hand, LD_{50} of naphthazarin and duroquinone towards transformed cells were by 2–5 times lower as compared to the nontransformed ones (Fig. 3).

The reduction of ferricyanide by whole cells is catalyzed by NADH dehydrogenase of the plasma membrane which is also associated with NADH oxidase activity [4–6]. We have found, that nontransformed, FLK and Mi-18 cells reduce ferricyanide with corresponding rates of 1.0 ± 0.2, 0.9 ± 0.2 and 2.5 ± 0.5 nmol ferricyanide · min^{-1} · $(10^6$ cells$)^{-1}$. The inhibition of this reaction by daunorubicin is analogous for all cells tested, showing a CI_{50} close to 20 μM in all cases (Fig. 4).

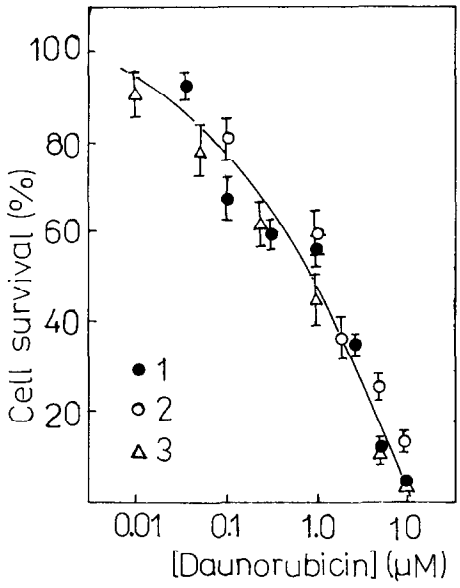

Fig. 2. Survival of fibroblasts after 24 h of growth in the presence of various concentrations of daunorubicin. Nontransformed cells (1), Mi-18 (2) and FLK (3).

4. DISCUSSION

The data of the present work indicate that the transformation of fibroblasts by BLV is accompanied by an increase of their prooxidant properties, i.e. increase of contents of prooxidant enzymes (NAD(P)H oxidases and cytochrome *c* reductases) while the antioxidant activity was almost unchanged (Table I). This is in contrast to most described cases where the activities of antioxidant enzymes were markedly decreased [7–11]. We think that the data of Table I may also predict insignificant changes in activities of other enzymes related to the glutathione cycle. It was established that during the transformation of fibroblasts, the activity of glutathione peroxidase parallels the changes in activity of superoxide dismutase, and the activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) parallels that of glutathione reductase [12]. The increase in NAD(P)H oxidase activities may be related to the stimulating role of low concentrations of reactive oxygen species in cell proliferation [24,25], since transformed cells usually proliferate more rapidly. These changes in enzyme activity increased the rates of redox cycling of naphthazarin and duroquinone and, evidently, their toxicity to

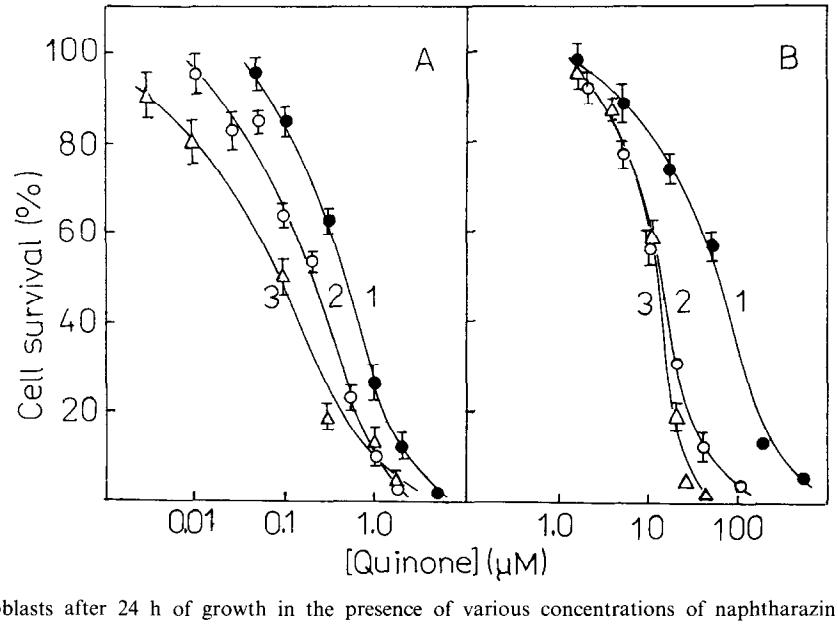

Fig. 3. Survival of fibroblasts after 24 h of growth in the presence of various concentrations of naphtharazin (A) and duroquinone (B). Nontransformed cells (1), Mi-18 (2) and FLK (3).

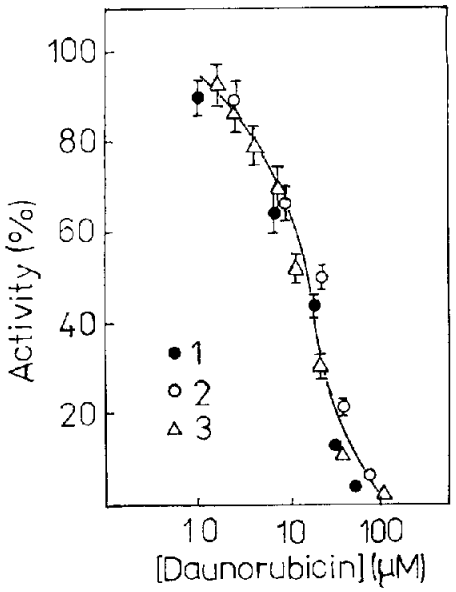

Fig. 4. The effect of daunorubicin on ferricyanide (0.125 mM) reduction by nontransformed (1), Mi-18 (2) and FLK (3) cells.

transformed cells (Fig. 3). However, the transformation has not affected the toxicity of daunorubicin (Fig. 2) which was as high as that of naphthazarin (Fig. 3) despite a much lower rate of redox cycling. Thus another mechanism of daunorubicin toxicity prevails in this case. It seems that the latter was also not affected by an increased content of DT-diaphorase in transformed cells (Table I). That rules out any specific contribution of DT-diaphorase, e.g. formation of alkylating quinomethide after a two-electron reduction [3] to the toxicity of daunorubicin. Besides, it was shown that the reduction of daunorubicin by DT-diaphorase is accompanied by the redox cycling of hydroquinone leading to the formation of activated oxygen species [21], rather than by other reactions. The analogous prooxidant activity of DT-diaphorase was demonstrated during the reduction of naphthazarin [26]. It seems that the prevailing mechanism of daunorubicin toxicity in this case is the inhibition of plasma membrane NADH dehydrogenase, since its equal toxicity to transformed and nontransformed cells (Fig. 2) parallels the patterns of inhibition (Fig. 4). This conclusion is supported by studies on hepatocytes, their simian virus-transformed line and hepatoma [4], where the efficiency of inhibition of their plasma membrane NADH dehydrogenase by adriamycin was parallel to its cytotoxicity.

It was shown that isolated NAD(P)H-oxidizing flavoenzymes reduce anthracyclines markedly slower than quinones with more positive single-electron reduction potentials, e.g. naphthazarin [27,28]. Analogous data were obtained for the reduction of quinones by hepatocytes, where their cytotoxicities paralleled the rates of their redox cycling [29]. The data of the present work indicate that if cytotoxicity of anthracyclines is higher than expected from the rates of their redox cycling, it may be related to the inhibition of plasma membrane NADH dehydrogenase.

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