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THE CYTOSKELETON AS A TARGET IN QUINONE TOXICITY

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The exposure of mammalian cells to toxic concentrations of redox cycling and alkylating quinones causes marked changes in cell surface structure known as plasma membrane blebbing. These alterations are associated with the redistribution of plasma membrane proteins and the disruption of the normal organization of the cytoskeletal microfilaments which appears to be due mainly to actin cross-linking and dissociation of α-actinin from the actin network. The major biochemical mechanisms responsible for these effects seem to involve the depletion of cytoskeletal protein sulfhydryl groups and the increase in cytosolic Ca^{2+} concentration following the alkylation/oxidation of free sulfhydryl groups in several Ca^{2+} transport systems. Depletion of intracellular ATP is also associated with quinone-induced plasma membrane blebbing. However, ATP depletion occurs well after the onset of the morphological changes, and thus it does not seem to be causatively related to their appearance. Thiol reductants, such as dithiothreitol, efficiently prevent the oxidation of cytoskeletal protein thiols, the increase in cytosolic free Ca^{2+} concentration and cell blebbing induced by redox cycling, but not alkylating, quinones. These results demonstrate that alkylating and redox cycling quinones cause similar structural and biochemical modifications of the cytoskeleton by means of different mechanisms, namely alkylation and oxidation of critical sulfhydryl groups.

KEY WORDS: Quinone, cytoskeleton, plasma membrane, bleb, calcium, actin, thiol.

INTRODUCTION

Plasma membrane blebbing (formation of multiple protrusions on the cell surface) is a common phenomenon during the development of cytotoxicity which often precedes cell death. Bleb formation has been described both *in vivo*[1,2] and *in vitro*[3-6] after exposure of animals, tissues, or cells to a variety of toxic conditions. The structural alterations underlying plasma membrane blebbing have not yet been clarified. However,it is generally believed that damage to cytoskeletal structure and organization occurs under these conditions. This view is supported by the demonstration that two cytoskeletal toxins, cytochalasin B and phalloidin, which interfere with the polymerization status of actin, cause bleb formation.[7,8]

Several biochemical mechanisms have been proposed to play a crucial role in inducing cytoskeletal alterations and plasma membrane blebbing during toxic cell injury. These include direct interaction of the toxin with cytoskeletal structures,[7]

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mitochondrial damage,[5] ATP depletion,[5] and disruption of thiol and Ca^{2+} homeostasis.[3,4] However, our knowledge of the mechanisms by which these alterations can result in surface blebbing is still incomplete.

We have recently investigated the cytoskeletal alterations in various freshly-isolated or cultured mammalian cells exposed to menadione (2-methyl-1,4-naphthoquinone). As a result of the oxidative stress generated during the metabolism of this quinone, microfilaments aggregate to form clusters and patches of actin in the perimembrane region.[9] In addition, there is a dissociation of α-actinin from the actin network and increased proteolysis of actin-binding protein(s).[10] The latter two effects appear to be directly associated with the increase in cytosolic free Ca^{2+} which occurs during the metabolism of toxic concentrations of this quinone.[11]

In this report, we provide structural evidence for the dissociation of plasma membrane and α-actinin from the actin microfilaments during quinone-induced bleb formation and compare the effects of redox cycling and alkylating quinones on surface structure, cytoskeletal protein thiols, cytosolic Ca^{2+} concentration, and ATP level in hepatocytes.

MATERIALS AND METHODS

Materials

Menadione, *p*-benzoquinone, Triton X-100, EGTA, DTNB, NBD-phallacidin, peroxidase-linked peanut lecitin and Quin-2/AM were obtained from Sigma. Collagenase (grade II) was from Boehringer-Mannheim. Culture media and fetal calf serum were obtained from Flow. Monoclonal anti-α-actinin antibodies and sheep anti-mouse IgG fluorescein-linked antibodies were obtained from Amersham. Monoclonal anti-EGF receptor antibodies were from Bio-Makor. Polyclonal antifibronectin antibodies were obtained from Biogenex. Polyclonal anti-β_2 microglobulin and anti-collagen were from Dako.

Isolation, Culture and Fractionation of Cells

Isolated hepatocytes were prepared from male Sprague-Dawley rats by collagenase perfusion of the liver as described by Moldeus *et al.*[12] and incubated at 10^6 cells/ml in Krebs-Henseleit buffer supplemented with 20 mM Hepes, pH 7.4. All incubations were performed in round-bottomed rotating flasks under an atmosphere of 95% O_2/5% CO_2. CG5 (human breast carcinoma) cells were cultured in Dulbecco's Modified Eagle's medium. K562 (human erythroleukemia) and THP (human myelomonocytic leukemia) cells were cultured in RPMI 1640 medium supplemented with non-essential amino acids. All cell culture media were supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin.

All treatments were performed in phosphate-buffered saline, containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.3. Menadione and *p*-benzoquinone were dissolved in dimethylsulfoxide (DMSO) and added to the media in a volume of DMSO not exceeding 0.2% of the total volume. Dithiothreitol (DTT) was dissolved in the same medium used for cell incubation.

For preparation of the cytoskeletal fraction, cells were extracted in a buffer containing 40 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 50 mM *Tris*/HCl, pH 7.5 and 1% Triton

X-100 and subsequently centrifuged at 4000 × g for 20 min to remove the soluble, non-cytoskeletal material. After washing, the pellet was solubilized in 8 M urea/1% SDS and processed for biochemical assays.

Structural Investigations

Light microscopy. Plasma membrane blebbing was evaluated as the percentage of cells exhibiting multiple surface protrusions. For sialoprotein detection, cells were fixed with 3.7% formaldehyde in phosphate buffer, pH 7.4, for 10 min at room temperature and treated with neuraminidase (type X, Sigma) in phosphate buffer, pH 6, for 30 min. Cells were then incubated with peroxidase-labelled peanut lecitin (Sigma) diluted in phosphate buffer (60 μg/ml) for 60 min at room temperature. Peroxidase activity was detected using a H_2O_2-diaminobenzidine solution. For collagen IV detection, cells were fixed as described above and treated with 0.5% Triton X-100 in phosphate buffer, pH 7.4, for 5 min at room temperature. Type IV collagen was visualized by the PAP technique. Briefly, cells were incubated with primary mouse antibodies for 10 min followed by washing and reincubation with rabbit anti-mouse antibodies for 10 min. An additional 10 min incubation was performed with linking goat anti-rabbit antibodies and rabbit peroxidase/antiperoxidase complex. Peroxidase activity was detected as described above.

Electron microscopy. Cells were fixed with 1.5% glutaraldehyde in cacodylate buffer for 30 min. For scanning electron microscopy, after fixation cells were washed twice in the same buffer used for fixation, dehydrated in graded ethanols, critical-point dried with CO_2 and gold-coated by sputtering. The samples were examined in a Philips 515 scanning electron microscope. For freeze-fracture analysis, unfixed cells were incubated for 30 min at 37°C with 25% glycerol, centrifuged at 1000 rpm for 10 min, put on carriers and quickly frozen in Freon 22, partially solidified by cooling with liquid nitrogen. The mounted carriers were then transferred into a Baizers BAF 300 freeze-etch unit, cleaved at − 100°C at a pressure of 2–4 × 10^{-7} mbar, shadowed with 2.5 μm of carbon-platinum (at an angle of 45°) and replicated with 20 nm of carbon. Cells were digested overnight by Clorox, and the replicas were mounted on naked 300 mesh grids and examined with a Zeiss electron microscope.

Fluorescence microscopy. For detection of actin and α-actinin, cells grown on coverslips were fixed in 3.7% formaldehyde in phosphate buffer, pH 7.4, for 10 min at room temperature. After washing, cells were treated with 0.5% Triton X-100 in the same buffer for 5 min at room temperature and then incubated with monoclonal anti-α-actinin antibodies, followed by anti-mouse IgG antibodies, or NBD-phallacidin. For detection of surface distribution of fibronectin, β_2 microglobulin and EGF receptor, cells were incubated with the specific antibodies before fixation in 3.7% formaldehyde and processed as described above.

Biochemical Assays

Cytoskeletal protein sulfhydryl groups were monitored as described by Dimonte *et al.*[13] Cytosolic free Ca^{2+} was assayed in accordance with Nicotera *et al.*,[11] using the fluorescent indicator Quin-2. Intracellular ATP was measured by the method of

Debetto and Bianchi,[14] and protein concentration was assessed as described by Lowry *et al.*[15]

RESULTS AND DISCUSSION

Biochemical Mechanisms Responsible for Quinone-Induced Bleb Formation

Cytotoxic concentrations of menadione cause bleb formation in freshly-isolated rat hepatocytes (Figure 1) and a variety of other mammalian cells.[16] Surface blebbing invariably precedes cell death, as monitored by the leakage of cytosolic enzymes or uptake or Trypan blue.

Early work from our laboratories suggested a close relationship between menadione-induced disruption of thiol and calcium ion homeostasis and surface blebbing.[3] The generation of active oxygen species during the metabolism of menadione in hepatocytes leads to the oxidation and depletion of intracellular glutathione (GSH) and protein sulfhydryl groups.[13,17] Among the protein thiols affected by this process are those located in actin and other cytoskeletal proteins (Figure 1). Oxidative cross-linking of actin to form high-molecular weight aggregates has been found to be closely associated with the appearance of surface blebs.[9] Moreover, the oxidation of free sulfhydryl groups in several Ca^{2+}-ATPases impairs the physiological regulation of intracellular Ca^{2+} homeostasis and causes a sustained increase in cytosolic free Ca^{2+} concentration[11,17] (Figure 1). Ca^{2+} is known to play a central role in regulating the organization of the cytoskeleton and its association with the inner surface of the

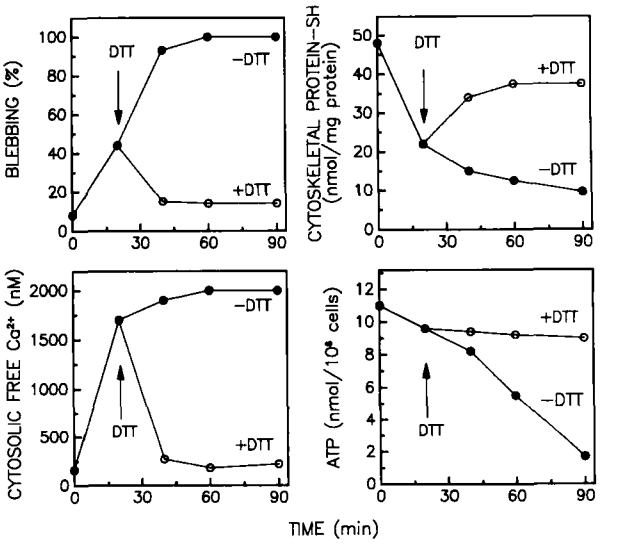

FIGURE 1 Menadione-induced surface blebbing, depletion of cytoskeletal protein sulfhydryl groups, increase in cytosolic free Ca^{2+} and loss of ATP in freshly-isolated hepatocytes. Hepatocytes (10^6 cells/ml) were incubated with 200 μM menadione and, at different time points, samples were taken for the measurement of the various morphological and biochemical parameters. Where indicated, a portion of the cells was harvested by centrifugation after 20 min incubation, washed and incubated in fresh medium lacking menadione but containing 2 mM dithiothreitol (DTT). One experiment typical of four is shown.

plasma membrane.[18] It is generally accepted that a toxic rise in cytosolic Ca^{2+} may activate secondary processes, leading to the disruption of normal cytoskeletal structure and generation of surface abnormalities.[19] This assumption is supported by the demonstration that incubation of isolated cells with the Ca^{2+} ionophore A23187 in the presence of extracellular Ca^{2+} results in bleb formation.

It has recently been proposed that ATP depletion may represent the critical event in bleb formation in cultured hepatocytes exposed to "chemical hypoxia".[5] During the metabolism of menadione in rat hepatocytes, the intracellular level of ATP declines progressively (Figure 1), most likely due to decreased ATP synthesis resulting from the collapse of the mitochondrial membrane potential and the inhibition of the glycolytic pathway.[20] However, it is unlikely that ATP depletion is the triggering event in surface blebbing caused by menadione. In fact, time-course experiments clearly demonstrate that ATP depletion follows, and does not precede, cell blebbing (Figure 1).

Under certain conditions (i.e. the removal of the toxic agent), the appearance of surface abnormalities may be reversible. The addition of the thiol reductant dithiothreitol (DTT) to menadione-treated cells prevented and, to some extent, reversed the depletion of cytoskeletal protein thiols and the increase in cytosolic free Ca^{2+} (Figure 1). These effects were associated with protection against menadione-induced blebbing. Taken together, these results suggest that menadione-induced bleb formation is associated with the oxidative modification of thiol groups in cytoskeletal proteins which are reversible upon treatment with thiol reductants.

Studies performed in freshly-isolated rat hepatocytes have demonstrated that certain substituted benzoquinones cause cytotoxicity without inducing oxidative stress.[21]

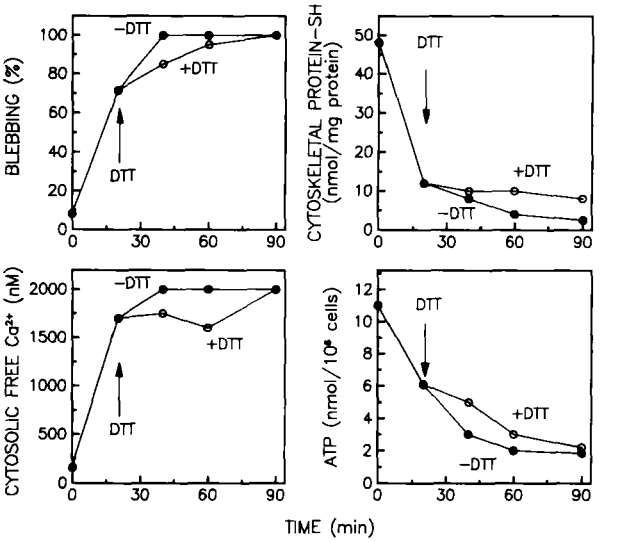

FIGURE 2 *p*-Benzoquinone-induced surface blebbing, depletion of cytoskeletal protein sulfhydryl groups, increase in cytosolic free Ca^{2+} and loss of ATP in freshly-isolated hepatocytes. Hepatocytes (10^6 cells/ml) were incubated with 150 μM *p*-benzoquinone and, at different time points, samples were taken for the measurement of the various morphological and biochemical parameters. Where indicated, a portion of the cells was harvested by centrifugation after 20 min incubation, washed and incubated in fresh medium lacking p-benzoquinone but containing 2 mM dithiothreitol (DTT). One experiment typical of four is shown.

This is observed with *p*-benzoquinone, which promotes extensive surface blebbing, depletion of cytoskeletal protein thiols, increase in cytosolic free Ca^{2+}, and ATP depletion (Figure 2). These alterations are almost identical to those caused by the metabolism of the redox cycling quinone, menadione. However, the addition of the thiol reductant, DTT, to *p*-benzoquinone-treated cells did not result in any significant protection (Figure 2). On the other hand, the inclusion of DTT in the incubation medium prior to the addition of *p*-benzoquinone resulted in complete protection (not shown). These results demonstrate that both redox cycling and alkylating quinones cause depletion of cytoskeletal protein thiols and elevation of cytosolic free Ca^{2+} concentration in hepatocytes, and that these effects are closely related to the appearance of surface blebs. However, the different sensitivity to DTT of *p*-benzoquinone-treated cells as compared to menadione-treated cells suggests that different mechanisms (alkylation *vs* oxidation) are responsible for these alterations.

Structural Characteristics of Quinone-Induced Bleb Formation

Figure 3A illustrates cultured CG5 cells exhibiting surface blebs following incubation with a cytotoxic concentration of menadione. Blebs appear as protrusions arising from the plasma membrane which grow in size with concomitant loss of fine structural details and microvilli.

An increased lateral diffusion of surface receptors associated with blebbing has been demonstrated and postulated to reflect major alterations in the relationship between the cytoskeleton and the inner surface of the plasma membrane.[22] Studies performed in various cell types have demonstrated that the actin network is not associated with the plasma membrane directly but via a series of actin-binding proteins including α-actinin.[23] Dissociation of the actin microfilaments from their anchoring domains would lead to the generation of sites of weakness where blebs could be formed.

The cytochemical localization of actin and the actin-binding protein, α-actinin, provides structural evidence for this dissociation in menadione-treated cells. As illustrated in Figure 3B, C, and D, blebs formed following menadione treatment are completely devoid of actin but appear to contain detectable amounts of α-actinin. This observation is strictly in keeping with the demonstration that α-acinin is released from the actin network in human platelets exposed to menadione.[10] Interestingly, this process appears to be Ca^{2+}-dependent. In fact, conditions which prevent the increase in cytosolic free Ca^{2+} during the metabolism of menadione efficiently protect against the dissociation of α-actinin from the cytoskeleton.[10] These findings are also consistent with the demonstration that Ca^{2+} regulates the binding of actin to α-actinin *in vitro* and that a sustained increase in cytosolic free Ca^{2+} above the physiological level results in the dissociation of this protein from microfilaments.[24]

The modification of the cytoskeletal protein localization within the blebs is also associated with a different expression of other integral proteins. This is easily observed when the distribution of intramembrane particles is studied by means of freeze-fracture analysis of menadione-treated cells. As illustrated in Figure 3E, the exoplasmic fracture face of the bleb is virtually devoid of intramembrane particles, whereas the protion of the plasma membrane not surrounding the bleb contains numerous randomly distributed particles. Detailed immunocytochemical localization of several membrane proteins has revealed that sialoproteins, collagen IV, fibronectin and β_2 microglobulin are only localized in the portions of plasma membrane not affected by

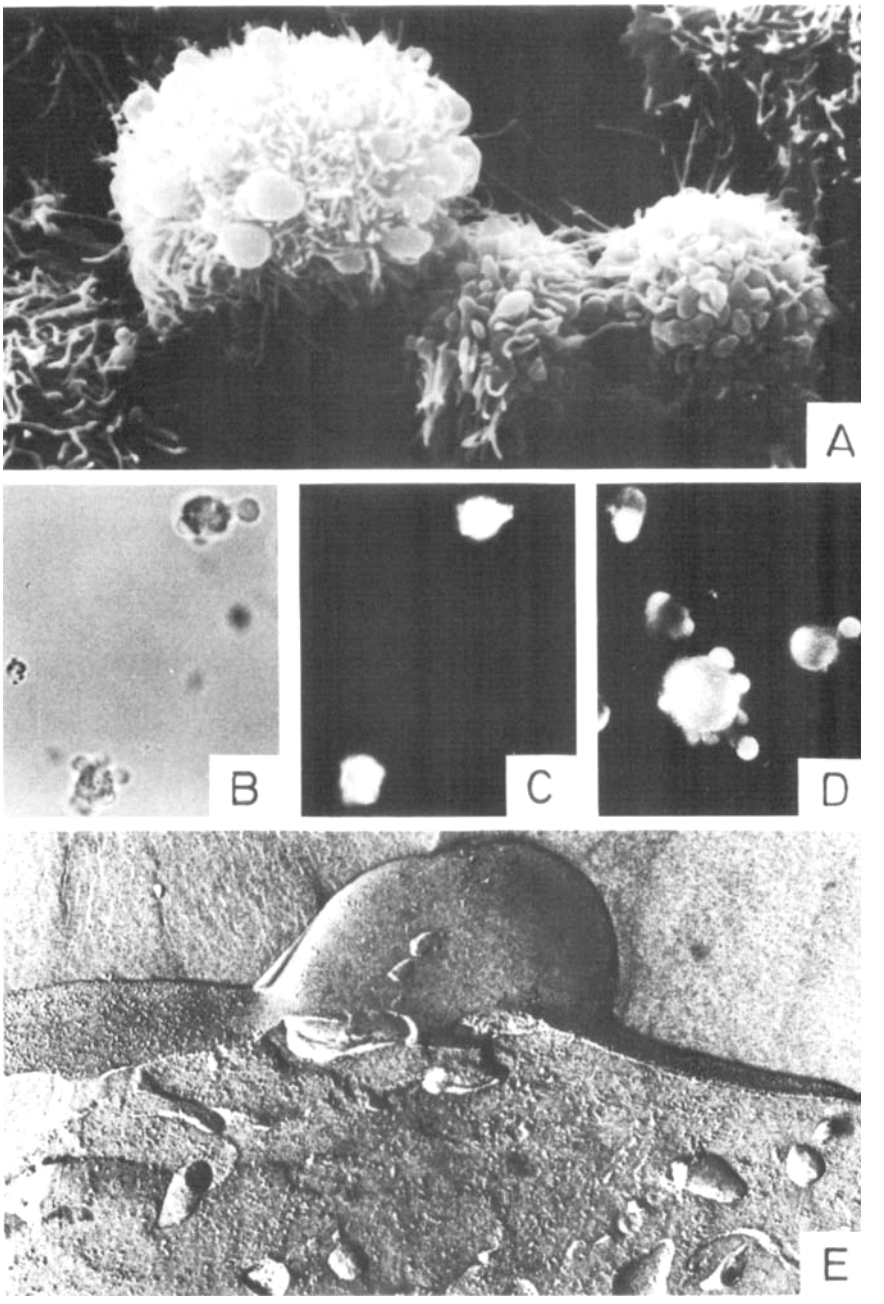

FIGURE 3 Morphological, structural and cytoskeletal alterations induced by menadione in cultured mammalian cells. **Panel A:** Scanning electron microscoopy of CG5 cells exhibiting numerous surface blebs following incubation with 200 μM menadione for 60 min. **Panels B and C:** Brightfield and fluorescence staining of actin in THP cells incubated with 200 μM menadione for 60 min. **Panel D:** Immunofluorescence staining of α-actinin in THP cells incubated with 200 μM menadione for 60 min. **Panel E:** Freeze-fracture analysis of K562 cells incubated with 200 μM menadione for 60 min.

TABLE 1
Distribution of Surface Proteins in Menadione-Exposed CG5 Cells

Proteins	Unaffected plasma membrane	Bleb-surrounding plasma membrane
Sialoproteins	+	−
Collagen fraction IV	+	−
Fibronectin	+	−
β_2 microglobulin	+	−
EGF receptors	+	+

CG5 cells were treated with 200 μM menadione for 60 min and then processed for microscopic visualization of the indicated proteins as described in Methods. (+) indicates a positive, and (−) a negative, immunocytochemical reaction in the portion of the plasma membrane indicated.

the blebbing process. The biochemical mechanisms involved in this redistribution of membrane proteins have not been investigated in detail. However, it is conceivable that cytoskeletal alterations similar to those causing bleb formation may be involved.

CONCLUSIONS

Taken together, our findings demonstrate that the administration of cytotoxic concentrations of both redox cycling and alkylating quinones to mammalian cells is associated with marked cytoskeletal alterations. These appear to result from the direct modification of cytoskeletal protein thiols (by oxidation or alkylation) as well as from the increase in cytosolic Ca^{2+} observed in quinone-exposed cells. The calcium ion seems to exert its effect either directly, by promoting the dissociation of polypeptides from the cytoskeleton, or indirectly through the activation of Ca^{2+} dependent proteases. The resulting formation of "sites of weakness", where the cytoskeleton has dissociated from the plasma membrane, may lead to the production of surface blebs.

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