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Adriamycin Induces Protein Oxidation in Erythrocyte Membranes

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Abstract: Adriamycin is an anthracycline antineoplastic agent whose clinical effectiveness is limited by severe side effects, including cardiotoxicity. A current hypothesis for adriamycin cardiotoxicity involves free radical oxidative stress. To investigate this hypothesis in a model system, we applied the technique of immunochemical detection of protein carbonyls, known to be increased in oxidized proteins, to study the effect of adriamycin on rat erythrocyte membranes. Erythrocytes obtained from adriamycin-treated rats demonstrated an increase of carbonyl formation in their membrane proteins. Yet, in separate experiments when adriamycin was incubated with rat erythrocyte ghosts, there was no significant increase of membrane protein carbonyls detected. In contrast, isolated erythrocytes incubated with an adriamycin-Fe^{3+} complex exhibited a robust carbonyl incorporation into their membrane proteins in a time-dependent manner. The level of carbonyl formation was dependent upon the concentration of Fe^{3+} known to form the adriamycin-Fe^{3+} complex. When the time course between protein carbonyl formation and lipid peroxidation was compared, protein carbonyl detection occurred earlier than lipid peroxidation as assayed by thiobarbituric acid reactive substances formation. These results are consistent with the notion that oxidative modification of membrane proteins may contribute to the development of the acute adriamycin-mediated toxicity.

Adriamycin is an anthracycline derivative and is one of the most frequently used antineoplastic agents in the treatment of leukaemias and solid tumours (Weiss *et al.* 1986). The therapeutic mechanism of action of adriamycin is thought to result from its capability to either intercalate between the bases of helical DNA or inhibit topoisomerase II, thereby inhibiting DNA replication and/or transcription (Crooke *et al.* 1973; Gabbay *et al.* 1976; Tewey *et al.* 1984; Potmesil *et al.* 1987). Clinical effectiveness is restricted due to dose-limiting toxic side effects, such as myelotoxicity, alopecia, gastrointestinal disturbances and irreversible cardiomyopathy (Lefrak *et al.* 1973; Arena *et al.* 1975). The cardiotoxic effects of adriamycin may appear either in an acute phase or a delayed phase (Villani *et al.* 1986). The acute phase is characterized by electrocardiogram abnormalities and contractile impairment, while the delayed cardiotoxicity resembles congestive heart failure that is poorly responsive to digitalis therapy (Monti *et al.* 1986; Timour *et al.* 1988; Steinhertz *et al.* 1991). Although the pathology has been well described and documented, the mechanism of induced cardiotoxicity by adriamycin is not fully understood.

The adriamycin-induced acute cardiotoxicity has a rapid onset (minutes to hours) and persists as long as the drug is detectable in cardiac tissue (Monti *et al.* 1986; Timour *et al.* 1988). One hypothesis for the mechanism for the acute toxicity is the involvement of free radicals. Adriamycin has been demonstrated to be a potent generator of free radicals by two distinct mechanisms. An enzymatic pathway may occur in which adriamycin, in the quinone form, accepts an electron from reactions catalyzed by NADH dehydrogenase, xanthine oxidase, or NADPH cytochrome P-450 reductase leading to a semiquinone free radical. Upon recycling of the semiquinone to the quinone, an electron is transferred to molecular oxygen, creating superoxide radical anion (Handa & Sato 1975; Goodman & Hochstein 1977; Bachur *et al.* 1978). A second mechanism for adriamycin free radical generation may involve an adriamycin-Fe^{3+} complex (Myers *et al.* 1982; Sugioka & Nakano 1982; Nakano *et al.* 1984; Gutteridge 1984; Zweier 1984). This complex undergoes redox cycling and reduces molecular oxygen to reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radical (Olson & Mushlin 1990). Conceivably, these reactive ocygen species could then damage membranes or macromolecules with subsequent myocardial injury.

In clinical practice adriamycin is administered via intravenous injection, which presents erythrocytes as a possible target to interact with the drug. The ability of adriamycin to interact with erythrocyte membranes has been demonstrated by several authors (Mikkelsen *et al.* 1977; Shinohara & Tanaka 1980; Myers *et al.* 1982; Arancia *et al.* 1988; Miura *et al.* 1991). Mikkelsen *et al.* (1977) have demonstrated that binding of adriamycin to red blood cells

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changes the architecture of erythrocyte plasma membranes. Adriamycin-Fe^{3+} complex has been demonstrated to bind spectrin, the major peripheral erythrocyte membrane protein (Mikkelsen *et al.* 1977). As a result of these studies, the possibility that adriamycin induces oxidative damage in the blood has been proposed. Many studies have demonstrated lipid peroxidation of erythrocyte membranes as a consequence of the interaction with adriamycin-derived free radicals (Myers *et al.* 1982; Nakano *et al.* 1984; Miura *et al.* 1991 & 1994). Adriamycin-derived free radicals conceivably could damage membrane proteins as well as lipids. However, oxidative modification of erythrocyte membrane proteins by adriamycin has not been reported yet. It is reported that free radicals, generated by adriamycin, may induce oxidative modification of proteins as demonstrated recently by adriamycin-Fe^{3+}-inactivation of rabbit muscle creatine kinase *in vitro* (Thomas *et al.* 1994).

Oxidative damage of proteins results in chemical modification of a variety of amino acid residues; protein carbonyls formed by oxidation of several amino acids side chains are often employed as a marker of protein oxidation (Levine 1983; Farber & Levine 1986; Stadtman 1993; Butterfield & Stadtman 1997; Aksenov *et al.* 1997). This report documents that adriamycin is able to cause oxidative modification of erythrocyte membrane proteins.

Materials and Methods

Chemicals. Adriamycin HCl (manufactured by Pharmacia) was purchased from the University of Kentucky Hospital Pharmacy. Sodium citrate, NaCl, Na_2HPO_4, NaH_2PO_4, EDTA, phenylmethylsulfonyl fluoride, iron (III) chloride, deferoxamine mesylate, thiobarbituric acid, electrophoresis reagents and iron (II) sulfate were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and drug treatments. All procedures involving rodents were approved by the University of Kentucky Institutional Animal Care and Use Committee. Rats were housed in a temperature- and humidity-controlled, 12-hr light/12-hr dark environment. All rodents were fed commercial rat chow and water *ad libitum*. *In vivo* studies were performed using adult male Sprague-Dawley rats weighing 350-400 g. Two groups of 6 animals received adriamycin (10 mg/kg intraperitoneally×2 every third day) or saline according to the same schedule (Mimnaugh *et al.* 1983; Floyd *et al.* 1986). On day four, the rats were anaesthetized by pentobarbital injection and blood was collected by exsanguination. For *in vitro* studies blood was collected from 6 male Sprauge-Dawley rats. In all groups, collected blood was mixed in a 3.8% sodium citrate solution (9 parts blood to 1 part sodium citrate) as an anticoagulant and used for erythrocyte ghost preparation.

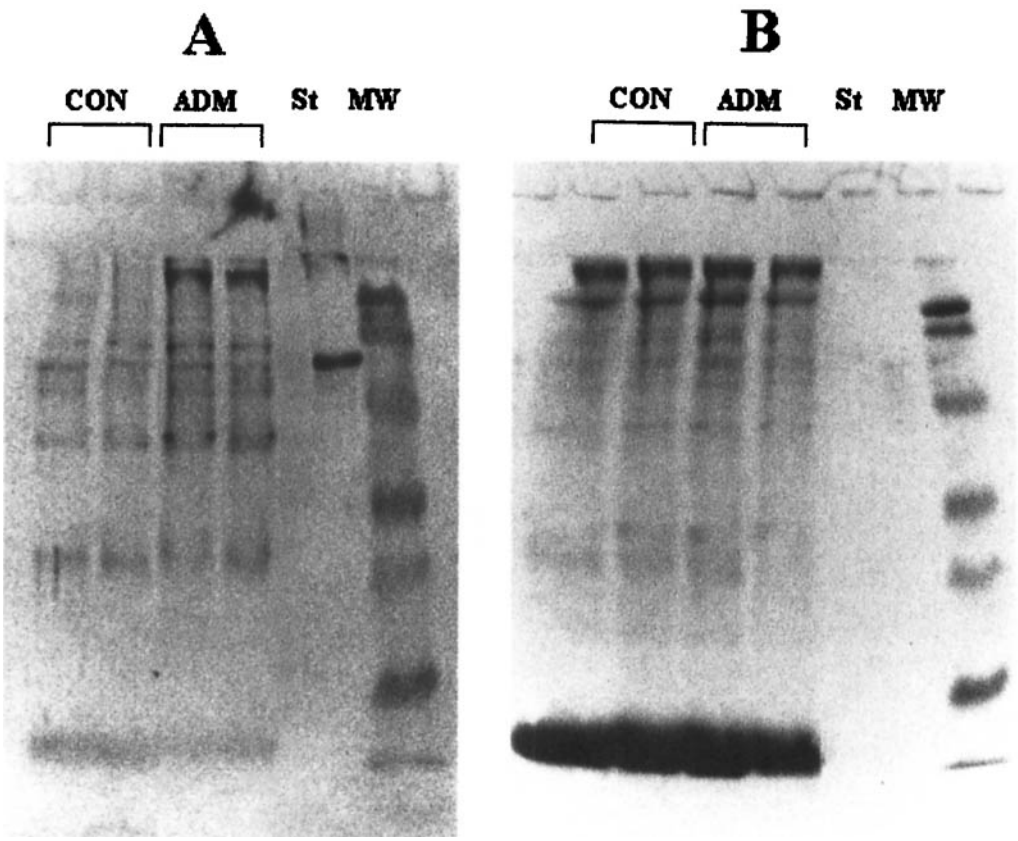

Fig. 1. Typical result of immunochemical detection for carbonyl formation in erythrocyte membranes from control (CON) and adriamycin-treated (ADM) rats. (A) anti-dinitrophenylhydrazone staining of control and adriamycin-treated rat erythrocytes. (B) Coomassie Brilliant Blue staining of control and adriamycin-treated rat erythrocytes. St=oxidized BSA standard (10.9 nmol carbonyl/mg protein); Mw=molecular weight standards.

Preparation of erythrocyte membranes. Erythrocyte membranes were prepared as described by Hoefner *et al.* (1997) with minor alterations. Briefly, whole blood containing sodium citrate was centrifuged for 5 min at 1200×*g*; plasma and buffy coat were replaced with 299 mOsm phosphate buffered saline (PBS). Following two additional washes, the haematocrit was adjusted to 50% and the cells were chilled in an ice bath. Erythrocyte membranes (ghosts) were prepared by adding cold 20 mOsm PBS (containing 0.5 mM phenylmethylsulfonyl fluoride and 1mM EDTA) followed by gentle mixing. Following a 5 min. cold incubation, ghosts were pelleted by centrifugation at 14,000×*g* for 15 min. at 4°. The supernate was removed, followed by one wash with cold 299 mOsm PBS, which resulted in pink ghosts.

Erythrocyte membrane treatment and incubation. Erythrocyte membranes were incubated in 10 mM HEPES buffer, pH 7.4, with either 50 μM adriamycin (ADM)/25 μM $FeCl_3$, or vehicle (double deionized water) at 37° for 0, 30, 60, and 90 min. The final concentration of erythrocyte membrane protein in each preparation was 4 mg/ml. Incubations of erythrocyte ghosts with 50 μM ADM alone, 25 μM $FeCl_3$ alone, 50 μM ADM /10 μM $FeCl_3$, and 1 mM H_2O_2/ 25 μM $FeSO_4$ were performed under the same experimental conditions. Ten μM deferoxamine mesylate was added to terminate the reactions, and the samples were immediately frozen for future analysis.

Electrophoresis and Western blot analysis. To demonstrate the scale of protein oxidation to erythrocyte membranes, an Oxidized Protein Detection Kit (Oxyblot, ONCOR Gaithersburg, MD, USA) was utilized. The basis for this procedure is the immunochemical detection of protein carbonyls following derivatization with 2,4-dinitrophenylhydrazine (Levine *et al.* 1990 & 1994). The samples were derivatized by 2,4-dinitrophenylhydrazine solution and neutralized with a 2M Tris containing 30% glycerol and 19% 2-mercaptoethanol. The samples (4 μg of protein per lane) were then loaded onto the gel for electrophoresis. Oxidized bovine serum albumin (BSA), containing an established concentration of carbonyls (10.95 nmol of carbonyls/mg of protein) was treated with 10 mM 2,4-dinitrophenylhydrazine and loaded as a standard with each set of samples. For the standard preparation the BSA (Standard for Gel Filtration Chromatography, Cat# A3581, Sigma) was dissolved in deionized water at 2 mg/ml and oxidized by Fe^{2+}/H_2O_2 (100 mM/1 mM subsequently) for 2 h at 37°. The reaction was stopped with deferoxamine mesylate and the small molecular weight substances were removed from the protein by passage through a Sephadex G-25 desalting column. The concentration of carbonyl groups per mg of protein was determined by colorimetric carbonyl assay (Levine 1983). Polyacrylamide gel electrophoresis (SDS-PAGE) was performed in mini-slabs (0.75×60×70 mm, 12% total acrylamide) using a modified procedure by Laemmli (1970). Following electrophoresis, the gels were stained with Coomassie brilliant blue or transferred onto nitrocellulose for immunoblotting. Western blotting was performed according to the procedure adapted from Glenney (1986). The transfer buffer contained Tris-Glycine pH 8.5 with 20% methanol. Following Western blotting, the membrane was blocked with a 3% BSA (in PBS with sodium azide, 0.01%, and Tween-20 0.2%) for 1 hr at room temperature. The transfer of proteins was complete within two hours. The primary antibody was a rabbit anti-dinitrophenylhydrazone (1:150 working dilution) supplied by ONCOR. The secondary antibody (anti-rabbit IgG conjugated with alkaline phosphatase, Sigma Chemical Co.) was diluted in a blocking solution (1:15000 working dilution) and incubated with the membrane for 1 hour at 37°. Following each incubation the membrane was washed in buffer (PBS with 0.01% sodium azide and 0.2% Tween 20) for 10 min. at room temperature. The washed membrane was developed using a 5-bromo-4-chloro-indolyl phosphate/nitro blue tetrazolium (BCIP-NBT) solution (SigmaFast tablets, Sigma).

Our results showed that the oxidation of BSA with hydrogen peroxide and iron (Fenton reagent) increases the intensity of anti-dinitrophenylhydrazone staining. Different amounts of oxidized BSA standard were loaded on the gel to analyze the relationship between the density of anti-DNP stain and the amount of protein carbonyls loaded. The dependence between the amount of protein carbonyl and the density of immunostaining was found to be linear within the range from 0.5 pmol to 2.4 pmol of carbonyl loaded.

Lipid peroxidation in erythrocyte membranes. Erythrocytes were incubated with adriamycin and iron as previously described. At time points of 0, 30, 60, and 90 min., 0.5 mg portions (130 μl) were removed for analysis. Lipid peroxidation was determined on erythrocyte membranes from control and adriamycin-Fe^{3+} treatment by measuring thiobarbituric acid reactive substances (Buege & Aust 1978).

Image analysis. Western blots and Coomassie brilliant blue stained gels were digitized and quantified by computer assisted imaging using MCID/M4 software supplied by Imaging Research Inc. (Ontario, Canada).

Statistical analysis. Statistical comparisons were made using ANOVA followed by Student Newman-Keuels' post hoc test for multiple comparisons. Significance was set at $P<0.01$.

Results

To investigate the possibility of oxidative modification to erythrocyte membrane proteins as a result of adriamycin administration, erythrocyte ghosts were prepared from control or adriamycin-treated rats, and changes of protein carbonyl content were determined by the semi-quantitative analysis of the Western blots stained with anti-DNP anti-

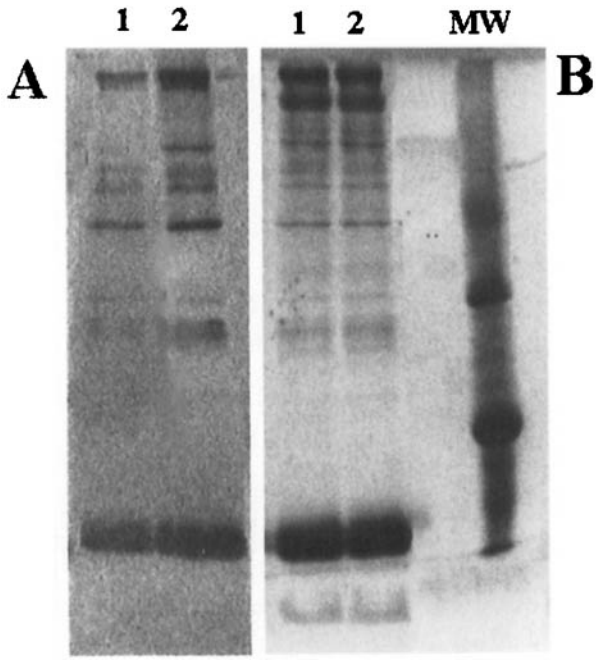

Fig. 2. Typical result of immunochemical detection for carbonyl formation in control and adriamycin-Fe^{3+}-treated erythrocyte membranes. (A) anti-dinitrophenylhydrazone staining of control (1) and 50 μM adriamycin/25 μM $FeCl_3$-treated pink erythrocyte ghosts (2) after 60 min. of incubation. (B) Coomassie Brilliant Blue staining of control (1) and 50 μM adriamycin/25 μM $FeCl_3$-treated pink erythrocyte ghosts (2) after 60 min. of incubation.

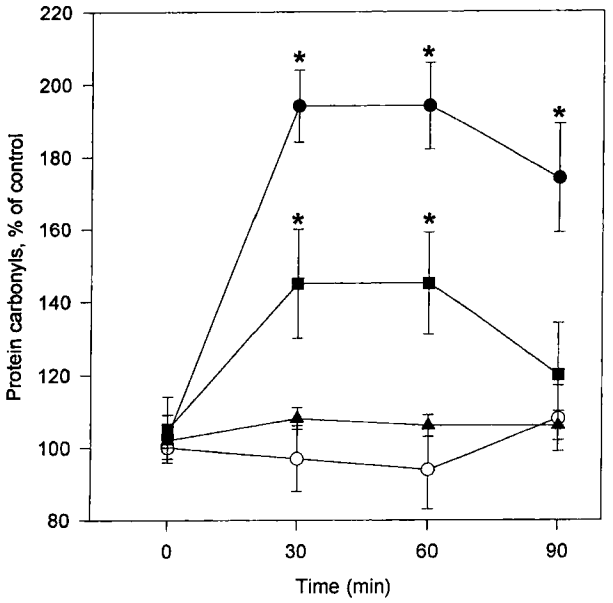

Fig. 3. Time-course of protein oxidation in erythrocyte membranes induced by adriamycin-Fe^{3+} complex: the effect of different Fe^{3+}concentrations. Open circle: control; closed circle: 50 μM adriamycin /25 μM $FeCl_3$; closed box: 50 μM adriamycin /10 μM $FeCl_3$; closed triangle: 50 μM adriamycin. Data are presented as mean±S.E.M., N=6, *P≤0.01 versus control.

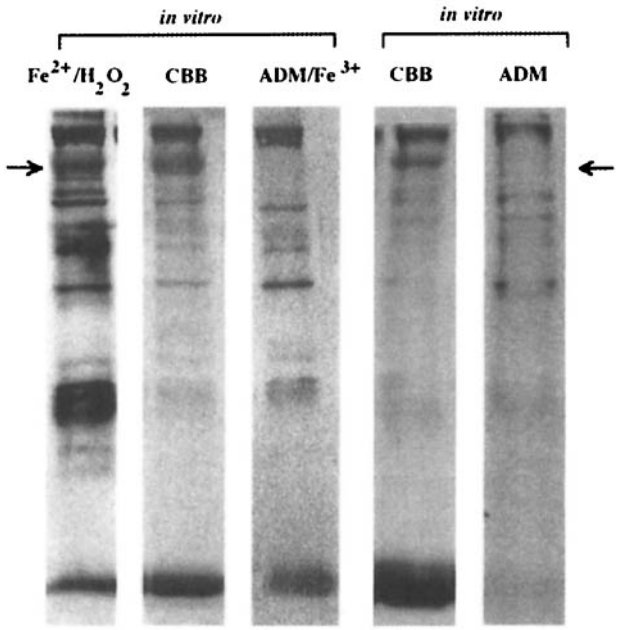

Fig. 4. Comparison of typical immunochemical detection (anti-dinitrophenylhydrazine staining) and Coomassie Brilliant Blue (CBB) stain for erythrocytes incubated with 1 mM H_2O_2/ 25 μM $FeSO_4$, or 50 μM adriamycin /25 μM $FeCl_3$ for 60 min. and erythrocytes obtained from adriamycin-treated (ADM) rats. Arrows indicate the protein band not oxidized by adriamycin-Fe^{3+}.

body. To confirm that the protein pattern is equal in control and adriamycin-treated samples, we performed the Coomassie Brilliant Blue staining in parallel with the transfer. The results of the Coomassie Brilliant Blue staining, of adriamycin-treated erythrocyte ghost preparations indicated that no loss of protein occurred, suggesting that potential degradation of oxidized proteins was not operative. The comparison of the integrated intesities of anti-dinitrophenylhydrazone staining in controls and adriamycin-treated samples demonstrated a significant increase of protein carbonyl content in the erythrocyte membranes obtained from rats injected with adriamycin. Protein carbonyl content in erythrocyte membranes of adriamycin-treated rats was found to be 208%±28% (mean±S.E.M., P<0.01) that of controls (fig. 1A,B,).

Our *in vitro* experiments demonstrate the inability of adriamycin alone to induce the oxidative damage. When isolated erythrocyte membranes were treated with adriamycin alone, there was no significant increase of carbonyl content. In order to model the oxidative effect observed *in vivo*, an adriamycin/ Fe^{3+} complex, a known adriamycin generator of free radicals, had to be added to the suspension of erythrocytes. Only under these conditions was the increase of membrane protein carbonyl formation observed (fig. 2). After 60 min. of incubation the increased carbonyl content was determined to be 194%±10% (mean±S.E.M., P<0.01) as that of controls (fig. 3). The results in fig. 3 also demonstrate the carbonyl formation occurred in a time-dependent manner. In addition, as illustrated in fig. 3, the magnitude of carbonyl content was dependent upon the concentration of Fe^{3+} added to adriamycin.

Probing of *in vivo* or *in vitro* Western blots from erythrocyte membranes with anti-dinitrophenylhydrazone antibodies typically showed that the pattern of oxidized protein was not identical to the pattern obtained after Coomassie Brilliant Blue staining of the gel (fig. 4). One of the major membrane proteins bands, of approximate 90 kDa MW,

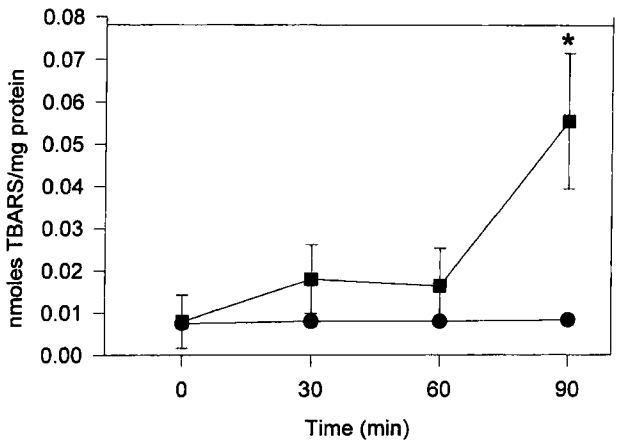

Fig. 5. The effect of adriamycin/$FeCl_3$ upon thiobarbituric acid reactive substances formation (TBARS) when incubated with erythrocyte membranes. Closed circle: control; closed box: 50 μM adriamycin/25 μM $FeCl_3$. Data are presented as mean±S.E.M., N=6, *P≤0.01 versus control.

probably Band 3, did not display significant increased carbonyl content either in the *in vitro* or *in vivo* experiments. In contrast with erythrocytes incubated with 1 μM H_2O_2 and 25 μM Fe^{2+}, the pattern of oxidized proteins obtained was the same as the Coomassie staining.

Consistent with the results obtained by Miura *et al.* (1991), we observed that the adriamycin-Fe^{3+} complex is able to induce lipid peroxidation in erythrocyte membranes. As illustrated in fig. 5, 50 μM adriamycin/25 μM Fe^{3+} caused continuous accumulation of thiobarbituric acid reactive substances formation during the total period of incubation (90 min.). The thiobarbituric acid reactive substances formation content increased from 0.008 (±0.006) (S.E.M.) nmoles per mg of protein at the zero time point to 0.055 (±0.016) (S.E.M.) nmoles per mg of protein after 90 min. of incubation.

Discussion

There is considerable agreement that the signs and symptoms of adriamycin toxicity may be related to its ability to generate free radicals. Much evidence supports the observation of membrane modification as a result of lipid peroxidation by adriamycin (Myers *et al.* 1977; Wang *et al.* 1980; Doroshow 1983; Nakano *et al.* 1984; Singal *et al.* 1985; Miura *et al.* 1994; Siveski-Iliskovic *et al.* 1994). Previous studies have demonstrated lipid peroxidation to be detrimental to erythrocyte membrane structure and function (Shinohara & Tanaka 1980; Arancia *et al.* 1988). However, our results demonstrate that oxidative modification to membrane proteins is also a consequence of adriamycin treatment, suggesting that membrane modification by adriamycin is not solely dependent upon the occurrence of lipid peroxidation. This observation may be a vital component regarding the mechanism of erythrocyte altered structure and function observed during adriamycin administration (Arancia *et al.* 1988).

Our *in vivo* observations have demonstrated that the oxidative modification of erythrocyte membrane proteins occurs upon the administration of adriamycin in rats. This finding is consistent with the report of Floyd *et al.* (1986), who observed a 4-fold increase of dihydroxybenzoic acid-reactive products in the blood from rats treated with adriamycin. Based upon the data obtained using this analytical procedure, which is sensitive to *in vivo* $OH^{\cdot}$ production, the authors proposed that adriamycin administration induces $OH^{\cdot}$-mediated oxidative damage in different tissues.

Numerous studies have suggested that the ability of adriamycin to generate free radicals requires its chemical modification (Handa & Sato 1975; Goodman & Hochstein 1977; Bachur *et al.* 1978; Olson *et al.* 1981; Sugioka & Nakano 1982; Nakano *et al.* 1984; Gutteridge 1984; Zweier 1984). The results of our *in vitro* experiments consistently showed that adriamycin alone is unable to induce oxidative damage in red blood cell membranes. When adriamycin was incubated with rat erythrocyte ghosts, no significant increase of protein carbonyl formation has been observed. However, when adriamycin/Fe^{3+}complex was incubated with erythrocyte ghosts, a robust carbonyl formation was observed in membrane proteins. These results suggest that when injected into the blood stream adriamycin undergoes the modification by one of possible mechanisms such as, an iron-dependent or enzymatic transformation. After an injection, adriamycin might be reduced by several enzymatic systems to the semiquinone radical, which not only donates electrons to oxygen to yield superoxide thus leading to hydrogen peroxide, but also reacts directly with H_2O_2 to yield $OH^{\cdot}$ (Thornally & Dodd 1985). Alternatively, adriamycin might possibly react with iron in the blood. Zweier (1984) demonstrated that adriamycin can abstract iron from ferritin, which suggest that bound iron may contribute to the drug's free radical production. As a result of this modification of adriamycin, the probable production of free radicals may cause damage to membrane bound proteins.

The oxidative modification of erythrocyte membrane proteins by adriamycin and H_2O_2 / Fe^{2+} were compared in separate experiments. Our results demonstrated the ability of H_2O_2 / Fe^{2+} to exhibit a different pattern of oxidized membrane proteins than those observed with adriamycin *in vivo* or adriamycin/Fe^{3+} *in vitro*. In particular, the band-3 protein of the erythrocyte membrane was not oxidized by either adriamycin or adriamycin/Fe^{3+}; however, when incubated with H_2O_2 / Fe^{2+}, this protein band was found to be anti-dinitrophenylhydrazone positive. This result may be due to the ability of adriamycin to bind to many different membrane components, especially proteins. Following the binding of adriamycin to the membrane proteins, and the subsequent formation of free radicals, protein targets in the vicinity of these free radicals would be the probable targets for oxidative modification. In contrast, the erythrocytes incubated with H_2O_2 / Fe^{2+} did not display any specificity to protein targets, which may be due to the lack of H_2O_2 / Fe^{2+} receptor binding to the membrane. Alternately, the ability of the latter to induce lipid peroxidation could account for widespread protein modification, in contrast to the more targeted oxidation induced by adriamycin.

Since lipid peroxidation products such as 4-hydroxy-2-trans-nonenal or malonaldehyde contain carbonyl functionalities, our findings do not rule out the possibility that lipid oxidation products could be causative agents responsible for the oxidatively modified membrane proteins (Subramanian *et al.* 1997). However, the *in vitro* data reported in this study suggest that protein oxidation is a parallel mechanism occurring in concert with lipid peroxidation, with the magnitude of protein oxidation manifesting at an earlier interval than the detection of lipid modification.

The oxidation of erythrocyte membrane proteins by adriamycin may contribute to the development of its acute cardiac toxicity. As proposed by Jian *et al.* (1993), free radical reactions in the blood may lead to abnormality of haemorheological properties and may damage certain tissue cells, including cardiomyocytes, indirectly by reducing the supply of oxygen and nutrients. Oxidative modification of membrane proteins by adriamycin-derived free radicals may compro-

mise membrane functions and induce morphological changes of the erythrocytes. The ability of adriamycin to affect the erythrocyte morphology has been demonstrated previously by the work of Arancia *et al.* (1988), who demonstrated morphological changes in the membranes of human red blood cells when incubated with adriamycin. Scanning and freeze-fracture electron microscopy revealed both changes in erythrocyte shape and in the membrane ultrastructure. It has been reported that the activity of several membrane-bound enzymes may be modified by adriamycin (Kessel 1979; Dasdia *et al.* 1979; Murphree *et al.* 1981; Caroni *et al.* 1981; Demant & Jensen 1983). The adriamycin-mediated oxidative damage of erythrocytes may lead to the decrease of their ability to transport oxygen. Changes in the morphology of red blood cells, induced by their interaction with adriamycin, might affect their rigidity and flexibility. Along with the impairment of cardiac function by adriamycin, the oxidative damage of erythrocytes induced by adriamycin-derived free radicals may contribute to the development of acute cardiotoxicity, known to be one of the major toxic side effects of adriamycin therapy. Studies to test this possibility are in progress.

Acknowledgements

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References

Aksenov, M., M. Aksenova, J. Carney & D. A. Butterfield: Oxidative modification of glutamine synthetase by amyloid beta peptide. *Free Rad. Res.* 1997, **27**, 267–281.

Arancia, G., A. Molinari, P. Crateri, A. Calcabrini, L. Silvestri & G. Isacchi: Adriamycin-plasma membrane interaction in human erythrocytes. *Eur. J. Cell. Biol.* 1988, **47**, 379–387.

Arena, E., M. Arico, F. Biondo, N. Dusonchet, L. Gebbia, F. Gerbasi, R. Sanguedolce & L. Rausa: Analysis of some probable factors responsible for adriamycin-induced cardiotoxicity. In: *2nd International Symposium on Adriamycin*. Belgium European Press, Medikon, 1974. pp 160–172.

Bachur, N. R., S. L. Gordon & M. V. Gee: A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res.* 1978, **38**, 1745–1750.

Buege, J. A. & S. D. Aust: Microsomal lipid peroxidation. *Meth. Enzym.* 1978, **152**, 302–310.

Butterfield, D. A. & E. R. Stadtman: Protein oxidation processes in aging brain. In: *The aging brain*. Eds.: M. P. Mattson and J. W. Geddes. JAI Press, Greenwich, 1997, 161–191.

Caroni, P., F. Villani & E. Carafoli: The cardiotoxic antibiotic doxorubicin inhibits the Na^+/Ca^{2+} exchange of dog heart sarcolemmal vesicles. *Fed. Eur. Biochem. Soc. Lett.* 1981, **130**, 184–186.

Crooke, S. T., V. H. Duvernay, L. Galvan & A. Prestakyo: Structure-activity relationship of anthracycline relative to effects on macromolecular syntheses. *Mol. Pharmacol.* 1973, **14**, 290–298.

Dasdia, T., A. DiMarco, M. Goffredi, A. Minghetti & A. Necro: Ion level and calcium fluxes in HeLa cells after ADR treatment. *Pharmacol. Res. Commun.* 1979, **11**, 19–29.

Demant, E. J. & P. K. Jensen: Destruction of phospholipids and respiratory activity in pig-heart submitochondrial particles induced by an adriamycin-iron complex. *Eur. J. Biochem.* 1983, **132**, 551–556.

Doroshow, J. H.: Effect of anthracycline antibiotics on oxygen radical formation in rat heart. *Cancer Res.* 1983, **43**, 460–472.

Farber, J. M. & R. L. Levine: Sequence of peptide susceptible to mixed function oxidation. Probable cation binding site in glutamine synthetase. *J. Biol. Chem.* 1986, **261**, 4574–4578.

Floyd, R. A., R. Henderson, J. J. Watson & P. K. Wong: Use of salicylate with high pressure liquid chromatography and electrochemical detection (LCED) as a sensitive measure of hydroxyl free radical in adriamycin treated rats. *J. Free Rad. Biol. Med.* 1986, **2**, 13–18.

Gabbay, E. J., D. Crier, R. E. Reimer, R. Levy, S. W. Pearce & W. D. Wilson: Interaction specificity of the anthracycline with deoxyribonucleic acid. *Biochemistry* 1976, **15**, 2062–2070.

Glenney, J. R.: Antibody probing on western blots have been stained with India ink. *Anal. Biochem.* 1986, **156**, 315–318.

Goodman, J. & P. Hochstein: Generation of free radicals and lipid peroxidation by redox cycling of adriamycin and daunomycin. *Biochem. Biophys. Res. Commun.* 1977, **77**, 797–803.

Gutteridge, J. C.: Lipid peroxidation and possible hydroxyl radical formation stimulated by the self-reduction of doxorubicin-iron (III) complex. *Biochem. Pharmacol.* 1984, **33**, 1725–1728.

Handa, K. & S. Sato: Generation of free radicals of quinone group containing anticancer chemical NADPH microsome system as evidenced by initiation of sulfite oxidation. *Gann* 1975, **66**, 43–47.

Hoefner, D. M., M. E. Blank & D. F. Diedrich: The anion transporter and a 28 kDa protein are selectively photolabeled by p-azidobenzylphlorizin under conditions that alter RBC morphology, flexibility, and volume. *Biochim. Biophys. Acta.* 1997, **1327**, 231–241.

Jian, M., F. Jiajung, G. Zhengren, H. Chen, Y. Bo, W. Fong, W. Duoning & S. Shufen: A new hypothesis about the relationship between free radical reactions and hemorheological properties *in vivo*. *Med. Hyp.* 1993, **41**, 516–520.

Kessel, D.: Enhanced glycosylation induced by adriamycin. *Mol. Pharmacol.* 1979, **16**, 306–312.

Laemmli, U. K.: Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970, **227**, 680.

Lefrak, E. A., J. Pitha, S. Rosenheim & J. A. Gottlieb: A clinicopathologic analysis of adriamycin cardiotoxicity. *Cancer* 1973, **32**, 302–314.

Levine, R. L.: Oxidative modification of glutamine synthetase I. *J. Biol. Chem.* 1983, **258**, 11823–11827.

Levine, R. L., D. Garland, C. N. Oliver, A. Amici, I. Climent, A. G. Lenz, B. W. Ahn, S. Shaltiel & E. R. Stadtman: Determination of carbonyl content in oxidatively modified proteins. *Meth. Enzym.* 1990, **186**, 464–487.

Levine, R. L., J. A. Williams, E. R. Stadtman & E. Shacter: Carbonyl assays for determination of oxidatively modified proteins. *Meth. Enzym.* 1994, **233**, 346–357.

Mikkelsen, R. B., L. Peck-Sun & D. H. Wallach: Interaction of adriamycin with human red blood cells: A biochemical and morphological study. J. *Mol. Med.* 1977, **2**, 33–40.

Mimnaugh, E. G., M. A. Trush & T. E. Gram: Enhancement of rat heart microsomal lipid peroxidation following doxorubicin treatment *in vivo*. *Cancer Treat. Rep.* 1983, **67**, 731–733.

Miura, T., S. Muraoka & T. Ogiso: Effect of ascorbate on adriamycin-Fe^{3+}-induced lipid peroxidation and DNA damage. *Pharmacology & Toxicology* 1994, **74**, 89–94.

Miura, T., S. Muraoka & T. Ogiso: Lipid peroxidation of rat erythrocyte membrane induced by adriamycin-Fe^{3+}. *Pharmacology & Toxicology* 1991, **69**, 296–300.

Monti, E., F. Piccinini, F. Villani & L. Favalli: Myocardial contractility and heart pharmacokinetic of adriamycin following a single administration in rat. *Cancer Chemother. Pharmacol.* 1986, **18**, 289–291.

Murphree, S. A., T. R. Tritton, A. Smith & C. Sartorelli: Adriamycin-induced changes in the surface membrane sarcoma 180 ascites cells. *Biochim. Biophys. Acta.* 1981, **649**, 317–324.

Myers, C. E., L. Gianni, C. B. Simone, R. Klecker & R. Greene: Oxidative destruction of erythrocyte membrane catalyzed by the doxorubicin-iron-complex. *Biochemistry* 1982, **21**, 1707–1713.

Myers, C. E., W. P. McGuire, R. H. Liss, I. Ifrim, K. Grotzinger & R.C. Young: Adriamycin: The role of lipid peroxidation in cardiac toxicity and tumor response. *Science* 1977, **197**, 165–167.

Nakano, H., K. Ogita, J. C. Gutteridge & M. Nakano: Inhibition by the protein ceruloplasmin of lipid peroxidation stimulated by an Fe^{3+}-ADP-adriamycin complex. *Fed. Eur. Biochem. Soc. Lett.* 1984, **166**, 232–236.

Olson, R. D., R. C. Boert, J. G. Gerber & A. S. Niles: Mechanism of adriamycin cardiotoxicity: Evidence for oxidative stress. *Life Sci.* 1981, **29**, 1393–1401.

Olson, R. D. & P. S. Mushlin: Doxorubicin cardiotoxicity: analysis of prevailing hypothesis. *J. Fed. Amer. Soc. Exp. Biol.* 1990, **4**, 3076–3086.

Potmesil, M., M. Israel, R. Sehardi, T. W. Sweatman, S. Kirschenbaum & R. Silber: Structure and biological activity of N-substituted anthracyclines: Correlation with drug-DNA topoisomerase II interaction. *Proc. Amer. Assoc. Cancer Res.* 1987, **28**, 262.

Shinohara, K. & K. R. Tanaka: The effects of adriamycin on human red blood cells. *Hemoglobin* 1980, **4**, 735–745.

Singal, P. K., R. J. Segstro, R. P. Singh & M. J. Kutryk: Changes in lysosomal morphology and enzyme activities during the development of adriamycin-induced cardiomyopathy. *Can. J. Cardiol.* 1985, **1**, 139–147.

Siveski-Iliskovic, N., N. Kaul & P. K. Singal: Probucol promotes endogenous antioxidants and provides protection against adriamycin-induced cardiomyopathy in rats. *Circulation* 1994, **89**, 2829–2835.

Stadtman, E. R.: Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Ann. Rev. Biochem.* 1993, **62**, 797–821.

Steinhertz, L. J., P. G. Steinhertz, C. C. Tan, G. Heller & L. Murphy: Cardiac toxicity 4 to 20 years after completing anthracycline therapy. *J. Amer. Med. Assoc.* 1991, **266**, 1672–1677.

Subramanian, R., F. Roediger, B. Jordan, M. P. Mattson, J. N. Keller, G. Waeg & D. A. Butterfield: The lipid peroxidation product, 4-hydroxy-2trans-nonenal, alters the conformation of cortical synaptosomal membrane proteins. *J. Neurochem.* 1997, **69**, 1161–1169.

Sugioka, K. & M. Nakano: Mechanism of phospholipid peroxidation induced by ferric ion-ADP-adriamycin-coordination complex. *Biochim. Biophys. Acta* 1982, **713**, 232–236.

Tewey, K. M., G. L. Chen, E. M. Nelson & L. F. Liu: Intercalative anti-cancer drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem.* 1984, **359**, 9182–9187.

Thomas, C., A. C. Carr & C. C. Winterbourn: Free radical inactivation of rabbit muscle creatine kinase: catalysis by physiological and hydrolyzed ICRF-187 (ICRF-198) iron chelates. *Free Rad. Res.* 1994, **21**, 387–397.

Thornally, P. J. & N. F. Dodd: Free radical production from normal and adriamycin-treated rat cardiac sarcosomes. *Biochem. Pharmacol.* 1985, **34**, 669–674.

Timour, Q., P. Nony, J. Lang, M. Lakhal, T. Veronique & G. Faucon: Doxorubicin concentration in plasma and myocardium and their respective roles in cardiotoxicity. *Card. Drug. Ther.* 1988, **1**, 559–560.

Villani, F., E. Monti, F. Piccinini, L. Favalli, E. Lanza, A. Rozza & P. Poggi: Relationship between ECG changes and myocardial morphological alterations induced by adriamycin in rat. *Tumori* 1986, **72**, 339–344.

Wang, Y. M., F. F. Masenat, J. C. Kimball, C. A. Gleiser, M. K. Ali, M. W. Kaufman & J. V. Eys: Effect of vitamin E against adriamycin-induced toxicity in rabbits. *Cancer Res.* 1980, **40**, 1022–1027.

Weiss, R. B., G. Sarosy, K. Clagett-Carr, M. Russo & B. Leyland-Jones: Anthracycline analogs: the past, present, and future. *Cancer Chemother. Pharmacol.* 1986, **18**, 185–197.

Zweier, J. L.: Reduction of O_2 by iron-adriamycin. *J. Biol. Chem.* 1984, **259**, 6056–6058.