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Chemical-Induced Cytotoxicity in Platelet Rich Plasma Isolated from Rats

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ABSTRACT *: The elevation of intracellular calcium in various tissues due to oxidative stress induced by either menadione or adriamycin has been well documented. The increase of calcium level in platelets results in aggregation of platelets. To test the hypothesis that chemically induced calcium elevations can play a role in platelet aggregation, we have studied the effects of menadione and adriamycin on aggregation of platelets isolated from female rats. Treatment with menadione and adriamycin to platelet rich plasma (PRP) appeared to induce platelet aggregations up to 60%, as determined by aggregometry. However, exposure of PRP to menadione or adriamycin led to a loss of viability, as measured by lactate dehydrogenase (LDH) leakage. Morphological studies of platelets revealed that, when PRP was treated with menadione, aggregates of platelets were not observed and the numbers of platelets were decreased significantly. This suggests that menadione and adriamycin decreased turbidity by inducing platelet lysis rather than platelet aggregation. These cellular toxicities induced by menadione or adriamycin was not correlated with oxygen consumption rate but with depletion of protein thiols, suggesting that protein thiols might play an important role in chemical-induced platelet toxicity.*

Key Words *: Adriamycin, Menadione, Cytotoxicity, Thiols, Platelets*

I. INTRODUCTION

The involvement of platelets in hemostasis and their production of thrombus implies an important pathophysiological role for platelets in mammalian circulatory systems (Mustard and Packman, 1979; Frojmovic and Milton, 1982). This hemostatic balance is broken by damage to the vessels or pathological factors. Furthermore, excess aggregation of platelets can be induced, which results in vessel diseases (Thompson and Harker,1987).

In all mammalian cells, the cytosolic calcium level is approximately 10,000-fold less than the extracellular calcium concentration in plasma (Nicotera *et al.*, 1992). Normally, unstimulated platelets maintain a low cytosolic calcium concentration and a steep plasma membrane calcium gradient (Brass, 1984). However, when activated by agonists (thrombin, collagen, etc.), a significant increase in cytosolic calcium is observed (Johnson *et al.*, 1985; Kovacs *et al.*, 1989). An increase in the level of cytoplasmic calcium is considered the primary event leading to platelet shape change due to cytoskeletal rearrangement, the secretion of granule contents, and finally aggregation of platelets (Rink *et al.*, 1982; Rink and Sage, 1990).

Both menadione and adriamycin have a quinone moiety and both have been used therapeutically--menadione for hypothrombinemia and adriamycin for certain types of cancer. These two substances are redox-active chemicals which are metabolized by flavoprotein reductase to semiquinones, and then, in the presence of molecular oxygen, are converted back to quinones via oxidation. During this redox cycle process, molecular oxygen is converted to a superoxide anion radical. The superoxide anion radical is metabolized further to hydrogen peroxide and other reactive oxygen species (Monks *et al.*, 1992). Numerous studies have shown that

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treatment with menadione or adriamycin leads to cytotoxicity in certain tissues, such as liver and heart, through oxidative stress induced by the redox cycling of the quinone moiety of these substances (Doroshow, 1986). In these tissues, two important toxic effects of menadione and adriamycin include alterations in cytoskeleton morphology and increases in cytosolic calcium (Solem *et al.*, 1994; Thor *et al.*, 1982).

Our laboratory originally hypothesized that, in platelets, the redox active chemicals would cause similar elevations in intracellular calcium, leading to a change in platelet shape, possible excessive platelet aggregation. However, instead of inducing platelet aggregation, our previous research has shown that treatment of menadione to blood platelets appeared to cause cell lysis, as manifested by lactate dehydrogenase (LDH) leakage (Kim *et al.*, 1996).

In this study, we determined whether other redox-active chemical, adriamycin, could also induce platelet lysis. In addition, we have investigated the possible mechanism for chemical-induced platelet lysis using platelet rich plasma (PRP) system isolated from rats.

II. EXPERIMENTAL METHODS

1. Materials and Animals

The following chemicals were purchased from Sigma (St. Louis, USA): sodium citrate, adriamycin, menadione, Wright's stain and thrombin. Female Sprague Dawley rats (Yuhan Pharmaceutical Co., Korea) weighing 200 to 250 gm, were used. Prior to experiments, animals were housed for at least 3 or 4 days in the laboratory animal facility in polypropylene cages. The lighting in the animal room was regulated by an automatic control switch such that lights were on from 7 am to 7 pm and off from 7 pm to 7 am. Water was provided ad libitum throughout the experiments.

2. Preparations of platelets

Animals were sacrificed under light ether anesthesia. Blood collected from the abdominal aorta and anticoagulated with sodium citrate (3.8%, 1:9) was centrifuged for 15 min at 150 g at room temperature. Platelet rich plasma (PRP) was obtained from the supernatant resulting from this relatively low g-force centrifugation. Platelet poor plasma (PPP) was obtained from the supernatant of a 20 minute, 1,500 g centrifugation of the blood cell residue resulting from the first spin. Throughout all experiments, the platelet number was adjusted to 5×10^8 platelets/ml by diluting PRP with PPP.

3. Measurement of platelet aggregation

Platelet aggregation was induced by addition of 1.2 units of thrombin to the platelet preparations. Aggregation was measured by platelet turbidity, with 0% aggregation calibrated as the absorbance of PRP and 100% aggregation calibrated as the absorbance of PPP or suspension buffer. PRP in a silicon-coated aggregation cuvette was stirred at 1,200 rpm for 1 min prior to addition of menadione or adriamycin. Dimethyl sulfoxide (DMSO) and distilled deionized water (DDW) were used as the vehicles for menadione and adriamycin, respectively, such that the final concentration of solvent in the cuvette's incubation medium (PRP) was 0.5%. This concentration was shown to have no effect on either platelet aggregation induced by thrombin or platelet lysis induced by menadione and adriamycin. Changes in turbidity were detected by a Lumi-aggregometer (Chrono-log Corp., USA).

4. Observation of platelet morphology

Morphological changes of platelets by menadione and thrombin were observed by a modified thin smear method (Yun *et al.*, 1985). After incubation with these compounds, PRP was thinly smeared on glass slides and dried quickly in the air. The glass smears were fixed in methanol for 30 sec and stained with a Wright stain for 5 min. After stabilizing by the addition of phosphate buffer (pH 6.6), the glass smears were dried in an oven at 37°C. The smears were subjected to examination under an ordinary light microscope using an oil immersion objective lens (magnification: 1,000x).

5. Biochemical assays

Leakage of lactate dehydrogenase (LDH) from platelets was measured by spectrophotometry. After various times of incubation of the PRP with menadione or adriamycin, the incubation medium was centrifuged. A 0.05 ml aliquot of resulting supernatant was added to 2 ml of Tris-EDTA-NADH buffer (pH 7.4) and then incubated for 10 min at 37°C. The decrease of absorbance at 340 nm as NADH was converted to NAD was measured (Bergmeyer *et al.*, 1965).

Protein thiol concentrations were measured by a modified assay based on the colorimetric method of Di Monte *et al.* (1984a). One ml of chemical-treated PRP was centrifuged at 10,000g for 20 sec, and the supernatant was discarded. The pellet was precipitated and washed with 5% perchloric acid , and was finally suspended in 2.5 ml of Tris-EDTA buffer (0.5 M Tris-HCl, 5 mM EDTA, pH 7.6). 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB; 250 uM final concentration) was then added and, after 20 min, the absorbance was measured at 412 nm. Protein thiol levels were calculated on the basis of a glutathione calibration curve.

Oxygen consumption was measured polarographically with a Clark oxygen electrode (Yellow Springs Instrument Co., Inc. Model 5300) in magnetically stirred sample chambers at 37°C. PRP was preincubated for at least 5 min to saturate samples with air. Initial rate of oxygen consumption was determined from time of addition of menadione or adriamycin through 3 min post treatment.

III. RESULTS

In order to determine the effect of redox-cycling chemicals on platelet aggregation, platelet rich plasma (PRP) was treated with 0.25 mM of either menadione or adriamycin (Fig. 1). Pretreatment with menadione or adriamycin leading to changes in the shape of the platelets (as indicated by a turbidity increase), followed by decreases in turbidity, were observed. At higher concentrations of menadione or adriamycin, dose-dependent changes in the shape of the curve were observed and reaction times to reach maximum were considerably shortened as well. However, the maximum decreases in turbidity induced by these chemicals were no greater than 50-60% (data not shown). This was unexpected because it was known that most agonists, such as thrombin, collagen, etc., can induce 100% aggregation of platelets. Furthermore, no platelet aggregates were visible in the PRP solutions following pretreatment with either menadione or adriamycin. Based on these observations, it was postulated that platelet aggregation was not the cause of the observed decreases in turbidity induced by menadione and adriamycin. Instead, it was postulated that menadione-induced and adriamycin-induced cell lysis might be the cause.

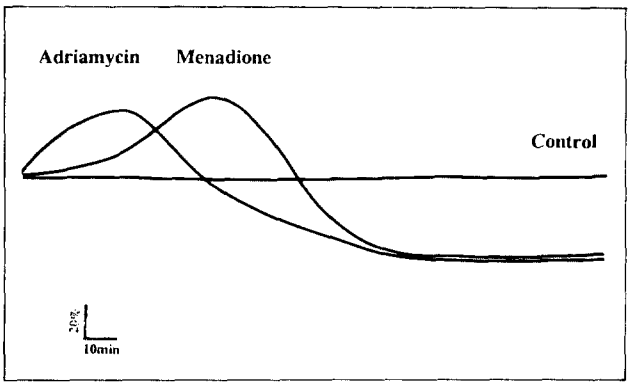

Fig. 1. Effect of menadione and adriamycin on turbidity changes in aggregometer under platelet rich plasma isolated from rats. Methods to prepare platelet rich plasma (PRP) were described in the methods section. Each platelet suspension was incubated with either 0.25 mM menadione or adriamycin. The X-axis represents the time of incubation and the Y-axis represents the percentage change in turbidity.

In order to determine whether these redox-cycling chemicals induce platelet lysis, the effect of menadione or adriamycin on platelet lactate dehydrogenase (LDH) leakage in PRP was studied (Fig. 2). When PRP was incubated with menadione or adriamycin, LDH leakages were increased in a time-dependent manner. Incubation with either 0.25 mM adriamycin or 0.25 mM menadione resulted in a 90% LDH release at 120 min, indicating 90% cell lysis.

The effect of a quinone substance on platelet morphology in PRP was also studied. Alterations of platelet morphology by treatment with menadione were observed under light microscopy through the thin smear method (Fig. 3). Control PRP contained

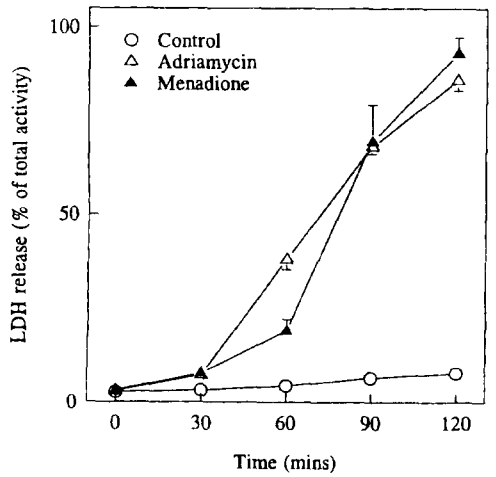

Fig. 2. Lactate dehydrogenase (LDH) leakage from platelets by menadione and adriamycin. All treatments were same as described in Figure 1. Values are means ± SEM of three experiments using platelet preparations from three animals.

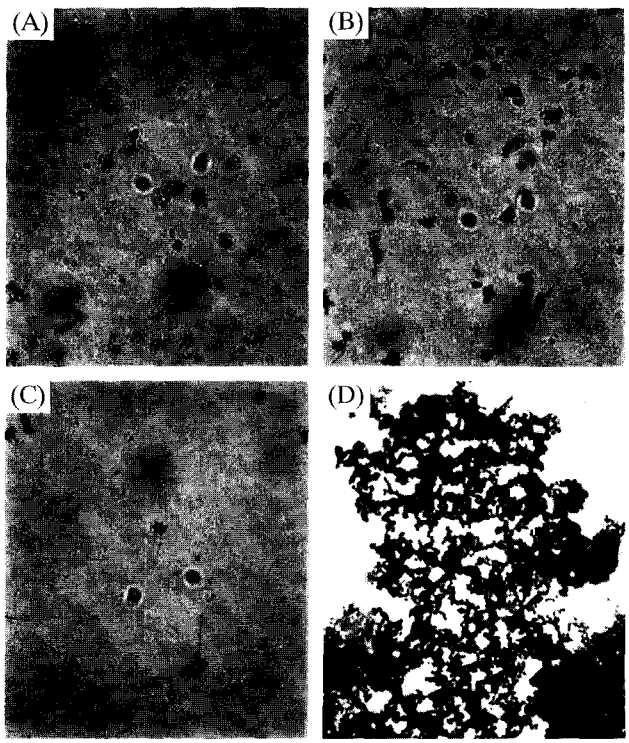

Fig. 3. Alterations in platelet morphology by treatment with menadione and thrombin. PRP was incubated with A) dimethyl sulfoxide, B) 0.25 mM menadione for 60 min, C) 0.25 mM menadione for 120 min, and D) 0.9 unit thrombin for 3 min, respectively. Morphology was examined using a thin smear method, which is described in the methods section.

well stained, spherical platelet granules in a relatively clear medium. PRP treated with menadione contained irregularly shaped platelets which appeared to be leaking, as evidenced by the vast number of stained granules observed in the medium itself. Furthermore, the platelet cell count appeared greatly reduced and there was no evidence of platelet aggregation. These menadione-induced decreases in cell number and absence of aggregation are consistent with a loss of cell viability, as evidenced by the LDH leakage illustrated in Fig. 2.

Previous studies have shown that menadione and adriamycin have redox-cycling capacities in microsomes isolated from liver or heart (Doroshow, 1986). Therefore, experiments were performed to determine if these chemicals could undergo redox cycling in platelets. The redox-cycling capacities of menadione and adriamycin were compared by measuring oxygen consumption rates in PRP (Fig. 4). The control group (PRP without menadione or adriamycin) resulted in minimal oxygen consumption rate. The oxygen consumption due to 0.25 mM menadione was increased significantly, as previously reported (Kim *et al.*, 1996). On the other hand, 0.25 mM adriamycin did not increase oxygen consumption significantly. Thus, the rate of oxygen consumption by adriamycin in intact platelets was significantly lower than that by menadione.

In order to determine if chemical-induced cytotoxicity was related to the level of cellular protein

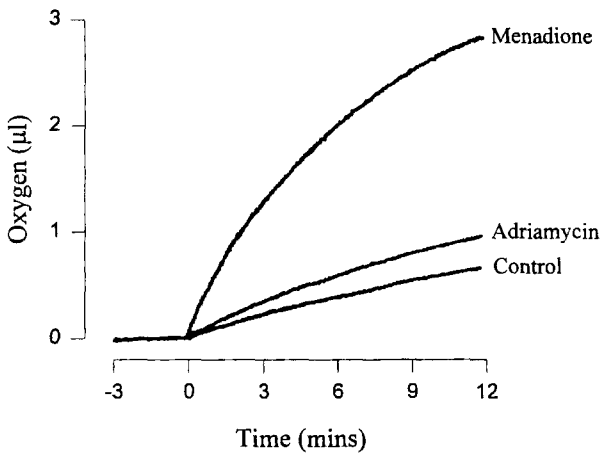

Fig. 4. Effects of menadione and adriamycin on oxygen consumption rate in platelet rich plasma (PRP). PRP was exposed to 0.25 mM menadione or adriamycin and oxygen consumption rate was measured by a Clarke oxygen electrode. Data in the figure are from one representative in vitro run; two other similar *in vitro* runs were also performed.

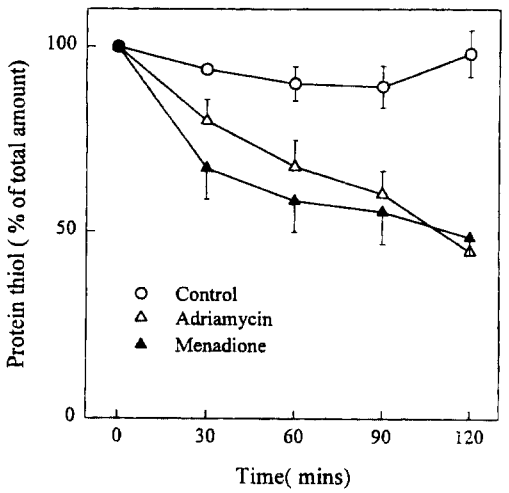

Fig. 5. Effect of menadione and adriamycin on protein thiol levels in platelets. All treatments were same as described in Fig. 2 and total protein thiol levels were determined as described in the methods section. Values are means ± SEM of three experiments from three animals.

thiols, total protein thiol levels were measured after addition of 0.25 mM menadione or adriamycin (Fig. 5). Treatment with menadione or adriamycin caused significant loss of protein thiols, resulting in more than 50% decrease during 2 hrs incubation. This protein thiol depletion correlates closely with the onset of toxicity to the platelet membrane (Fig. 2). These data suggest that the depletion of protein thiols may have been the critical step in manifesting damage to platelets.

IV. DISUCCION

It is well documented that both menadione and adriamycin can induce a change in cellular calcium homeostasis, which plays an important role in cell toxicity (Solem *et al.*, 1994; Thor *et al.*, 1982). The change in intracellular calcium homeostasis in platelets also plays a pivotal role in the regulation of cell function, which is related to changes in cell shape, granule release, and platelet aggregation (Brass, 1984; Siess, 1989). It was originally hypothesized that an increase in calcium concentration in platelets induced by menadione and adriamycin could lead to platelet aggregation, thereby resulting in excessive thrombus formation that could provoke cardio vascular disease. However, this is the second study to demonstrate that that treatment of adriamycin to platelets also led to cell lysis instead of cell aggregation. Several lines of evidence support this view: 1) unlike agonists, such as thrombin, the turbidity of PRP was decreased only 60% at high doses of adriamycin; 2) unlike thrombin, adriamycin treatment significantly decreased platelet cell count; 3) treatment with adriamycin resulted in intracellular LDH release into the incubation medium in a time-dependent manner.

The flavoprotein quinone reductase is responsible for the reduction of quinones into corresponding hydroquinones. These hydroquinones may undergo a conjugation reaction, which is the detoxifying mechanism for quinone-induced toxicity (Monks *et al.*, 1992; Thor *et al.*, 1982). Most cells have relatively abundant supplies of quinone reductase (Benson *et al*, 1980). However, our experiments show that platelets lack quinone reductase and that the well known quinone reductase inhibitor, dicoumarol, has no effect on menadione-induced cytotoxicity (data not shown). The observed lack of quinone reductase and dicoumarol-induced inhibition suggest the possibility, in contrast to other cell types, platelets will be especially susceptible to cytotoxic effects of redox-cycling substances, such as menadione and adriamycin.

Our previous work has reported that menadione-induced cytotoxicity to platelets was due to oxidative stress (Kim *et al.*, 1996). But the current work suggests that another pathway may be involved in chemical-induced cytotoxicity in platelets. Menadione or adriamycin may manifest its toxicity to platelets via protein arylation, as reported in some other cells (Miller *et al.*, 1985; Ross *et al.*, 1986; Stone *et al.*, 1996). The rationale for this conclusion is as follows: 1) 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), a compound which causes oxidative stress, but not protein arylation (Gant *et al.*, 1988), does not induce cytotoxicity to platelets (manuscript in preparation); 2) cellular toxicity induced by menadione or adriamycin is not correlated with oxygen consumption rate (Fig. 4), but with depletion of protein thiols (Fig. 5) . This chemical-induced damage possibly occurring by protein arylation could be very non-specific within cells. This deduction requires further investigation.

Our results also suggest that depletion of total cellular thiols may represent a crucial step in the process of chemical-induced injury to platelets. This hypothesis is supported by two lines of evidence: 1) only concentrations of menadione or adriamycin that deplete the total cellular thiol pool also result in cell lysis and 2) supplementing the incubation medium with a thiol source completely protects against the damage caused by menadione (data not shown). These observations in platelets are consistent with reports of chemical-induced cytotoxicity in isolated hepatocytes(Di Monte *et al.*, 1984b).

Our results suggest the need for further investigations into the mechanism of quinone-induced platelet lysis, since platelet lysis could be associated with promoting cardiovascular disease. The effect of cell lysis could ultimately result indirectly in aggregation, due to the release of platelet granules into the blood. These platelet granules are rich in platelet aggregation agonists, such as thrombin, ADP, etc., (Stormorken, 1984) and their release could cause secondary platelet aggregation. A more direct effect on cardiovascular systems following platelet lysis could be even more significant. Platelets have a well developed serotonin-uptake system which limits serotonin levels in the blood and therefore prevents excessive vasoconstriction (Vanhoutte, 1991; Nishio *et al.*, 1995). If quinone substances induce platelet lysis, serotonin will be released and vasoconstriction will occur. Further research should be undertaken to determine if platelet lysis is a possible cause of cardiovascular disease.

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