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Actin Filaments Disassembly: A Novel Step in the Genesis of Paraquat Toxicity?

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Paraquat (methyl viologen; 1,1'-dimethyl 4,4'-bipyridylium dichloride), a widely used broad-spectrum herbicide, induces known toxic effects on mammalian cells (Haley 1979). Bus at al. (1974) proposed that oxygen free radicals produced by paraquat react with membrane phospholipids to induce cell damages. A paraquat-induced lesion appears to be the excessive production of reactive oxygen species in response to the intracellular cyclic reduction-oxidation of the compound (Krall et al. 1988). Alternative views have also been advanced, such as the suggestion that paraquat injures cells by depletion of biological reductants, chiefly NADPH (Forman et al. 1980). Despite intensive investigation into paraquat toxicity, the cellular target site ultimately responsible for the herbicide cytotoxicity has not been identified.

The cytoskeleton plays a crucial role not only in cell shaping but also in many cellular functions. Our previuos study (Cappelletti et al. 1991) showed cytoskeletal alterations induced on cultured mouse fibroblasts by treatment with N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin which causes parkinsonism in primates (Langston et al. 1983). The toxic metabolite of MPTP is 1-methyl-4-phenylpyridinium ion (MPP^+), whose chemical structure resembles paraquat. Therefore it is reasonable to hypothesize that paraquat and MPTP share similar intracellular targets. In this study we present the effect of paraquat treatment on the cytoskeleton of cultured mouse fibroblasts. To identify a new possible step in the genesis of paraquat toxicity on mammalian cells, the organization of microfilaments, microtubules and intermediate filaments, was examined.

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MATERIALS AND METHODS

Swiss 3T3, an established cell line of mouse embryonic origin (Todaro and Green 1963) was maintained at 37 °C in Minimal Essential Medium (GIBCO BRL), supplemented with 10% fetal calf serum (Irwine,UK) in an atmosphere of 95% air/5% CO_2. Cells were plated on glass coverslips at a density of $2.5x10^3$ cells/cm^2 and treated, 3 days after, with paraquat (Sigma, St. Louis, MO) concentrations ranging from 10 to 640 μM. The viability test on treated cells was performed by examination of Trypan Blue dye exclusion and cells were counted with a haemocytometer counter. The cytoskeletal organization was studied on cells treated 15 min and 48 hr with 100 μM paraquat. The actin stress fibers and the microtubules distribution were analysed by indirect immunofluorescence technics (IIF) with monoclonal anti-actin and anti-atubulin antibodies (Amersham International plc, U.K.) as previously described (Cappelletti et al. 1991). Briefly, to detect actin filaments, cells were fixed in paraformaldehyde (3.7% in PBS) pH 7.4, at room temperature for 1 hr, and permeabilized in acetone at -20°C for 10 min, whereas to detect microtubules, cells were simultaneously fixed and permeabilized in methanol at -20°C for 10 min. Next, the cultures were incubated with a 1:100 dilution of mouse monoclonal antibodies in a humid atmosphere at 37°C for 1 hr, washed in PBS and incubated with a 1:50 dilution of goat anti-mouse IgG-Texas Red conjugates at 37°C for 45 min. The vinculin localization was investigated by IIF with monoclonal anti-vinculin antibodies (Boehringer Mannheim, Germany) according to Marchisio et al. (1984). The coverslips were viewed with a Zeiss Axioplasm microscope equipped with epifluorescent optics. Pictures were taken with an oil immersion objective (63x) on Ektachrome 400 Kodak.

RESULTS AND DISCUSSION

The herbicide paraquat results toxic for "Swiss 3T3" cell line. When cells were incubated for 48 hr with paraquat concentrations ranging from 10 to 640 μM, a dose-dependent decrease in viability was observed (Fig. 1), with a half maximal inhibition value of 160 μM. Therefore to evaluate the starting events evoked by the herbicide on a cell population in good viability conditions, the analysis of the cytoskeleton involvement in paraquat toxicity was carried out on cells exposed 15 min and 48 hr to 100 μM paraquat.

The cytoskeleton organization was studied by IIF with specific antibodies. Cultured fibroblasts have prominent sheaths of actin filament bundles, also called stress fibers, which run the length of the cells (Lazarides and Weber 1974). Fig. 2a shows the

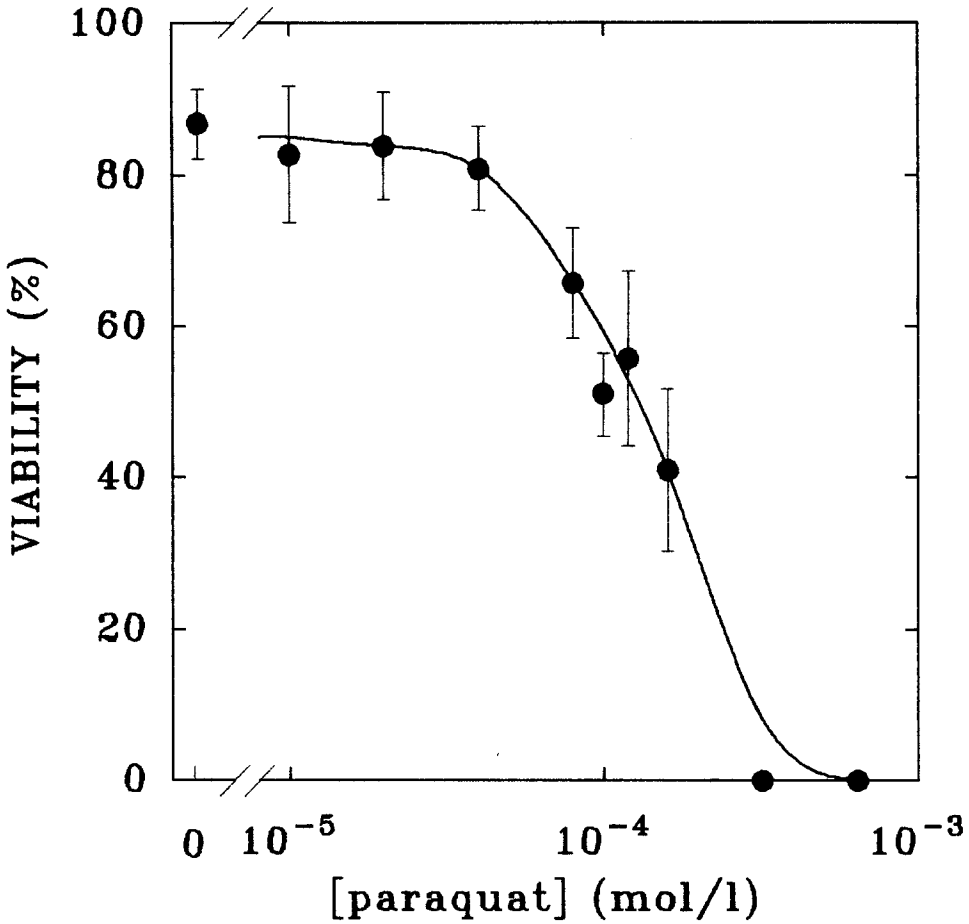


Figure 1. Cell viability test on "Swiss 3T3" cells exposed 48 hr to paraquat.

conventional organization of stress fibers in control cells stained with monoclonal anti-actin antibody. The paraquat treatment induces a clear modification of actin filaments in 50-60% of the cells after 15 min (Fig. 2b), whereas the effect was complete after 48 hr (Fig. 2c). Apparently, the alterations in the actin cytoskeleton observed by fluorescence microscopy primarily reflect only changes in the organization of the F-actin in the treated cells. In normal cultured fibroblasts, actin filament bundles are anchored to the membrane in specialized regions called adhesion plaques (Chen and Singer 1982). Among the cytoskeletal proteins localized in these anchorage regions, vinculin was the first thought as a linkage protein (Geiger et al.

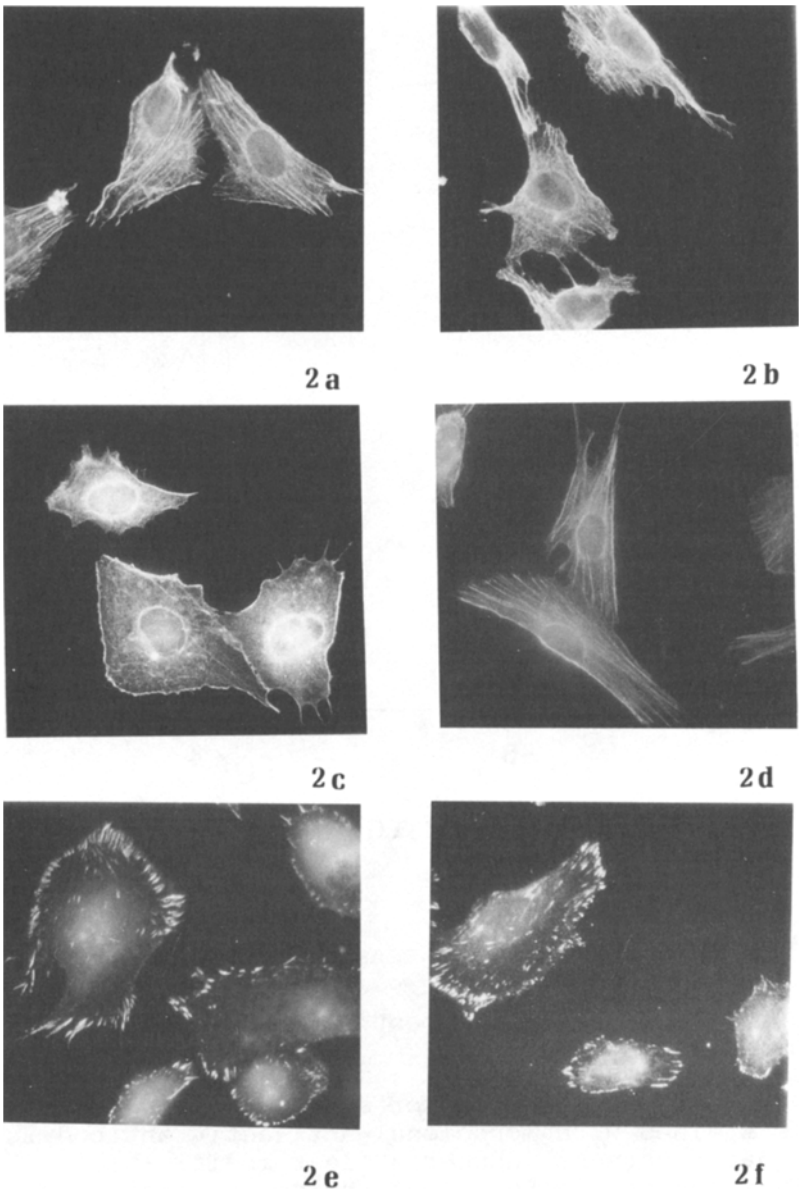
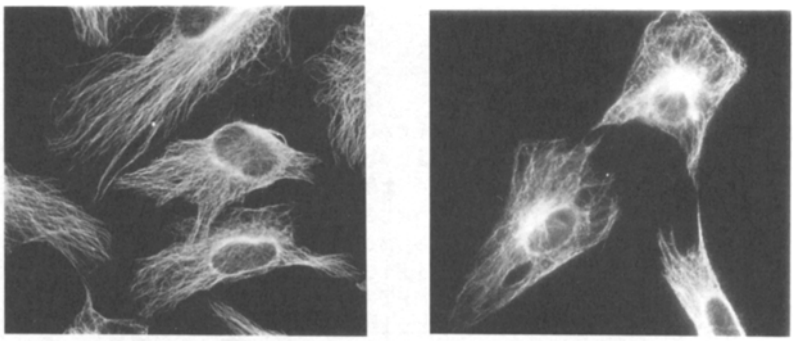


Figure 2. Indirect immunofluorescence of actin (a,b,c,d,) and vinculin (e,f) on "Swiss 3T3" cells. a,e: control cells; b: cells exposed 15 min to 100 μM paraquat; c,f: cells exposed 48 hr to 100 μM paraquat; d: cells incubated 24 hr with control medium after 48 hr exposure to paraquat. Magnification x 1575



3a 3b

Figure 3. Indirect immunofluorescence of tubulin on "Swiss 3T3" cells. a: control cells; b: cells exposed 48 hr to 100 μM paraquat. Magnification x 1575.

1980). Based on prior knowledge that the vinculin-containing adhesion plaques may act as initiation centers for actin bundles formation (Geiger et al 1984), the localization of vinculin was investigated in control (Fig. 2e) and paraquat treated cells (Fig. 2f). Since the number and size of adhesion plaques stained with anti-vinculin antibody were unmodified after treatment, we suggest that the observed microfilaments disruption was not related to a significant vinculin modification induced by paraquat. The observed alterations were not linked to morphological changes and resulted completely reversible when the cell cultures treated for 48 hr with paraquat were then incubated 24 hr (Fig.2d) in culture medium without the herbicide. Besides microfilaments, the two main filament types that make up the cytoskeleton are microtubules and intermediates filaments. All microtubule functions are based on the ability of tubulin to polymerize and on the resistance to depolymerization. Paraquat resulted ineffective to induce modifications of microtubule organization. In fact, the microtubule network, stained with monoclonal anti-atubulin antibody, appeared clear and well organized both in control (Fig. 3a) and in paraquat treated cells (Fig. 3b). Afterward we studied the organization of intermediate filaments, which comprise several subunit proteins expressed in tissue specific patterns. The subunit vimentin is expressed in a wide variety of mature cell type, as well as in immature stages of cell differentiation, such as "Swiss 3T3" cells (Lazarides 1982). According to the suggestion that structural interactions exist between microtubules and some classes of intermediate filaments (Geiger and Singer 1980), IIF experiments with monoclonal anti-

vimentin antibody showed that vimentin network was not different between control and treated cells (data not shown).

This study clearly demonstrates that paraquat toxicity on "Swiss 3T3" cell line involves modifications of cytoskeletal elements. The three main components of the cytoskeleton were investigated, but only microfilaments appeared altered. The disappearance of the actin bundles results as a specific step in the genesis of paraquat toxicity. Since MPTP induces both microfilament and microtubule modifications, we suggest that paraquat and the neurotoxin act in a different way on "Swiss 3T3" cytoskeleton. Moreover, since the effect on actin filaments was reversible, a transient perturbation in the levels of some cellular componets may be involved in the paraquat mechanism of action. Actin disassembly may be explained by the lipid peroxidation hypothesis of paraquat toxicity (Bus et at 1974). The oxygen free radicals generated in the intracellular cyclic reduction and reoxidation of paraquat induce the peroxidation of membrane phospholipids and, consequently, structural and functional alterations of the plasma membrane. Microfilaments disorganization may result from modifications in the membrane anchorage regions, where the adesion plaques proteins are covalently bound to lipids. Further studies are in progress to clarify if the disassembly of actin filaments is related to one of the previously described intracellular effect of paraquat, such as the production of oxygen free radicals (Krall et al 1988) and the depletion of biological reductants (Forman et al 1980),or additional mechanisms of paraquat toxicity on mammalian cells are involved.

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