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Paraquat Modulates the Differentiation of C2C12 Cells to Myotube

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Abstract—Paraquat is one of herbicides, known to have toxicity in animals and human. It has been reported that paraquat injures lungs and also induces abnormal differentiations in muscle cells. This suggests the possibility that paraquat perturbs the differentiation of muscle. In this study, we examined the effects of paraquat on the differentiation of C2C12 cells, mouse myoblast. In presence of paraquat, C2C12 cells morphologically changed to myotube even in the undifferentiating condition. The amount of myosin heavy chain (MHC), one of the differentiation markers of muscle in C2C12 cells increased with the concentration of paraquat. Because Paraquat produces reactive oxygen species (ROS), effect of ROS on the differentiation of C2C12 cells were examined. The addition of ascorbic acid did not repress the differentiation of C2C12 cells by paraquat. H_2O_2 also did not induce the differentiation of C2C12 cells. These results suggest that paraquat perturbs the differentiation of C2C12 cells in a ROS-independent manner.

Keywords: paraquat, muscle, C2C12 cells, differentiation

INTRODUCTION

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride, Fig. 1) is one of herbicides. Paraquat has been known to have strong toxicity in human (Christakis-Hampsas *et al*., 1998; Gear *et al*., 2001; Wesseling *et al*., 2001; Rahman *et al*., 2007) affecting lungs, liver, skin, etc. However, nowadays paraquat is sparingly used all over the world, especially in developing countries.

One of important toxic effects of paraquat in mammals is the induction of Parkinsonism. 1-methyl-4-phenylpyridinium (MPP^+), the structure which is similar to paraquat, has been known to induce Parkinsonism (Langston *et al*., 1984a, b). MPP^+ is a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and occurs as an impurity. Because paraquat and MPP^+ have similar

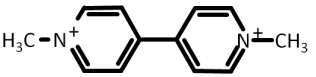

Fig. 1. The structure of paraquat.

structure, paraquat also is concerned with Parkinson's disease (Sanchez-Ramos *et al*., 1987). It is also known that paraquat activates Mitogen-activated protein kinase (MEK)—extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) in brain neuroblasts (Niso-Santano *et al*., 2006). These kinases are known to differentiate cells to macrophages (Li *et al*., 2008) and adipocytes (Kim *et al*., 2007), suggesting the importance of these kinases in the differentiation of these kind of tissues. These kinases play important role in the differentiation of muscle (Mauro *et al*., 2002; Ishrath *et al*., 2002).

The toxicity of paraquat is dependent on the reactive oxygen species (ROS) produced by it. ROS produced by paraquat injures certain tissues. Lungs are the main target organ injured by ROS originated from paraquat (Manktelow, 1967). Paraquat induces an abnormal differentiation of the cells in the lungs of monkey (Fukuda *et al*., 1985), suggesting the possibility that paraquat induces the abnormal differentiation of muscle cells.

C2C12 cells are isolated from muscle of mouse, and able to differentiate to skeletal muscle cells (Yaffe and Saxel, 1977). C2C12 cells have been often used to study the differentiation of muscle *in vitro*. In this study, we examined the effect of paraquat on the differentiation of C2C12 cells to understand how paraquat perturbs the differentiation of muscle.

MATERIALS AND METHODS

Samples

Paraquat was purchased from Wako (Osaka, Japan). C2C12 cells were obtained from Cell Bank, RIKEN BioResource Center (Tsukuba, Japan).

Cell culture

C2C12 cells were maintained under 5% CO_2 at 37°C in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (JRH bioscience, Lenexa, KS), 100,000 unit/L penicillin and 10 mg/L streptomycin (Nakarai Tesque, Kyoto, Japan).

To study the effect of paraquat on the differentiation of C2C12 cells, C2C12 cells were plated at 5×10^4 cells/well, and cultured for 24 hours. Then the medium was changed to DMEM with 10% fetal bovine serum and paraquat (0.1~1000 ng/mL, Wako). The cells under culture were photographed and harvested at specific intervals and examined.

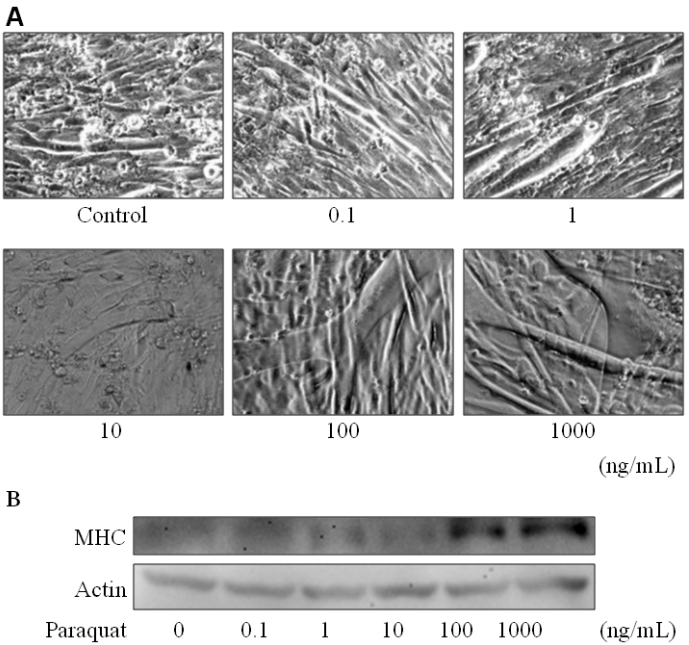

Fig. 2. Effect of paraquat on the differentiation of C2C12 cells. C2C12 cells were cultured in 10% FBS-containing DMEM with each concentration of paraquat for 12 days. (A) Effect of paraquat on the morphology of C2C12 cells. (B) Effect of paraquat on the amount of myosin heavy chain in C2C12 cells.

Western blotting

After culture under specific conditons, C2C12 cells were harvested and washed twice with phosphate-buffered saline and lysed in the buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM HaCl, 1% Triton-X 100, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride, 2.0 μg/mL aprotinin and 1 mM pervanadate. The whole-cell lysate was incubated at 4°C for 30 minutes and then centrifuged at 12,000 × g for 30 minutes. The supernatant was mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The mixture was loaded onto 8% SDS-PAGE gel, and electrophoresis was performed under reducing conditions. The sample was then electrotransferred onto a PVDF membrane (Millipore, Billerica, MA). The blotted membrane was probed for monoclonal mouse anti-myosin heavy chain (MHC) antibody M4276 (Sigma, St. Louis, MO), anti-actin antibody produced in rabbit A2066 (Sigma). The secondary antibody was horseradishperoxidase-conjugated anti-mouse immunoglobulin (Ig) G, and the detection of each protein was performed using ECL Plus Western Blotting Detection System (GE Healthcare,

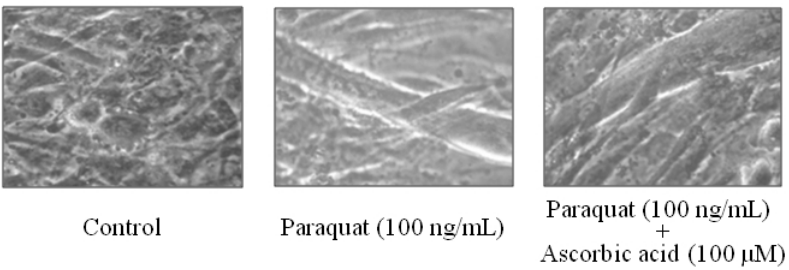

Fig. 3. Effect of ascorbic acid and paraquat on the morphology of C2C12 cell. C2C12 cells were cultured in DMEM with 10% FBS containing 100 ng/mL of paraquat and 50 μg/mL ascorbic acid for 12 days. Ascorbic acid did not reduce the perturb of differentiation by paraquat.

Buckinghamshire, UK) and Lumino Imaging Analyzer FAS-1000 (Toyobo, Osaka, Japan).

Measurement of TBARS level in C2C12 cells

Thiobarbituric acid reactive substance (TBARS) is an index of lipid peroxidation and oxidative stress levels. TBARS level in C2C12 cells were measured with a commercial kit (Cayman Chemical, Ann Arbor, MI) according to the method of Yagi (1976).

RESULTS AND DISCUSSION

C2C12 cells were cultured for 12 days in the medium containing 0.1~1000 ng/mL paraquat under similar condition. The morphology of C2C12 cells changed to muscle cell-like condition depending on the concentration of C2C12 cells. Furthermore, C2C12 cells became thicker depending on the concentration of paraquat (Fig. 2). Especially the morphology of C2C12 cells changed to myotube in 100 and 1000 ng/mL paraquat.

The amount of MHC in C2C12 cells, the differentiation maker of muscle increased depending on paraquat concentration in the medium.

Paraquat produces ROS to show its toxicity. So it is important to know the relationship between ROS and the differentiation of C2C12 cells. When C2C12 cells were cultured in presence of both 100 ng/mL paraquat and 100 μM ascorbic acid, the differentiation of C2C12 cells by paraquat was not suppressed by ascorbic acid (Fig. 3). Moreover, H_2O_2 (0.1~100 μM) did not show any effect on the differentiation of C2C12 cells (Fig. 4). To discuss the role of ROS produced by paraquat on the differentiation of C2C12 cells, it is important to measure ROS level in each sample. In this study, TBARS level was measured as a marker of ROS level. There was no significant difference between control and 100 ng/mL paraquat-add cells in TBARS level (data not shown). The concentration of paraquat in this study was low when compared with other studies as ROS was not produced very much in the present study. These results suggest that paraquat

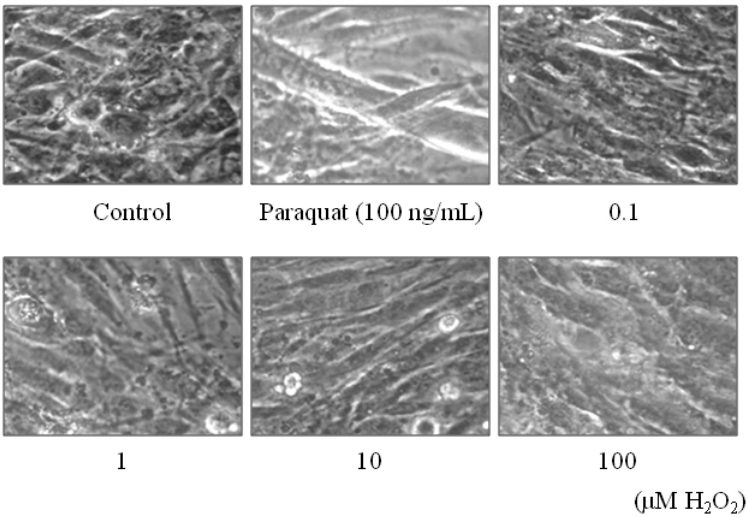

Fig. 4. Effect of H_2O_2 on the morphology of C2C12 cells. C2C12 cells were cultured in DMEM with 10% FBS containing paraquat or each concentration (0.1~100 μM) of H_2O_2 for 12 days. H_2O_2 did not induce the differentiation of C2C12 cells in any concentration administrated in this study.

perturbs the differentiation of muscle in a ROS-independent manner.

When paraquat induces inflammation in the lungs, the differentiation of cells to muscle was noticed (Fukuda *et al*., 1985). This suggests the possibility that paraquat perturbs the differentiation of muscle cells. Usually C2C12 cells differentiate in the medium with 2% house serum (Yaffe and Saxel, 1977). However, in this study C2C12 cells showed morphological change to myotube and increasing MHC level with 100 or 1000 ng/mL paraquat even in the medium with 10% FBS under similar conditions. These results suggest that paraquat perturbs the differentiation of C2C12 cells to myotube.

The activity of paraquat as a herbicide is dependent on its ROS-producing activity for weed control. In many cases, the toxicity of paraquat is dependent on the production of ROS from paraquat in mammals. For instance, it has been reported that paraquat and MPP^+, the structure of which is similar to paraquat, are the causes of Parkinson disease-like symptoms (Hertzman *et al*., 1990; Jimenez-Jimenez *et al*., 1992; Wang *et al*., 1992; Hubble *et al*., 1993; Liou *et al*., 1997). ROS induces apoptosis in nerve cells of *Substantia nigra*. It is important to know the role of ROS produced by paraquat to clarify the perturbing-mechanism of muscle-differentiation. In our study, ascorbic acid, one of anti-oxidative substances showed no effect on the differentiation perturbed by paraquat. H_2O_2, one of ROS did not cause any differentiation. Moreover, 100 ng/mL of paraquat did not increase TBARS level when compared with control. These results suggest that paraquat induces differentiation on C2C12 cells in ROS-independent manner.

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