INVOLVEMENT OF CYCLIN D1, ERK AND JNK ON DBP-INHIBITED CELL PROLIFERATION AND DIFFERENTIATION IN RAT EMBRYONIC LIMB BUD CELLS

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Introduction

The micromass cell culture method for rat embryonic cells, developed by Flint (1), has been extensively used as an *in vitro* test for developmental toxicants. Di-n-butyl phthalate (DBP) is mainly used as a coalescing aid in latex adhesive, and also used as a plasticizer in cellulose plastics and a solvent for dyes. Several studies using mice (2) and rats (3) have demonstrated that DBP was embryolethal and capable of producing various defects. Previously, we reported that DBP and its metabolite, mono-butyl-phthalate (MBuP), inhibited cell proliferation and differentiation of cultured rat embryonic limb bud cells in a dose dependent manner. In the present study, we examined the role of cell cycle regulators on DBP-inhibited cell proliferation and differentiation in limb bud cells. The activity of Erk1/2, JNK and expression of cyclin D1 are downregulated in limb bud cells with DBP treatment. These results demonstrate that DBP inhibited cell proliferation and differentiation through pathway involving Erk, JNK and cyclin D1.

Methods and Materials

Cell Culture

Cell culture was prepared according to the method of Flint and Orton (4). Cell proliferation and differentiation was measured by neutral red and alcian blue staining, respectively.

Cell Cycle Analysis

At days 1, 2, and 4, cultures were harvested and collected. Cells were fixed in 80% ethanol. After addition of propidium iodide solution and RNase, the samples were incubated for 30 min at 37℃ and analyzed with a FACScan flow cytometer (Becton Dickinson. Franklin Lakes, NJ).

Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Cells were solubilized with lysis buffer and boiled. An equal amount of protein was electrophoresed and proteins were transferred onto a nitrocellulose membrane and processed for immunoblotting. Blots were incubated with the rabbit polyclonal anti-cyclin D1, -p-Erk1/2, -p-JNK antibodies (Santa Cruz Biotechnology). Blots were further incubated with horseradish peroixdase-conjugated secondary antibody, and specific bands were visualized by chemiluminescence (ECL, Amersham International).

Results and Discussion

Cell Proliferation and Differentiation

The effect of DBP on rat embryonic limb bud cell proliferation is illustrated in Fig. 1. Following 96 hrs of treatment with DBP, proliferation and differentiation of the limb bud cells

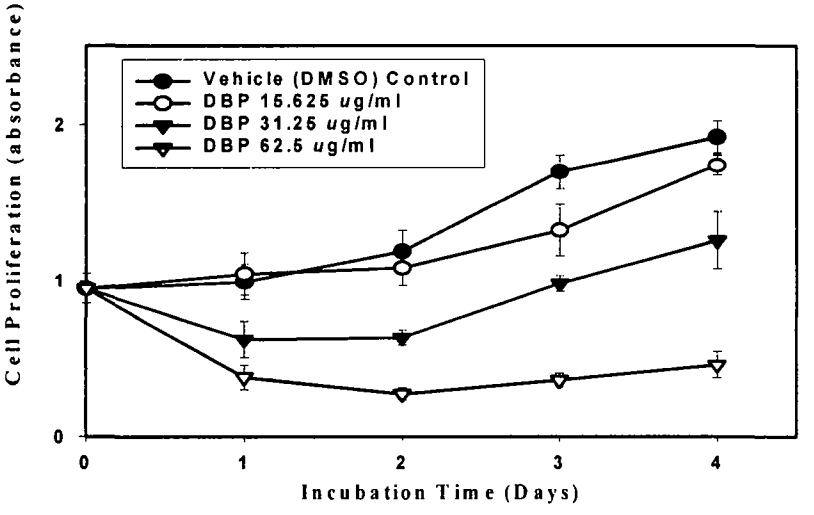
were inhibited in a dose dependent manner (Fig. 2). The concentrations showing a 50% inhibition of proliferation and differentiation were 25.54 μg/ml (91.75 μM) and 21.21 μg/ml (76.20 μM), respectively.

Cell Cycle Analysis

DBP slightly accumulated cells in G1 phase of the cell cycle after 1, 2, and 4 day culture in rat embryonic limb bud cells [Fig. 3 (A)].

Western Blot Analysis

Fig. 3 (B) showed that DBP downregulated activation of JNK, Erk1/2 and expression of cyclin D1 protein in limb bud cells. MAP kinases including Erk and JNK play an important role in a variety of cellular responses including proliferation, development, differentiation, cell cycle and apoptosis of cells (5). Cyclins are involved in the regulation of cell cycle and cyclin D control cell entry



from G1 to S phase. There are three known mammalian isoforms of cyclin D (types D1, D2, and D3). Each type has its own pattern of expression in different cell types (6). Further studies are needed to investigate the pathway.

Fig. 1. Proliferation in Limb Bud Cells Over a 4 Day Period.

Limb bud cells were cultured at a density of 2×10^7 cells/ml, and treated with the indicated concentration of DBP. Cell proliferation was quantified by neutral red assay on each day of a 4 day culture period. Each point represents the mean ± SD of duplicate determinations, conducted in four to eight replicate wells per experiment.

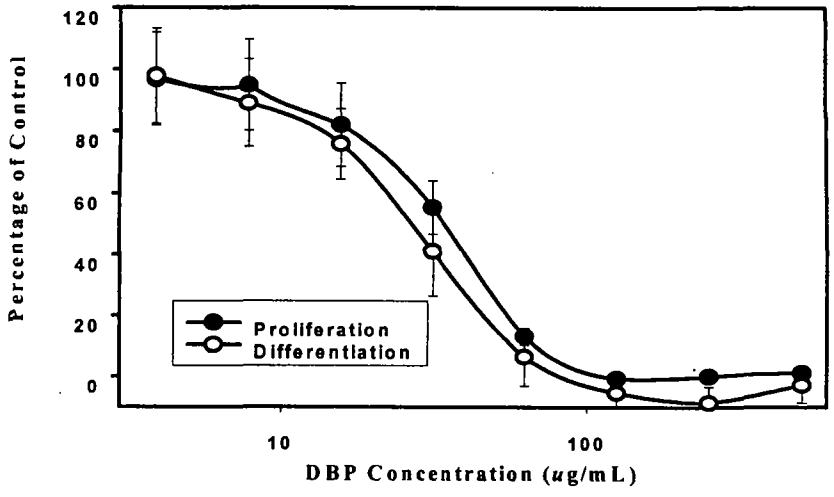
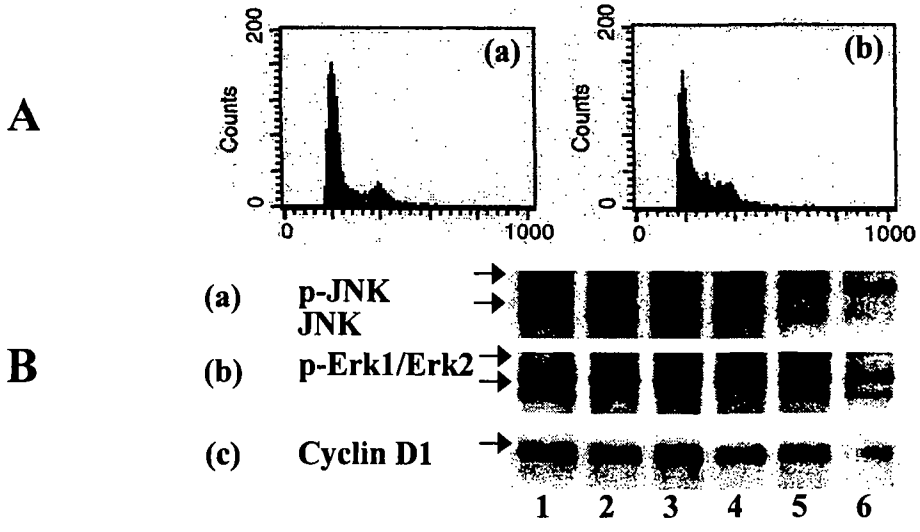


Fig. 2. Effect of DBP on the Proliferation and Differentiation of Limb Bud Cells.



Each point represents the mean ± SD of duplicate determinations from three separate experiments

Fig. 3. Effect of DBP on cell cycle and activation of JNK, Erk1/2 and expression of cyclin D1 in rat embryonic limb bud cells.

Cells were treated with DBP. After 1, 2, and 4 days, cells were harvested and carried out (A) cell cycle and (B) western blot analysis ; A. (a) vehicle, day 2 (b) DBP treated, day 2 ; B. (a) p-JNK, (b) p-Erk1/Erk2, and (c) cyclin D1 protein level. Lane 1, 3, and 5 denote vehicle (DMSO) treated: days 1, 2, 4, respectively, lane 2, 4, and 6 denote DBP (31.25 *u*g/ml) treated: days 1, 2, 4, respectively.

Acknowledgements
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