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Cytolytic Activities of Taxol on Neural Stem Cells

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Stem cells have been the subject of increasing scientific interest because of their utility in numerous biomedical applications. Stem cells are capable of renewing themselves; that is, they can be continuously cultured in an undifferentiated state, giving rise to more specialized cells of the human body. Therefore, stem cells are an important new tools for developing unique, *in vitro* model systems to test drugs and chemicals and a potential to predict or anticipate toxicity in humans. In the present study, *in vitro* cultured F3 immortalized human neural stem cell line and *in vivo* adult Sprague Dawley rats was used to evaluate the cytotoxicity of anticancer drug paclitaxel. *In vitro* apoptotic activity of paclitaxel was evaluated in F3 cell line by a MTT assay and DAPI test. The cell death was induced with the treatment of 20 nM paclitaxel and chromatin degradation was detected by DAPI staining, which was analyzed by fluorescent microscope. *In vivo* studies, we also observed nestin immunoreactivity on subventricular zone, which is stem cell rich region in the adult brain of the SD rat. Immunofluorescent staining result shows that pixel intensities of nestin were decreased in a dose dependent manner. These results suggest that paclitaxel is able to induce cytotoxic activity both in F3 neural stem cell line and neural stem cell in SD rat brain.

Key Words: Paclitaxel, F3 human neural stem cell, Cytotoxicity

INTRODUCTION

Stem cells are defined by two essential abilities: first, they are able to generate identical copies, or self-renew, and they give rise to different cell types. Differentiation is the process whereby cells acquire new morphological and functional characteristics (Theise and Krause, 2002). *In vivo*, differentiation governs the establishment of somatic cell lineages from germ layer precursors during mammalian development. However, the process is also prevalent in tissue repair and maintenance during postnatal and adult life, such as in differentiation of bone marrow stem cells into functional erythroid and myeloid blood cells (Ramalho-Santos et al., 2002, Sato et al., 2003).

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Stem cells can be classified into two major categories, according to their developmental status: embryonic and nonembryonic, or adult, stem cells. Embryonic stem cells are pluripotent cells, isolated from the inner cell mass of the blastocyst-stage mammalian embryo (Martin, 1981; Nagy et al., 1990). Pluripotent cells are capable of giving rise to most tissues of the organism, including the germ line during development (Muller et al., 2000; Reubinoff et al., 2000; Xu et al., 2002; Rambhatla et al., 2003).

Adult stem cells (ASCs), also known as mesenchymal stem cells or multipotent adult progenitor cells, are specialized cells found within many tissues of the body where they function in tissue homeostasis and repair. Multipotent cells are precursor cells capable of differentiation into several different cell types but not all cell types in the organism like pluripotent cells. Multipotent ASCs can be harvested from organs, grown in culture as a homogeneous adherent population of fibroblast-like cells, and induced to differentiate into multiple cell types (Pitenger et al., 1999; Clarke et al., 2000; Yang et al., 2002; Zhao et al., 2003).

Neural stem cells (NSCs), derived from hippocampus and other germinal centers of the brain, have been isolated and defined as cells with the capacity of self-renewal and multi-lineage differentiation. These cells possess the utilizing potential to develop the transplantation strategies and screen the candidate agents for neurogenesis in neurodegenerative diseases.

Paclitaxel is a chemotherapeutic agent extracted from the bark of the Pacific yew (Taxus breifolia). At the cellular level, paclitaxel interferes with mitotic spindle function by enhancing the rate and yield of microtubule assembly and preventing microtubule depolymerization resulting in G2/M arrest (Long et al., 1994; Rieder et al., 1994; Derry et al., 1995; Madiraju et al., 2005). Also they have demonstrated significant activity in clinical trials against a wide variety of tumors, including refractory ovarian cancer, metastatic breast cancer, non-small cell lung cancer, AIDS-related Kaposi's sarcoma, head and neck malignancies and other cancers (Rowinsky et al., 1995; Sabbatini et al., 2004; Han et al., 2005).

In this context, this study aimed to clarify the cytolytic effect of paclitaxel, with which a variety of carcinomas have been treated, on neural stem cells by using the cultured F3 immortalized human neural stem cell line *in vitro* and adult Sprague Dawley rats *in vivo*.

MATERIALS AND METHODS

1. Cell culture and morphology observation

F3 human neural stem cells were maintained in a DMEM that was supplemented with 5% fetal bovine serum. The cells were treated with various concentrations of paclitaxel for 24 h. And the cells were observed under phase-contrast microscopy.

2. *In vitro* cytotoxicity assay

F3 human neural stem cells were maintained in a DMEM that was supplemented with 5% fetal bovine serum. All of the cells were grown at 37℃ in a humidified atmosphere of 5% CO_2. Cytotoxicity was measured by the microculture tetrazolium (MTT) method. The cells were resuspended in 100 ml DMEM at 1×10^4 cells/ml after verifying cell viability by a trypan blue dye exclusion assay. One hundred ml of cell suspension were distributed into each well of a 96-well plate. After the treated cells were incubated for 24 h and 48 h, 50 ml MTT (1 mg/ml, Sigma, USA) was added into each well. The plates were incubated for 4 h. To dissolve formazan, 150 ml DMSO was added and the absorbance values of each well at 540 nm were read using an ELISA Reader (Molecular Device, Menlo Park, USA). Assays were performed at least three times, and data shown are representative of those assays.

3. DAPI stain

Cells grown in 12-well plates were fixed with paraformaldehyde for 20 min at 20℃. After blocking with PBS supplemented with 0.1% bovine serum albumin and 0.3% Triton X-100 for 40 min. Nuclei were then stained with DAPI (1 μg/ml) for 5 min. Fluorescence images were obtained by fluorescence microscopy.

4. Animals

Adult male Sprague-Dawley rats (Charles River Laboratories, St. Constanet, Quebec, Canada) weighting 250~300 g were used for the experiments. The animals were housed in groups of three in atemperature- and humidity-controlled room that was kept on a 12 h light/dark schedule. Food and water were available ad libitum throughout the experiment. All animal experiments were conducted in accordance with the guidelines set by the Committee on Animal Research at the University of British Columbia.

5. Tissue preparation and immunohistochemical analysis

After paclitaxel injection, the animals were deeply anesthetized and then transcardially perfused with heparinized cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (0.1 M PB, pH 7.4). The brains were removed from the skull and postfixed in the same fixative solution overnight and then placed in 30% sucrose for cryoprotection. The brains were then frozen in powdered dry ice and stored at 70℃. Coronal sections throughout the striatum were cut at 40 m intervals on a cryostat and stored in cryoprotectant solution. Free-floating sections were pro-

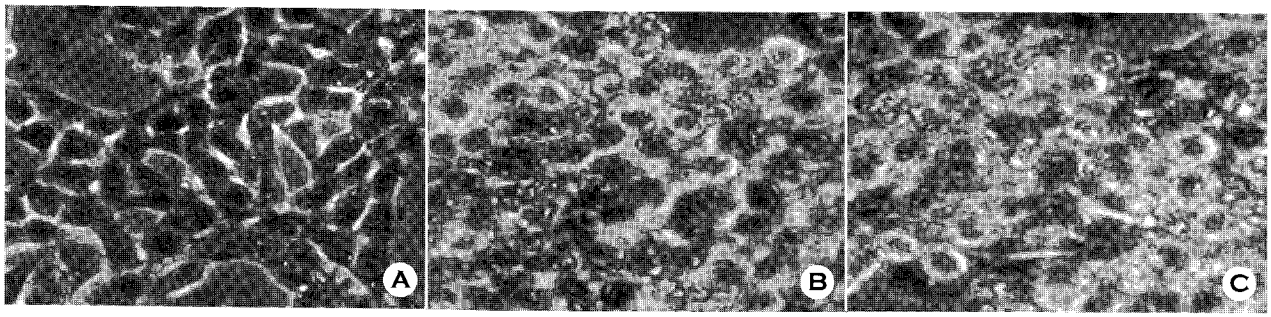

Fig. 1. Phase contrast image of F3 immortalized human neural stem cells treated with paclitaxel. The cells were treated with the control vehicle **(A)**, 50 nM paclitaxel **(B)**, 200 nM paclitaxel **(C)** for 24 h.

cessed for the immunohistochemistry as described previously (Ryu et al., 2004). Briefly, endogenous peroxidase was quenched with 3% hydrogen peroxide in 0.1 M phosphate-buffered saline (PBS) and sections were incubated in blocking solution containing 10% normal goat serum and 0.2% Triton X-100 in 0.1 M PBS for 1 h. The sections were then incubated overnight at 4℃ with the following primary antibodies. Sections were rinsed three times with PBS, incubated at room temperature for 2 h with fluorescein-conjugated secondary antibody. Stained sections were examined under a Zeiss Axioplan 2 fluorescent microscope (Zeiss, Jena, Germany).

6. Statistical analysis

All of the assays were set up in triplicate. The results were expressed as the mean ± SD. Statistical analysis was determined by a Students *t*-test.

RESULTS AND DISCUSSION

So as to examine closely any morphological changes associated with paclitaxel for the cultured F3 human neural stem cells, the cells were treated 50 nM and 200 nM paclitaxel samples, subsequently cultured them in media containing 1% FBS for 24 hrs, and then observed them under phase-contrast microscope. The normal cellular morphology was intactly maintained in the control group, whereas the concentration-dependent changes in the cellular morphology were significant in the experimental group treated with paclitaxel (Fig. 1).

In order to demonstrate that such the cellular morphological changes are associated with the cytotoxicity of

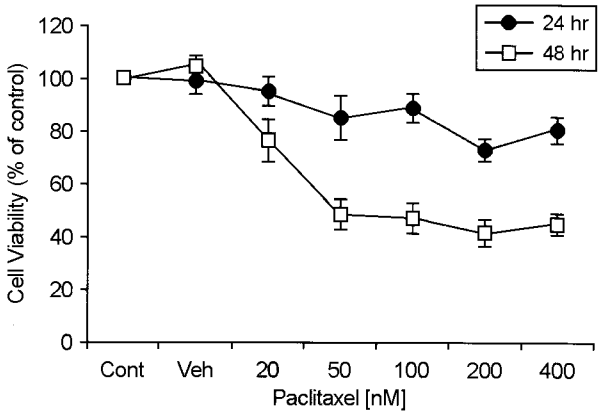

Fig. 2. Cytotoxic effects of pacritaxel on F3 immortalized human neural stem cells. The cells were incubated with various concentrations of pacritaxel for 24 h and 48 h. Cytotoxicity was measured by MTT assay. The results are presented as mean ± SD for triplicate.

paclitaxel, MTT assay was performed to determine its cytotoxicity. Prior to determining its cytotoxicity results in the F3 human neural stem cells, the relationship between the known cell count and measured O.D. values (absorbance) was ascertained to check that it reflects the stability of MTT assay and the number of cell survivals well. The viable cells to be screened with Tryphan blue exclusion technique were seeded onto a 96 well microculture plate at six different plating densities: 1.25×10^4, 2.5×10^4, 5×10^4, 1×10^5, 2×10^5 and 4×10^5 cells/well, followed by applying MTT assay to determine their absorbance. The standard curve of the relationship between the determined absorbance and cell count was drawn, which represented directly proportional relationship within the range measured, suggesting that the number of cell survivals was reflected correctly. Based upon this result, paclitaxel was added by density into a 96 well microculture plate where 1×10^4 cells had been seeded, and after 24 hrs and 48 hrs, MTT assay was per-

formed to determine the absorbance at 540 nm. At the same time, the determined absorbance was converted into % survival (Fig. 2). It was found that the F3 human neural stem cell inhibited cell growth as the concentration rate of paclitaxel. In addition, it was revealed that it inhibited cell growth more strongly after 48 hrs treatment than after 24 hrs. In other words, paclitaxel inhibited the growth of the F3 human neural stem cell depending on its concentration and treatment time.

To demonstrate the morphological changes in the nucleus if the cells associted with paclitaxel, DAPI staining was carried out with fluorescence microscope (Fig. 3). The degree of chromatin degradation increased as paclitaxel concentration rate and also over time. Moreover, the results of counting the nucleuses and graphing the percentage of damaged nucleuses in the entire ones also indicate the same results.

The results of *in vitro* experiment described above suggest that paclitaxel induces the apoptosis of the cultured F3 human neural stem cell depending on its concentration and

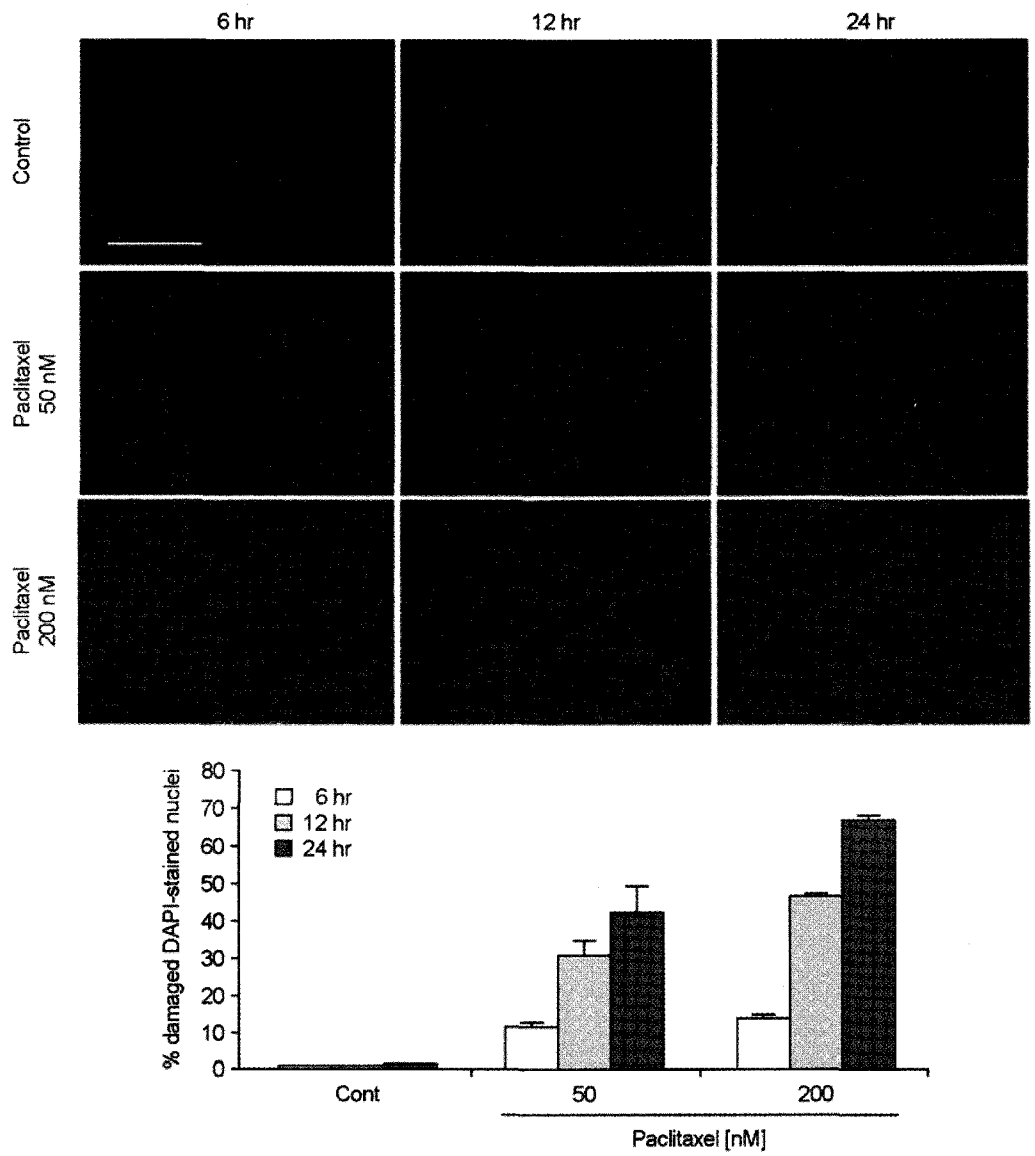

Fig. 3. Fluorescence microscopic appearance of DAPI-stained nuclei of paclitaxel-treated cells. The cell were incubated with various concentrations of paclitaxel for 6 h, 12 h, and 24 h. The cell stained with DAPI, and analyzed under a fluorescence microscope (×40) (A). The cells with condensed and fragmented nuclei were counted under a fluorescence microscope (×40). The results are presented as mean ± SD for triplicate.

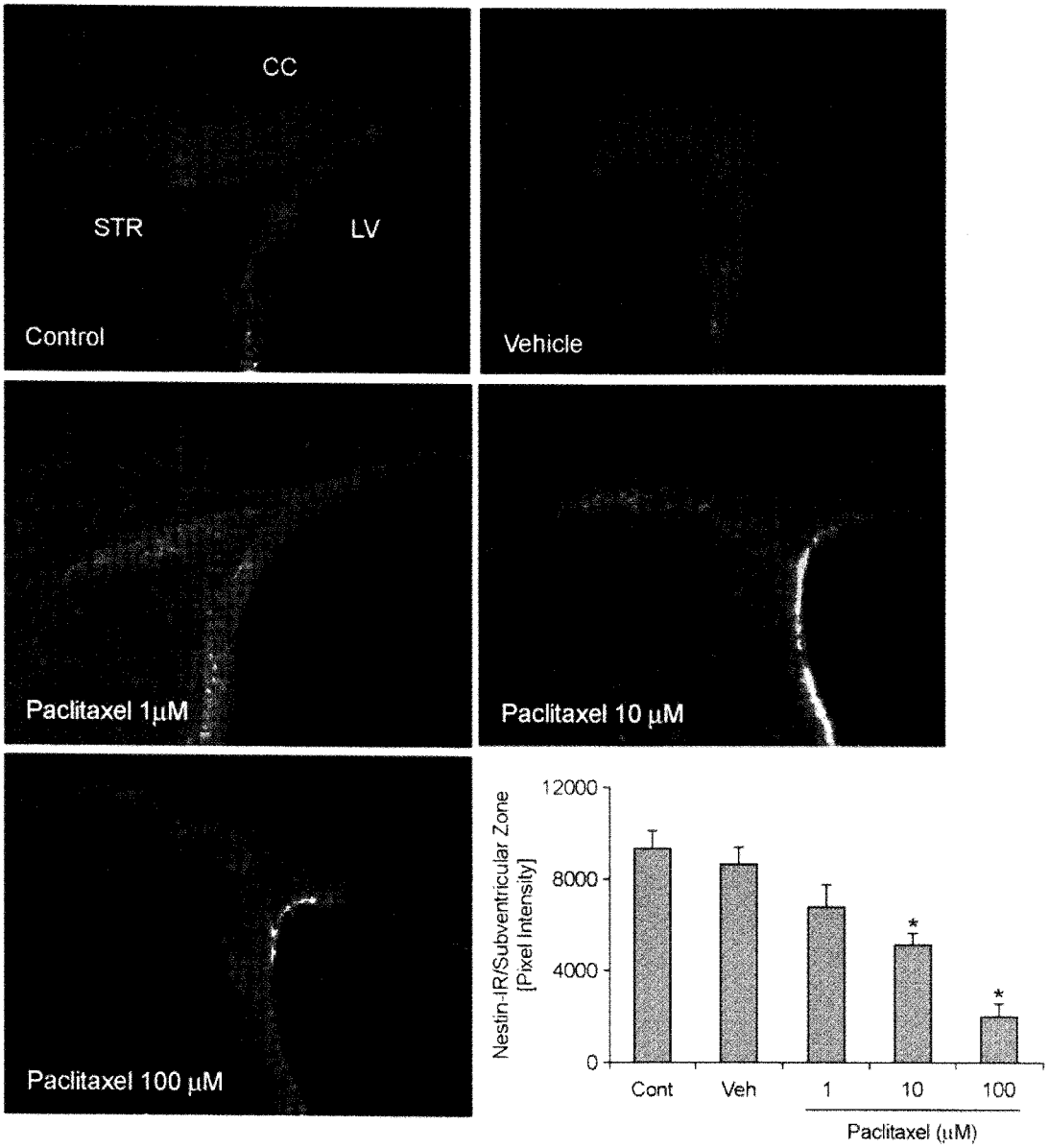

Fig. 4. Immunofluorescence micrographs of subventricular zone in the adult brain of the SD rat. Paclitaxel was treated to lateral ventricle of SD rat by stereotaxic injection. After 3 days, we measure nestin immunoreactivity on subventricular zone of brain section. The results are presented as mean ± SD for triplicate (LV: lateral ventricle, STR: striatum, CC: corpus callosum).

time. With the intention of verifying these results *in vivo*, an *in vivo* experiment was designed and performed.

For the *in vivo* experiment, adult Sprague Dawley rats weighing 250 g through 300 g were used. Regarding paclitaxel treatment, stereotaxic injection method was employed to apply single injection to cerebrolateral ventricle region. Three days after injection, the rats were sacrificed and the brain was fixed by means of transcardical perfusion, and the striatal area was frozen-sectioned at 40 μm thickness. To observe nestin as a neural stem cell marker in the sections, immunofluorescence staining was proceeded. By examiningly closely the subventricular zone area rich in neural stem cells in the brain, changes in the neural stem cell associated with paclitaxel were studied (Fig. 4). While the control group not treated with paclitaxel showed high-intensity fluorescence attributed to the stained nestins of neural stem cells in the subventricular zone, the group treated with paclitaxel exhibited the tendency that the fluorescent intensity decreased as the concentration of paclitaxel increased. This result showed that the neural stem cells

might be damaged by paclitaxel.

In conclusion, the above-mentioned results suggest that paclitaxel used as drug on various kinds of cancers may damage stem cells which are performed a critically important roles in human body. In other words, paclitaxel administered for the purpose of cancer treatment may destroy stem cells, and thus, cause the secondary cell toxicity. In summary, the use of stem cells to the field of drug toxicology has possibility to help develop a risk evaluation of drug exposure to human beings.

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