D609 induces vascular endothelial cells and marrow stromal cells differentiation into neuron-like cells[1]

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ABSTRACT

AIM: To investigate the effect of tricyclodecane-9-yl-xanthogenate (D609) on cell differentiation in vascular endothelial cells (VECs) and marrow stromal cells (MSCs). **METHODS:** Morphological changes were observed under phase contrast microscope. Electron microscope and immunostaining were used for VECs identification. The expressions of neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) were examined by immunohistochemistry. **RESULTS:** After 6 h of induction with D609, some VECs showed morphological changes characteristic of neurones. 9 h later, more VECs became neuron-like cells. About 30.8 % of VECs displayed positive NSE ($P<0.01$), while the expression of GFAP was negative. When MSCs were exposed to D609, the cells displayed neuronal morphologies, such as pyramidal cell bodies and processes formed extensive networks at 3 h. 6 h later, almost all of the cells exhibited a typical neuronal appearance, and 85.6 % of MSCs displayed intensive positive NSE, but GFAP did not express. **CONCLUSION:** D609 induces VECs and MSCs differentiation into neuron-like cells.

INTRODUCTION

Tricyclodecane-9-yl-xanthogenate (D609) exhibits a variety of biological functions, including antiviral, antitumor, anti-inflammatory, and anti-apoptosis activities[1-4]. Most of these activities have been largely attributed to the inhibitory effect of D609 on phosphatidylcholine-specific phospholipase C (PC-PLC). However, a recent research showed that D609 was a potent antioxidant and had the ability to inhibit ionizing radiation (IR) -induced cellular oxidative stress and protected the mice from IR-induced lethality[5].

Our previous study showed that D609 inhibited apoptosis induced by deprivation of survival factors in vascular endothelial cells (VECs)[6]. But, when subconfluent VECs were exposed to D609, some cells became neuron-like cells in morphology. This founding initiated us to study the role of D609 in inducing the cells differentiation into neurons. Besides, marrow stromal cells (MSCs) may be useful in the treatment of a wide variety of neural diseases, offering significant advantages over other "stem" cells. The marrow cells are readily accessible, grow rapidly in culture, and differentiate into neurons exclusively with use of a simple protocol. However, whether D609 can induce MSCs differentiation into neuron-like cells is not known. In this study we investigated the action of D609 in VECs

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and MSCs differentiation to provide evidence for its clinical application.

MATERIALS AND METHODS

Reagents M199 medium was purchased from Gibco BRL Co, Grand Island, NY. Fetal bovine serum (FBS) was obtained from Hycolon Lab Inc USA. Fibroblast growth factor (FGF) was extracted from bovine brains in our laboratory by the method of Lobb *et al*[7]. D609 was obtained from Funakoshi Inc, Tokyo, Japan. D609 was dissolved in ethanol and applied to cells. SABC-AP immunofluorescence kit was purchased from BoShiDe Co,Wuhan, China.

Cell cultures Human umbilical vascular endothelial cells (HUVECs) were obtained in our laboratory by the method of Jaffe *et al*[8]. The cells were cultured on gelatin-coated plastic dishes in M199, supplemented with 10 % FBS, and FGF 70 μg/L (as well as heparin 100 mg/L) at 37 ºC in 5 % CO_2+95 % air. All experiments were performed on cells from 10-20 passages. VECs were identified according to a protocol provided by Antonov *et al*[9]. Human bone marrow stromal cells (hBMSCs) were obtained from fracture volunteers with a protocol described by Friedenstein *et al*[10] and cultured on plastic dishes in M199, supplemented with 20 % FBS at 37 ºC in 5 % CO_2 and 95 % air. The MSCs were purified by the method of Huang *et al*[11].

Cell differentiation induction VECs were washed once with the medium and divided into two groups: cells cultured in the medium without serum and FGF (control group); D609-treated cells (5 mg/L, 10 mg/L, 15 mg/L) in the culture deprived of serum and FGF and added D609 (D609-treated group). On the other hand, MSCs were washed once with the medium and divided into two groups: cells cultured in the medium without serum (control group); D609-treated cells (5 mg/L) in the culture deprived of serum and added D609 (D609-treated group). The morphological changes of the cells were observed by phase contrast microscope (Nikon, Japan).

Immunohistochemistry To confirm the character of the neuron-like cells, we examined the expressions of neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) according to the instruction of SABC-AP immunofluorescence kit. VECs and MSCs after treatment were washed three times with 0.1 mol/L TBS, and fixed for 20 min with acetone at room temperature. Cells were blocked with normal goat serum for 20 min at room temperature. Then, primary antibodies (rabbit anti-NSE IgG or rabbit anti-GFAP IgG) were added and incubated in a humid chamber at 4 ºC overnight. After washing with 0.1 mol/L TBS, secondary antibodies (biotin-goat anti-rabbit IgG) were added and incubated at 37 ºC for 20 min. After washing with 0.1 mol/L TBS, cells were incubated with SABC-AP complexes for 20 min at 20 ºC-37 ºC. Finally the cells were incubated with BCIP/NBT (1:20/TBS) for 10-20 min at 20 ºC-37 ºC. The cells were observed and their photos were taken by phase contrast microscope . The positive cells were counted by the method of Jia *et al*[12]. For every point, the mean value was calculated from five random field observations of three replicate experiments, and a minimum of 50 cells per field were counted.

Statistical analysis Data were expressed as mean±SD and analyzed by *t*-test.

RESULTS

Identification of VECs and MSCs Electron microscope showed structures compatible with Weibel-Palade bodies that are found exclusively in vascular endothelium (Fig 1). The positive result from immunostaining of factor VIII related antigen ensured that the cells were VECs (Fig 2). The asymmetrical division, which is the characteristic of the stem cells, was observed clearly in the cultures obtained from bone marrow under phase contrast microscope. This finding showed that the adherent cells were MSCs.

Cell morphological changes After a 6-h of exposure to D609 15 mg/L, changes in morphology of some VECs were apparent. Generally, cytoplasm in

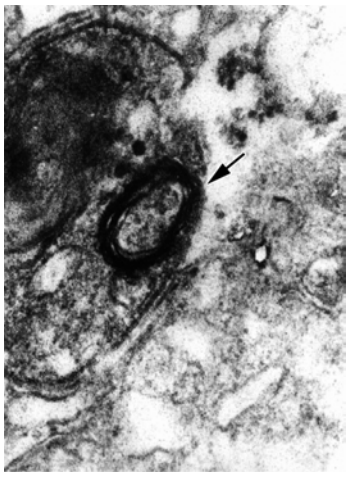

Fig 1. The electron microscopic photograph of VECs with Weibel-Palade bodies (arrow). ×50 000.

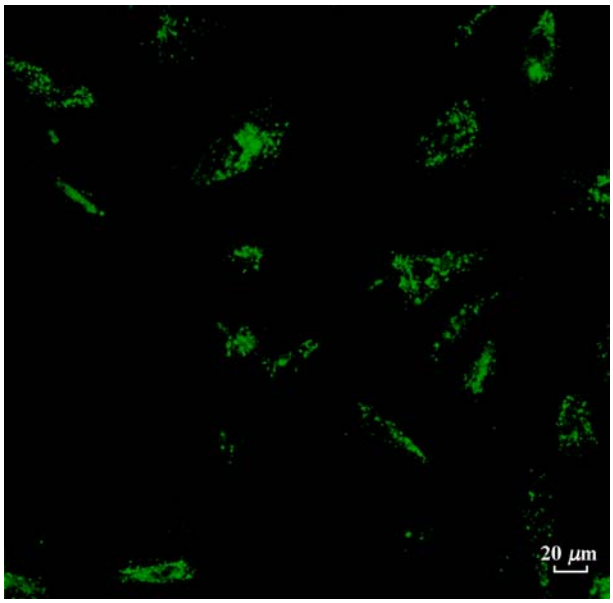

Fig 2. The positive result from immunostaining of factor VIII related antigen in VECs.

VECs retracted towards the nucleus, forming a triangular cell body and process-like extensions peripherally. These processes formed extensive networks locally. Some of the cell bodies became refractile, very similar to neurons. At the same time, a few of cells fell off and died. After 9 h, about 30.8 % of the cells exhibited a typical neuronal appearance (Fig 3B). There were no obvious morphological changes in the control groups (Fig 3A and Fig 3C). After a 3-h treatment with D609 5 mg/L MSCs started to change into neuron-like cells in morphology. After 6 h, 95 % of the cells exhibited a typical neuronal appearance (Fig 3D). When the cells were exposed to D609 10 mg/L, almost all of the cells floated in the medium and died. So we chose D609 5 mg/mL as the most proper concentration and the typical neuronal appearance remained for 3 d.

Immunohistochemistry assay The expression of neuronal marker NSE in the neuron-like cells was positive (Fig 4A), while GFAP did not express in VECs (Fig 4B). Furthermore, 30.8 % of VECs displayed positive NSE at 9 h. The effect of D609 was dose-dependent in VECs (P<0.01, Tab 1). We got the similiar results that MSCs could be induced to differentiate into neuron-like cells by D609 (Fig 4C). About 85.6 % of MSCs displayed intensive positive NSE at 6 h (P<0.01) (Tab 2), but the expression of GFAP was negative (Fig 4D).

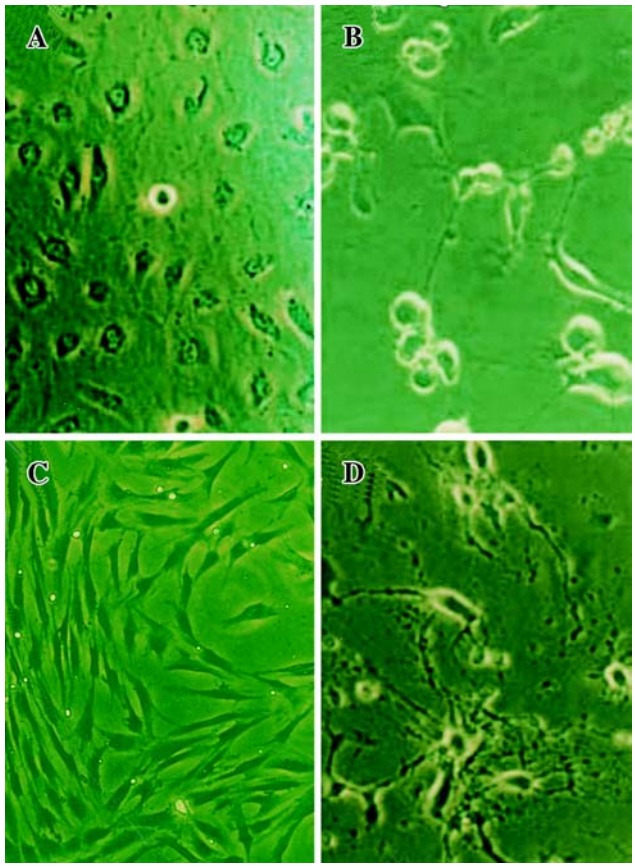

Fig 3. The morphological photographs of VECs and MSCs. (A) VECs cultured in the medium without FGF and serum. (control group) (B) VECs treated with D609 15 μg/mL in the medium deprived of FGF and serum. (D609-treated group) (C) MSCs cultured in the medium without serum. (control group) (D) MSCs treated with D609 5 mg/L in the serum free medium. The time of treatment was 6 h. (D609-treated group)×400.

Tab 1. The differentiation rate of VECs treated with D609. Positive cells remaining on the dishes were counted 9 h after deprivation of serum and FGF and treatment with D609. n=3. Mean±SD. [a]P>0.05, [b]P<0.05, [c]P<0.01 *vs* the 1st group.

Group	D609/mg·L^{-1}	Differentiation rate/%
1	0	0
2	5	1.4±1.2[a]
3	10	3.2±2.0[b]
4	15	30.8±1.6[c]

DISCUSSION

D609 has been used as a specific inhibitor of PC-

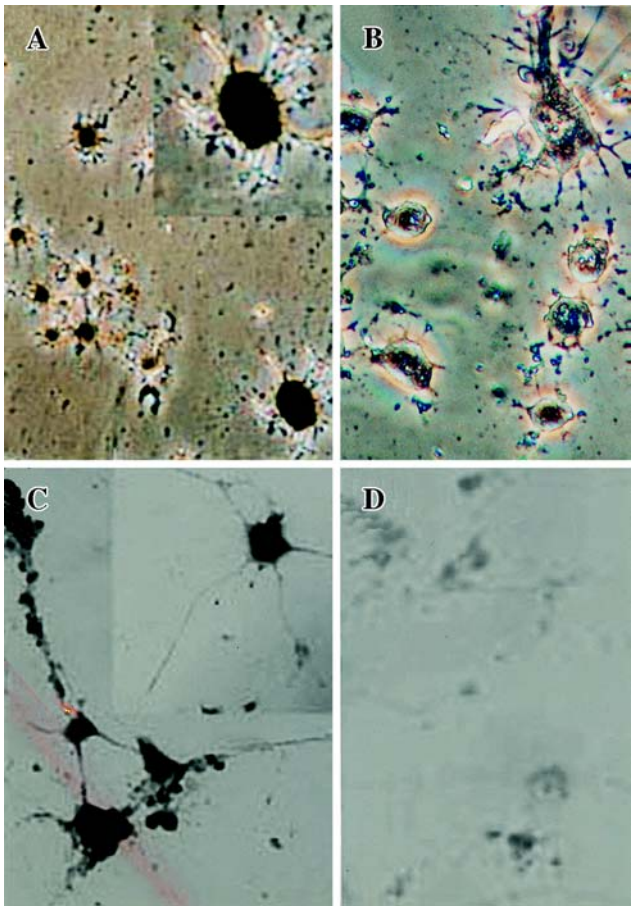

Fig 4. The differentiation of cells treated with D609 into neurons. (A) VECs treated with D609 exhibited NSE positive at 9 h. (B) VECs treated with D609 exhibited GFAP negative at 9 h. (C) MSCs treated with D609 exhibited NSE positive at 6 h. (D) MSCs treated with D609 did not express GFAP at 6 h. ×400.

Tab 2. Time course of D609 effect on MSCs differentiation rate at 5 mg/L. The positive cells remaining on the dishes were counted 3, 6, 9, and 72 h after the start of treatment. *n*=3. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* 0 h.

Time/h	Differentiation rate/%
0	0
3	4.8±2.0^{b}
6	85.6±1.2^{c}
9	86.3±2.1^{c}
72	83.8±1.4^{c}

PLC for about 10 years. Recently, it was reported that D609 was a potent antioxidant. Our previous results showed that D609 suppressed apoptosis induced by deprivation of survival factors in VECs by its inhibition on PC-PLC. But this function of D609 was performed when VECs became confluent. In this study, we found that when sub-confluent VECs were exposed to D609, these cells differentiated into neuron-like cells in morphology.

The experiment results showed that the expression of neuronal marker NSE was positive while the expression of GFAP was negative. The expression of NSE protein indicated that differentiated cells induced by D609 possessed some features of neurons. The data indicated that D609 could induce VECs and MSCs differentiation into neuron-like cells, not glial cells *in vitro*.

A recent finding indicates that VECs has the potential to differentiate into smooth muscle-like cells[13]. Our data showed that VECs could differentiate into neuron-like cells. These results suggest that VECs exhibit multipotentiality. At the same time, we found D609 could quickly induce MSCs differentiation into neuron-like cells. Then we asked what is the possible mechanism by which D609 could induce cells to differentiate into neuron-like cells? Is it due to the inhibition of PC-PLC or/and its antioxidant? Our previous study indicated that D609 could inhibit the activity of PC-PLC in VECs. At present, it was shown that D609 might function as a potent antioxidant, because some of the inducers which induce MSCs differentiation into neurons are antioxidants, such as b-mercaptoethanol (BME), butylated hydroxyanisole (BHA), and dimethyl sulphoxide (Me_2SO)[14]. A recent report showed that antioxidant activity was proposed as a generalized stimulus for cell differentiation during development[15]. In this study, D609 might induce VECs and MSCs differentiation into neuron-like cells by inhibiting the activity of PC-PLC, or by redox, or by both.

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