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The protective effect of curcumin in Olfactory Ensheathing Cells exposed to hypoxia

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ABSTRACT

Curcumin, a phytochemical component derived from the rhizomes of *Curcuma longa*, has shown a great variety of pharmacological activities, such as anti-inflammatory, anti-tumor, anti-depression and anti-oxidant activity. Therefore, in the last years it has been used as a therapeutic agent since it confers protection in different neurodegenerative diseases, cerebral ischemia and excitotoxicity. Olfactory Ensheathing Cells (OECs) are glial cells of the olfactory system. They are able to secrete several neurotrophic growth factors, promote axonal growth and support the remyelination of damaged axons. OEC transplantation has emerged as a possible experimental therapy to induce repair of spinal cord injury, even if the functional recovery is still limited. Since hypoxia is a secondary effect in spinal cord injury, this *in vitro* study investigates the protective effect of curcumin in OECs exposed to hypoxia. Primary OECs were obtained from neonatal rat olfactory bulbs and placed both in normal and hypoxic conditions. Furthermore, some cells were grown with basic Fibroblast Growth Factor (bFGF) and/or curcumin at different concentration and times. The results obtained through immunocytochemical procedures and MTT test show that curcumin stimulates cell viability in OECs grown in normal and hypoxic conditions. Furthermore, the synergistic effect of curcumin and bFGF is the most effective exerting protection on OECs. Since spinal cord injury is often accompanied by secondary insults, such as ischemia or hypoxia, our results suggest that curcumin in combination with bFGF might be considered a possible approach for restoration in injuries.

1. Introduction

Curcumin is a natural polyphenolic compound which is extracted from the rhizomes of *Curcuma longa* and it has been used in traditional Chinese and Indian medicine for centuries (Prasad et al., 2011). In recent years, a wide range of pharmacological activities of curcumin have been widely recognized, even for its peculiarity to be able to cross the blood-brain barrier because of its low molecular weight; therefore, many studies have focused on its therapeutic potential in the treatment of various diseases (Monroy et al., 2013). In fact, curcumin exhibits anti-inflammatory and anti-cancer properties (Aggarwal et al., 2009; Ye et al., 2015; Chen et al., 2016), besides being beneficial for the treatment of depression (Lopresti et al., 2012; Tizabi et al., 2014; Yu et al., 2015). In addition, curcumin has shown protection in a variety of neurological disorders, including Parkinson's disease (Mytri and Bharath, 2012; Tripanichkul et al., 2013), and other disorders associated with neuroinflammation, such as Alzheimer's disease (Hamaguchi et al., 2010; Huang et al., 2012; Yang et al., 2013; Tizabi et al., 2014), Huntington's disease (Chongtham and Agrawal, 2016), multiple sclerosis (Xie et al., 2011) and axonal degeneration from neuroinflammation (Tegenge et al., 2014). Local inflammation is one of the secondary processes, besides ischemia, hypoxia, delayed axonal loss, cell death, excitotoxicity (Rossignol et al., 2007) that are characteristic in spinal cord injury, which is a serious devastating condition among humans, leading to serious disability and death.

In recent years, many papers have reported promising effects on axonal regeneration and functional recovery after transplantation of neural stem cells, Schwann Cells and Olfactory Ensheathing Cells (OECs; Mackay-Sim and St. John, 2010; Mackay-Sim, 2005; Raisman and Li, 2007; Richter and Roskams, 2008). OECs are glial cells from the olfactory system that show numerous peculiarities making them candidates for transplantations in spinal cord injury (Wang et al., 2010; Tetzlaff et al., 2011) since are able to promote axonal regeneration (Franssen et al., 2007; Su and He, 2010; Takeoka et al., 2011), functional recovery (Ramon-Cueto et al., 2000; Boyd

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et al., 2005) and remyelination (Franklin, 2003; Lu et al., 2002; Boyd et al., 2005; Sasaki et al., 2011). This is explained because OECs are a source of growth factors (GFs) (Lipson et al., 2003; Woodhall et al., 2001), express adhesion molecules and several markers, such as GFAP, $p75^{NTR}$, S100, 04, vimentin, nestin (Ramon-Cueto and Avila, 1998; Pellitteri et al., 2010). In addition, OECs present a greater ability to intermingle with astrocytes (Lakatos et al., 2000) and to migrate to areas distal from the transplantation site, forming the gap between the lesion site and normal spinal cord (Li et al., 2012) compared with other cells.

This study was designed to investigate, in an *in vitro* model, the neuroprotective effect of a curcumin treatment on OECs exposed to hypoxia, which represents one of secondary injuries in lesioned spinal cord. Moreover, a mixture of bFGF and curcumin was used to tentatively rescue OECs survival/proliferation. The results, obtained through immunocytochemical procedures, were used to assess morphological modifications and MTT test to evaluate the viability of OECs.

2. Materials and methods

2.1. Animal care

Experiments were performed on 2-d old rat pups (P2; Harlan, Italy). Animals were kept in a controlled environment (23 ± 1 °C, 50 ± 5% humidity) with a 12 h light/dark cycle with food and water available *ad libitum*. Experiments were carried out in compliance with the Italian law on animal care no. 116 /1992 and in accordance with the European Community Council Directive (86 / 609 / EEC). All efforts were made to minimize animal suffering and to use the fewest number of animals possible.

2.2. OECs cultures

As previously described (Pellitteri et al., 2007), OECs were isolated from 2-d old rat pup (P2) olfactory bulbs. Briefly, pups were decapitated and the bulbs removed and dissected out in cold (+4 °C) Leibowitz L-15 medium (Sigma). Subsequently, they were digested in Medium Essential Medium-H (MEM-H, Sigma) containing collagenase and trypsin. Trypsinization was stopped by adding Dulbecco's Modified Eagle's medium (DMEM, Sigma) supplemented with 10% Foetal Bovine Serum (FBS, Sigma). Cells were resuspended and plated in flasks fed with fresh complete medium DMEM/FBS, 2mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 µg/ml). The antimitotic agent, cytosine arabinoside (10^{-5} M) was added 24 h after initial plating to reduce the number of dividing fibroblasts. A further procedure of purification was adopted following the method by Chuah and Au (1993): OEC cultures were processed to an additional step of transferring cells from one flask to a new one. This step reduces contaminating cells because they adhere more readily to plastic than OECs, consequently forming a cellular substratum upon which OECs attached. Hence, during trypsinization, the OECs were the first to detach and if the enzymatic digestion was carefully monitored and stopped at this stage, this manipulation leaves most of the contaminating cells on the plastic. The purity of OECs was verified using immunocytochemistry with p75 and S-100. The percentage of S-100/p75 positive cells in our cultures was about 85–90% (data not shown). In the last passage, OECs were plated on 25 cm^2 flasks and cultured in DMEM/FBS supplemented with bovine pituitary extract. Cells were incubated at 37 °C in DMEM and fed twice a week.

2.3. Treatment of cells

Purified OECs were replated on 14-mm diameter poly-L-lysine (PLL, 10 µg/ml, Sigma) coated glass coverslips at a final density of $0{,}3\times10^4$ cells/coverslip and grown in DMEM/FBS (Sigma). Some cells were cultured with the addition of bFGF (10 ng/ml; Sigma-Aldrich, Milan, Italy). Curcumin was obtained from Sigma and a stock solution (30 mM) was prepared in Dimethyl sulfoxide (DMSO); in some cultures, 24 h post-seeding, curcumin was added, at different concentrations (0.1, 0.5, 5 µM diluted in culture medium) and for different times (1, 3, 6 days). A mixture of bFGF (5 ng/ml) and curcumin (0,5 µM) was administered in some OEC cultures and maintained for 1, 3 or 6 days. Control cultures (Ctr) were grown in DMEM/FBS with the addition of DMSO (5 µl/ml) with no treatment.

2.4. Hypoxic condition

To establish hypoxia, condition cells were seeded on coated glass coverslips. When cells were perfectly attached, the coverslips were upturned. This procedure reduces oxygen concentration in the cellular environment, meaning, between the coverslips and the bottom of multiwells, as reported in our previous papers (Pellitteri et al., 2014, 2015).

2.5. Immunocytochemistry

After 1, 3 and 6 days OECs were processed through immunocytochemical procedures. Cells were fixed by exposing them to 4% paraformaldehyde in 0.1 M PBS for 30 min. After washing in PBS the cell membranes were permeabilized with 5% normal goat serum (NGS) in PBS containing 0.1% Triton X-100 (PBS-Triton) at room temperature for 15 min. OECs were incubated overnight with polyclonal antibody anti-S-100 (1:400; Dako). Successively, cells were incubated with the secondary Cy3 anti-rabbit antibody (1:200; Jackson ImmunoResearch, Laboratories, Inc) to visualize primary anti S-100. The immunostained coverslips were analyzed with a Zeiss fluorescence microscope and images were captured with an Axiovision Imaging System. A direct cell count was carried out to allow the evaluation of OEC number for each specific treatment in different grown conditions. No non-specific staining of cells was observed in control incubations in which the primary antibody was omitted.

2.6. Cellular viability

OECs were seeded in plastic multiwell (24) plates (Nunc) and grown both in normal and hypoxic condition. Cells were incubated at 37 °C in a humidified 5% CO_2–95% air mixture. At the end of curcumin and/or bFGF treatment time, cellular viability was evaluated by the 3-[4, 5-dimethylthiazol- 2-yl)-2,5-diphenyl] tetrazolium bromide (MTT, Sigma) reduction assay, a quantitative colorimetric method (Mosmann, 1983). Briefly, MTT was added to each multiwell with a final concentration of 1.0 mg/ml and placed for 2 h in a CO_2 incubator. Media were gently removed and MTT solvent (acid-isopropanol/SDS) was added, then cells were placed on an orbital shaker for 15 min. The absorbance was read by a multisKan reader at 570 nm. Results were expressed as the percentage MTT reduction of control cells.

2.7. Statistical analysis

For each culture condition a minimum of 4 experiments were carried out. Results were expressed as the percentage MTT reduction of control cells. Differences between culture conditions were assessed using one-way analysis of variance (one-way ANOVA) followed by post hoc Holm–Sidak test.

Values for each group were expressed as the mean ± S.D. Differences were considered statistically significant at $P < 0.05$.