EXPERIMENTAL STUDY

Low concentrations of morphine enhanced the neuroglia-like differentiation

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ABSTRACT

AIM: The present study was undertaken to evaluate the effects of different concentrations of morphine in chronic manner on neuroglial differentiation in PC12 cells.

METHODS: PC12 cells were cultured in RPMI 1640 culture medium including 0.02 % bovine serum albumin together with different concentrations of morphine for 12 days. Cytotoxicity was performed by lactate dehydrogenase assay. Cell death was performed by PI/Hoechst staining assay. Neuroglial differentiation was performed by Nestin, Tuj-1, MAP-2, S-100 and GFAP Immunocytochemistry assay.

RESULTS: Data showed that morphine either at low or high concentration activated opioid receptors, which resulted in a decrease of cytotoxicity and cell death and induction of Nestin, Tuj-1, MAP-2, Neurofilament-M (NF-M), GFAP and S-100 protein expression as compared in treated cells with the control (untreated cells) (p<005).

CONCLUSION: It can be concluded that low concentrations of morphine in chronic manner stimulate the neuroglial-like differentiation by activating protein expression and survival-promoting signaling in PC12 cells with opioid receptor-dependent mechanism (Fig. 8, Ref. 36). Text in PDF www.elis.sk.

KEY WORDS: morphine, naltrexone, neuroglial differentiation, PC12 cell.

Introduction

Morphine is an effective pain-relieving drug and can affect neurotransmitter release, reduction of intracellular cAMP and apoptosis in neuronal cells (1, 2).

Morphine binds to Mu receptor and modulates survival (3–5), apoptosis (6), cell growth (7), proliferation (7–13), and viability (9) of neural and tumoral cells (14–16). Morphine has an effective role in the development of CNS, neuritogenesis, and cell differentiation of neuroglial cells (18–22). Further, concerning neuritogenesis, chronic morphine has been found to exert two opposite effects that are concentration-dependent. Morphine, at 1mM concentration, inhibited neurite elongation in PC12 cells, cerebellar granule neurons, cerebellar neuroblasts, and embryonic dorsal root ganglion neurons with naloxone-dependent mechanism whereas, at 1pM, enhanced neurite elongation of PC12 cells, cultured rat spinal cord, cerebral cortical neurons with naloxone-independent mechanism (22–25).

This study was undertaken to determine whether chronic exposure of morphine stimulates neuronal differentiation in PC12 cells and whether that action dependent on the mu-opioid receptor.

Materials and methods

Cell culture

PC12 cells were grown in RPMI 1640 culture media (Gibco), supplemented with 10 % fetal bovine serum (FBS, Gibco), 1 % non-essential amino acid (NEAA; Sigma), 2 mM L-glutamine (Sigma), 100 IU/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) in 10-cm tissue culture dishes. The cultures were incubated at 37 °C in 5 % CO₂ medium was replaced every 2 days.

While cell cultures reached 70 to 80 % confluence, they were trypsinated using trypsin-EDTA 0.25 % (Sigma) and were subcultured at a density of 1×10⁴ cells/well in 24-well culture plates.

Morphine treatment

One day after plating the cells, cells were washed with phosphate buffer saline (PBS), pH: 7.4. There were two groups: group I; no preincubation with naltrexone hydrochloride (Sigma) (without naltrexone) and group II; preincubation with 100 nM naltrexone. There were six treatments in each group. PC12 cells were cultured in RPMI 1640 culture medium containing 0.02 % bovine serum albumin (BSA; Sigma) together with different concentrations of morphine sulfate (Temad, Iran). The treatments were 1×10⁻¹² M morphine (treatment 1), 1×10⁻¹⁰ M morphine (treatment 2), 1×10⁻⁸ M morphine (treatment 3), 1×10⁻⁶...
M morphine (treatment 4) and $1 \times 10^{-4}$ M morphine (treatment 5) and culture media alone (treatment 6) for 12 days. The cells were placed in the incubator at 37 °C with 5% CO₂.

**Cell cytotoxicity measurement**

Cell cytotoxicity was quantified by measuring the release of lactate dehydrogenase (LDH) from damaged or destroyed cells into the media. Cytotoxicity was measured with LDH Cytotoxicity Detection Kit (Roche, Germany). Cells were plated in 24 well culture plates with $1 \times 10^4$ cells/mL densities for 12 h. Then, cells were cultured by differentiation medium for 24 h. The colorimetry of LDH activity was measured by calculating the absorbance of samples at 492 nm using a plate reader (EL800; USA). All experiments were replicated independently at least 3 times. Within each experiment, we replicated each condition 4 times.

**Quantification of cell death incidence**

Hoechst / PI nuclear staining was carried out as previously described (26). Briefly, cells were plated in 24 wells culture plates with $1 \times 10^4$ cells/mL density for 12 h. Cells were then cultured in different treatment media for certain time. Then, cells were incubated for 15 min at 37 °C with Hoechst 33342 dye (10 mg/ml in PBS), washed twice in PBS PI (50 mg/ml in PBS) added just before microscopy. Cells were visualized using an inverted fluorescence microscope (Olympus IX-71, Japan). All experiments were independently replicated at least 3 times. Within each experiment, we replicated each condition 4 times.

**Immunofluorescence microscopy**

Cells were grown in 24 wells of cell culture plate in different treatment media. Then, cells were fixed in 4% Paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% TritonX-100/PBS for 5 min. Then, cells were incubated in PBS containing 0.5% bovine serum albumin and 0.1% Tween 20 for 30 min to reduce nonspecific binding, followed by overnight incubation at 4 °C with the following rabbit polyclonal Abs: Nestin (1:50; Chemicon); MAP-2 (1:50; Santa Cruz Biotechnology); Tuj-1 (1:40; Sigma), NF-M (1:1000; Chemicon, Inc, Temecula, CA), S-100 (1:5000; Dako) and GFAP (1:64; Sigma). After washing, Alexa Fluor 488-conjugated (green) secondary Abs (1:500) was applied for 1 h at room temperature. The slides were treated with anti-fade reagent (Molecular Probes, Inc) and examined for immunofluorescence with the Olympus microscope.
Results

Interestingly, morphine exhibited a neuron-like morphology with a short neurite outgrowth in PC12 cells at 12 days in culture (Fig. 1). In the group I, Morphine at low concentration (treatments 1 and 2) compared to the group II formed a neuroglial morphology in PC12 cells at 3 days in culture (Fig. 1). In the group I, at low concentration, morphine result in a heavy glial-like morphology with an expansion cell body and few neuron-like morphologies with a short neurite outgrowth in the culture dish. In the group I, Morphine in all treatment, especially in the treatment 4 and 5, compared to the group II, was likely to be postsynaptic. In these treatments, morphine resulted in colonization with disperse neuroglial network in cultured cells at 6 days in culture (Fig. 1).

Some studies suggest that morphine induces apoptosis of glial cells and neurons (27), whereas others suggest that morphine

Fig. 2. Effects of different concentration of morphine on cell cytotoxicity in cells. All data represented by mean ± SEM (p < 0.05).

Fig. 3. Effects of different concentration of morphine on cell death in cells. All data represented as the mean ± SEM (p < 0.05).
protects neurons from cell death (28). Morphine-induced changes in spines could be caused by loss of cells due to cell death. To check this possibility, we used double staining of PI and Hoechst (PI/H) together with cytotoxicity assay by LDH activity to detect cell death and cell cytotoxicity in two groups for 12 days cultured PC12 cells (Figs 2 and 3). At first, cells were exposed to different concentrations of morphine, then, cell cytotoxicity was measured by LDH assay at 1, 3, 6, 9 and 12 days after the exposure. In the group I, the results of this experiment showed that in treatments 1–3 the percentage of cell cytotoxicity was decreased compared to the treatments 4, 5 and 6. While in the group II, exposure of the cells to the different concentrations of morphine reversed the effect of cell cytotoxicity (Fig. 2). To induce cell death as positive controls, we treated such cultured cells with staurosporine (1 μM) for 6 h. In the first day, about ~92% of staurosporine-treated cells were apoptotic (Fig. 2, compared to the untreated). In contrast, as in untreated cells, the nuclei of morphine-treated cells were smooth, and such cells had little PI/H staining (Fig. 4). In the group I, in treatments 1–3 compared to staurosporine and untreated cells, the percentage of cell death was decreased (p<0.05) (Fig. 3). In the group II compared to the group I, in these treatments, the percentage of cell death were increased (p<0.05). On the other hand, in the group I compared to staurosporine treated and untreated cells, in treatments 4 and 5 the percentage of cell death was decreased (p<0.05). In the group II compared to the group I, in treatments 4 and 5 the percentage of cell death was decreased (p<0.05). To further examine the possible loss of cells, cultured cells were treated with morphine for 6–12 days, and studied. In the two groups, untreated cell bodies of neurons were not visually identified. The percentage of cell death and cell cytotoxicity for untreated cells was 100%, and they were completely dead in culture dishes (Figs 2 and 3). In the group I, the percentage of cell cytotoxicity and cell death for morphine-treated neurons in the treatments 1–5 compared to the untreated cells was decreased (p<0.05). These results indicate that the morphine-induced changes in cells were likely to be largely caused by suppression of cell death and cytotoxicity, and these acts of morphine depended on opioid receptors.

The state of differentiation of morphine-induced PC12 cells was characterized by immunocytochemistry at 6 and 12 days after the exposure. For this purpose, PC12 cells were maintained under serum deprivation-induced self-renewal conditions, or they were differentiated in the presence of a different concentration of morphine. For the immunocytochemistry experiments, cultures were stained with nestin (a neural progenitor marker), MAP-2, Tuj-1, NF-M (mature neural markers), and S-100 and GFAP (glial pro-
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Antibodies (Fig. 4). To further examine possible differentiated cells, cultured cells were treated with different concentration of morphine in two groups for 6 and 12 days, fixed, stained and the percentage of stained cells was mounted. The cell bodies of stained cells were visually identified, and the number of stained cells on total cells (stained and unstained cells) in 100 were counted.

In the group I, there were nestin-stained cells among the undifferentiated PC12 cells, whereas morphine-induced PC12 cells showed few staining with nestin (Fig. 5). In treatments 1 and 2 compared to the untreated cells (treatment 6), the density of nestin-stained cells was increased (p < 0.05). In treatments 3–5, there was no significant difference compared to the treatment 6.

In the group I, there were Tuj-1, MAP-2 and NF-M-stained cells among the undifferentiated PC12 cells, whereas morphine-induced PC12 cells showed a good staining with antibodies (Figs 6 and 7). In treatments 1 and 2 compared to the treatments 3–6, the density of Tuj-1, MAP-2 and NF-M-stained cells was increased (p < 0.05). In treatments 3–5 they were significantly increased compared to the treatment 6 (p < 0.05).

In the group I, there was no S-100 and GFAP-stained cells among the undifferentiated PC12 cells, whereas morphine-induced PC12 cells showed a heavy staining with antibodies (Fig. 8). In treatments 1 and 2 compared to the treatments 3–6, the density of S-100 and GFAP-stained cells was increased (p < 0.05). In treatments 3–5 they were significantly increased compared to the treatment 6 (p < 0.05). In the group II, preincubated cells by naltraxone resulted in reversed concentration effects of morphine on the differentiation of cells (p < 0.05).

In the group I, at low concentration, morphine resulted in a heavy glial-like or S-100 and GFAP-positive cell with an expansion of cell body and few neuron-like morphologies or Tuj-1, MAP-2 and NF-M positive cell with a short neurite outgrowth in culture dish compared to the group II and other treatments (p < 0.05). In the group I, Morphine in treatment 4 and 5, compared to the group II was likely to be postsynaptic and resulted in a coloniza-
tion with a disperse neuroglial network (GFAP positive cells were connected to MAP-2 positive cells) in cultured cells at 6 and 12 days in culture (Fig. 1).

**Discussion**

In the present study, we evaluated the effect of different concentrations of morphine on cell differentiation in PC12 cells. Meanwhile, we concluded that chronic exposure to low and high concentrations of morphine led to neuroglial differentiation with a short neurite elongation in PC12 cells with the opioid-dependent mechanism. Our data revealed that administration of low concentration of morphine increased cell differentiation morphology, especially the glial form, and neuroglial zone colonization in PC12 cells (Fig. 1). Indeed, chronic treatment with morphine or naltrexone profoundly altered the function of cell differentiation (Fig. 5). Our data revealed that administration of low and high concentrations of morphine could lead to cell death and cell cytotoxicity in PC12 cells.

Our results were confirmed by enhancing and suppressing the cell differentiation by pM and mM concentrations of morphine in PC12 cells in the previous study (22). The inhibitory effect of pre-treating with 100 nM naltrexone on neurite elongation suggests that this effect was induced by opioid receptor signal transduction. Serum deprivation decreased the density and increased cell death and cell cytotoxicity of PC12 cells. Morphine treatment alone increased the cell viability and decreased cell death and cell cytotoxicity of PC12 cells. The simple explanation is that these two changes are mediated through the activation of the opioid receptor. In addition, chronic treatment with morphine profoundly altered the morphology of PC12 cells. Previous in vivo studies showed that chronic morphine administration decreased the density of spines in rats in comparison to the untreated control rats, whereas naltrexone increased the density of spines (29–31). Morphine, at chronic exposure, can cause changes of neuron synaptic plasticity, neurogenesis and neuronal differentiation (32–35). Opioid with activation of its receptors had an important effects on the nervous system. For example, it has been confirmed that opioids affected the neuronal differentiation and some of them like cocaine can induce neuronal differentiation (25). The previous study showed that in the presence of NGF, chronic low concentrations of morphine exposure enhanced neurite elongation of spinal and cortical neurons via a naloxone-independent mechanism in a chronic manner (22, 25). Although, morphine at 1 μM concentration inhibits staurosporine-induced apoptosis by enhanced Hsp70 protein expression, its effects on neurite outgrowth at acute exposure is not clear (36).

However, the morphine-induced increase in the density of neurite-like structure can be caused by the enhancement of the growth of new spines. Our results indicate that opioids mainly affect the induction and stabilization of the formation and maturation of neuroglial network in cultured cells (Fig. 1). Furthermore, these morphological changes are accompanied by functional changes consistent with such morphological changes (Figs 1 and 5).

In addition, chronic treatment using a low concentration of morphine profoundly altered the glial-neuronal protein marker expression in PC12 cells. Data showed that low concentrations of morphine increased the density of S-100, GFAP, MAP-2, NF-M, and Tuj-1 positive cells in PC12 cells in comparison to the untreated control cells, whereas naltrexone decreased the density of stained cells (Fig. 5).

The latter might have a role in the pathological basis for drug addiction. In addition to acting on cell viability and cell differentiation in PC12 cells, morphine may act directly on synapses between neuroglial differentiated cells as shown in this study. The interaction between these two distinct mechanisms is not yet known.

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