Curcumin could increase the proliferation of subventricular zone (SVZ) originated NPCs via a MAP kinase signaling pathway1,23,32). However, SVZ-NPCs and spinal cord NPCs (SC-NPCs) have some differences involving gene expression and proliferation characteristics. Liu et al.19) reported different electrophysiological properties of mitogen-expansion between adult rat SC-NPCs and SVZ-NPCs. Also, Pfenninger et al.27) reported different gene expressions and proliferations between adult spinal cord ependymal cells and SVZ originated neural precursor cells.

Therefore, the objectives of this study are to evaluate the effect of curcumin on SC-NPC proliferation and to clarify the mechanisms of the MAP kinase signaling pathways in SC-NPCs.

MATERIALS AND METHODS

NPC cultures

To establish cultures of SC-NPCs, Sprague-Dawley rats weighing 250 g to 350 g were used. We measured proliferation rates of SC-NPCs after curcumin treatment at different dosage. The immuno-blotting method was used to evaluate the MAP kinase signaling protein that contains extracellular signal-regulated kinases (ERKs), p38, c-Jun NH2-terminal kinases (JNKs) and β-actin as the control group.

Results: Curcumin has a biphasic effect on SC-NPC proliferation. Lower dosage (0.1, 0.5, 1 μM) of curcumin increased SC-NPC proliferation. However, higher dosage decreased SC-NPC proliferation. Also, curcumin stimulates proliferation of SC-NPCs via the MAP kinase signaling pathway, especially involving the p-ERK and p-38 protein. The p-ERK protein and p38 protein levels varied depending on curcumin dosage (0.5 and 1 μM, p<0.05).

Conclusion: Curcumin can stimulate proliferation of SC-NPCs via ERKs and the p38 signaling pathway in low concentrations.

Key Words: Curcumin · Spinal cord neural progenitor cell · Mitogen activated protein kinase.
ing 250 g to 350 g were sacrificed and their spinal cords were extracted. The spinal cords were chopped using microscissors and incubated in a cocktail containing papain (0.1%, Worthington Biochemical Corp., Lakewood Township, NJ, USA), dispase (0.1%), DNase (0.01%), and MgSO4 (12.4%) in a Hanks balanced salt solution with glucose (0.45%) for 30 minutes at 37°C. The dissociated cells were cultured to form neurospheres in a neurobasal medium containing B-27, glutamin (2 mM/L), penicillin/streptomycin (0.1 g/mL), fibroblast growth factor-2 (20 μg/mL), and heparin (2 μg/mL). Every 5 days, the neurospheres were dissociated with accutase and NPCs were plated into 96-well plates or culture dishes containing culture medium. The NPCs between 5 and 25 passages were used for the experiments.

**NPC proliferation assay**

The NPCs were suspended in 100 μL of a neurobasal medium containing curcumin and cultured in a 96-well plate. In different experiments, various concentrations of curcumin (0.1–50 μmol/L) were used. A 20-μl cell proliferation assay solution containing the tetrazolium compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium), (CellTiter 96 Aqueous One Solution, Promega® Fitchburg, WI, USA) was added to a well containing cultured cells and incubated for 2.5 hours (hrs) at 37°C. The optical density was measured at 490 nm with a microplate spectrophotometer. The same volume of culture medium with the assay solution was used as a blank. The optical density at 490 nm after reaction with MTS showed a proportional correlation to the number of cells. To study the effect of the duration of curcumin, cells were seeded at a density of 1×104 cells in 96-well plates. After 48 hrs, the cells were treated with different concentrations of curcumin (0.1, 0.5, 1, 10, 20, and 50 μM).

**MAP kinase signaling pathway analysis**

We used an immunoblotting method for evaluating the MAP kinase signaling protein. The protein concentration was mixed with a RIPA buffer (25 mM Tris-Cl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Roche®, Mannheim, Germany) and immediately homogenized. The homogenate was centrifuged at 13000 rpm for 30 min at 4°C and the supernatants were determined using the BCA protein assay (Sigma®, St. Louis, MO, USA). Proteins were separated by a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Incubation was in a blocking solution of 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 1 hr at room temperature. The membrane was incubated with a 1 : 1000 dilution of mouse monoclonal phospho-ERK, phospho-p38, phospho-JNK (Santa Cruz®, CA, USA), β-actin (Sigma®, St. Louis, MO, USA) and mouse monoclonal anti-β-actin (Sigma Aldrich®, Saint Louis, MO, USA) overnight at 4°C, and then with a horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. The proteins were detected with chemiluminescence reagents. Immune-positive bands used an image J, version 1.46 r, computer-assisted image analyzer (National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis**

All statistical comparisons were computed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean± standard error of the means. Repeated measure analysis of variance was used to compare groups. Null hypotheses of no difference were rejected if p-values were less than 0.05.

**RESULTS**

**Curcumin increases the proliferation of SC-NPCs**

To study the effect of curcumin on the proliferation of SC-NPCs, SC-NPCs growing in 96-well plates were maintained in medium lacking or containing increased concentrations of curcumin, and the cell proliferation rate was quantified at different time points. After 48 hours of curcumin treatment, lower dosage (0.1, 0.5, 1 μM) of curcumin increased NPC proliferation, whereas high dosage (≥10 μM) caused a decrease in NPC proliferation (Fig. 1). The result shows a few percent improvement compared to each control group.

To evaluate the neurosphere formation, dissociated SC-NPCs were diluted in a culture medium and plated into 96-well plate dishes and cultured for 48 hrs. The cells were treated with 1 μM curcumin for 24 hrs. NPC proliferation and neurosphere formation of the curcumin treated group (Fig. 2A) were increased compared to the control group (Fig. 2B).

**The proliferation of NPC via the MAP kinase pathway**

We investigated curcumin treatment associated with MAP kinase signaling pathways, especially involving ERK, p38, and JNK proteins related to NPC proliferation. Immunoblot analysis was used on antibodies against phospho-ERK (p-ERK), phospho-p38 (p-p38), and phospho-JNK (p-JNK), and levels of β-actin.
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Our findings are the first study to show that SC-NPCs are more relevant to ERK and p38 protein associated MAP kinases. The ERK protein has been reported to be concerned with cell proliferation, survival and apoptosis, and the p38 protein played a vital role in cellular responses to external stress signals like anti-inflammatory reactions.

DISCUSSION

SCI has been thought to be irreversible and the cause of critical sequela. Many studies have reported a desire to minimize secondary injury and increase functional recovery from neurological deficits. Recently, NPC proliferation after SCI plays an important role in functional recovery from neurological dysfunction.

This study finds that curcumin stimulates NPCs proliferation through a MAP kinase signaling pathway, and especially involves the ERK protein and the p38 protein. Also, the effect is biphasic in that low concentrations stimulated cell proliferation, while high concentrations inhibited it.

Previous studies have documented that curcumin could affect proliferation of stem cells through diverse pathways. Kim et al. found that a low concentration of curcumin stimulates proliferation and stemness acting signals of pre-adipocytes by the phosphoinositide 3-kinase pathway, whereas it inactivates p38 kinases and JNK protein. Mujoo et al. reported that curcumin induces differentiation of embryonic stem cells through nitric oxide-cyclic signaling. Kim et al. documented that curcumin stimulates proliferation of embryonic NPCs and neurogenesis in the adult hippocampus. The study suggested that a MAP kinase signaling pathway is connected to NPC proliferation through ERKs and the p38 protein.

In the present study, we investigated the effect of curcumin on SC-NPCs and analyzed MAP kinase signaling. According to the results, SC-NPC proliferation increased with lower dosage (0.1, 0.5, 1 μM) of curcumin. But, higher dosage (10, 20, 50 μM) of curcumin decreased the NPC proliferation rate. These results are comparable with previous studies showing that curcumin stimulates NPC proliferation relative to the dosage. Consequently, for effective clinical applications, these results suggest that proper dosage is important. Therefore, in future studies, we expect that setting the appropriate curcumin-dosage with human cells will be very important.

We found that curcumin affects the proliferation of SC-NPCs, and likewise it affects SVZ-NPCs via the MAP kinase signaling pathway, as we analyzed MAP kinase proteins in this study. Especially, the MAP kinase signaling pathway was revealed as an important mechanism for most stem cell proliferation. There are many different types of MAP kinase and stem cells originated each organization was founded to have slightly different mechanism of MAP kinases. Our findings are the first study to show that SC-NPCs are more relevant to ERK and p38 protein associated MAP kinases.

Fig. 2. The images show that different cell proliferation between the control group and curcumin treated group. Microscopically, the curcumin treated group (A) had increased cell proliferation and neurosphere formation compared with the control group (B).

Fig. 3. Curcumin stimulates proliferation of NPCs via the MAP kinase signaling pathway, and ERK and p38 proteins are connected with this MAP pathway. A: Immunoblot analysis was used by antibodies against phospho-ERK, phospho-p38, phospho-JNK. The phospho-ERK protein level increased with curcumin dosage of 0.5, 1 μM and the phospho-p38 protein level also showed an increase. But phospho-JNK and β-actin protein levels show a marginal difference with curcumin dosage. Levels of β-actin were determined as a control against possible protein loading variability. B: Phospho-ERK and phospho-p38 protein levels increased via curcumin dosage-dependence (p<0.05). *Significant increased protein level compared with control group. NPC: neural progenitor cell, MAP: mitogen-activated protein, ERK: extracellular signal-regulated kinase, JNK: c-Jun NH2-terminal kinase.
inflammatory effects might promote the proliferation of NPCs. Our finding suggests these effects are relevant to curcumin effect NPC proliferation. We consider that this result would be helpful to more selective agonists for NPC proliferation.

However, this study has some limitations. First, this study involves only in-vitro experiments. The spinal cord is a complicated environment, so we need further in-vivo study to evaluate the curcumin effect in live tissue. Second, we used rat SC-NPCs in this experiment. The rat SC-NPC is different from the human SC-NPC, so the proper dosage could be different in humans.

CONCLUSION

This study suggests that curcumin stimulates NPCs proliferation through a MAP kinase signaling pathway, and especially involves the ERK protein and the p38 protein. Also, the effect is biphasic in that low concentrations stimulated cell proliferation, while high concentrations inhibited it. In future studies, we should evaluate the effect of curcumin and MAP kinase signaling in a SCI model.

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