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Diclofenac inhibits proliferation and differentiation of neural stem cells

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in clinical situations as anti-inflammatory, analgesic and antipyretic drugs. However, it is still unknown whether NSAIDs have effects on the development of the central nervous system. In the present study, we investigated the effects of NSAIDs on neural stem cell (NSC) proliferation and differentiation into neurons. In contrast to aspirin, naproxen, indomethacin and ibuprofen, treatment with diclofenac (10 μM) for 2 days induced the death of NSCs in a concentration-dependent manner. Diclofenac also inhibited the proliferation of NSCs and their differentiation into neurons. Treatment with diclofenac resulted in nuclear condensation (a morphological change due to apoptosis of NSCs) 24 hr after the treatment and activated caspase-3 after 6 hr, indicating that diclofenac may cause apoptosis of neuronal cells *via* activation of the caspase cascade. These results suggest that diclofenac may affect the development of the central nervous system.

Keywords: Diclofenac; Neural stem cells; Nonsteroidal anti-inflammatory drugs; Central nervous system; Apoptosis

1. Introduction

NSAIDs are widely used for the alleviation of pain, fever and inflammation, and for the prevention of myocardial infarction and stroke through the inhibition of platelet aggregation. On the other hand, NSAIDs have several side effects, such as gastrointestinal damage and platelet dysfunction, and can cause convulsions when co-administered with quinolone-derivative antibacterial drugs [1–3]. Although information about these side effects has been widely reported, little is known about the effect of NSAIDs on the development of the central nervous system.

NSCs, which are isolated from the embryonic brain, are multipotent and self-renewing progenitor cells, and in the central nervous system, these cells differentiate into neurons and glial cells. Recently, it has been reported that NSCs exist not only in the developing mammalian nervous system but also in the adult nervous system, and adult NSCs have been found in the two principal adult neurogenic regions; the hippocampus and the subventricular zone. In addition, it has been revealed that NSCs in adult hippocampus have the potential to become functional neurons, indicating that the plasticity of adult NSCs may contribute to the regenerative potential of the adult central nervous system [4–6]. Therefore, it might be possible to utilize NSCs for the therapy of neurodegenerative diseases, such as Parkinson's and Huntington's diseases, nerve injury, stroke and multiple sclerosis. In fact, clinical trials have recently been undertaken to evaluate these potentially beneficial therapies [7,8].

Although the proliferation and differentiation of NSCs have been studied, there have been few investigations of the effects of NSAIDs on the development of central nervous system, and on NSCs in particular. NSCs are useful materials for the investigation of the development of central nervous system *in vitro*, and therefore, it is important to investigate the effects of common therapeutic drugs on the differentiation and proliferation of NSCs as a means of predicting the potential risk of teratogenicity in the central nervous system.

In this report, we investigated the effects of NSAIDs on the proliferation and differentiation of mouse NSCs into

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Abbreviations: COX, cyclooxygenase; DMSO, dimethyl sulfoxide; LOX, lipoxygenase; MAP2, microtubule associated protein 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSAIDs, nonsteroidal anti-inflammatory drugs; NSCs, neural stem cells; PPAR-γ, peroxisome proliferator-activated receptor-γ.

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neurons. In contrast to other NSAIDs, diclofenac inhibited the differentiation of NSCs into neurons and inhibited the proliferation *via* the induction of apoptosis. This is the first report of the inhibitory effects of diclofenac on the proliferation and differentiation of neuronal cells in the central nervous system.

2. Materials and methods

2.1. Reagents and antibodies

Diclofenac, naproxen, indomethacin, acetylsalicylic acid (aspirin) and (*S*)-ibuprofen were purchased from Sigma-Aldrich. Anti-MAP2 polyclonal antibody was purchased from Biogenesis Ltd. Anti-cleaved caspase-3 (Asp175) polyclonal antibody was purchased from Cell Signaling Technology Inc.

2.2. Cell culture and induction of differentiation into neurons

Mouse NSCs were prepared according to the method of Kaneko *et al*. [9], and cultured in DMEM/F-12 (1:1) media (Gibco BRL) containing 25 μg/mL insulin, 100 μg/mL transferrin, 20 nM progesterone, 60 μM putrescine, 30 nM sodium selenite, 20 ng/mL epidermal growth factor and 20 ng/mL basic fibroblast growth factor at 37° under 5% CO_2. In this condition, NSCs form the neurosphere, as a state of undifferentiation (Fig. 1B). When cultured on poly-L-ornithine/laminin-coated plates (Biocoat, Becton Dickinson Labware), NSCs adhered to the plates, proliferated and extended their neurites (Fig. 1C).

The treatment with NSAIDs or vehicle DMSO was started when NSCs were scattered on the ornithine/laminin-coated plates. Two days after the treatment, NSCs were collected and used for morphological investigations and other experiments.

2.3. MTT assay

After NSCs were treated with NSAIDs for 2 days, MTT solution (Sigma-Aldrich) was added to the culture medium and the cells were incubated for 4 hr. SDS (20%) was then added, the cells were left for 5 hr at room temperature, and the absorbance at a wave length of 595 nm was measured.

2.4. Evaluation of differentiation

We used MAP2 as a marker of the differentiation of NSCs into neurons since it is specifically expressed in neurons [10]. NSCs were treated with diclofenac, naproxen or vehicle, and cultured on Lab-Tek II Chamber Slides (Nalge Nunc International) for 2 days. Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min, permeabilized in PBS containing 0.1% Triton X-100 for 30 min and rinsed with PBS. The fixed cells were incubated with anti-MAP2 rabbit polyclonal antibody (1:400) for 16 hr at 4°, and then visualized with Alexa Flour 488 goat anti-rabbit IgG antibody (Molecular Probes Inc.). Nuclear staining was performed by incubation with SYTOX™ Orange (Molecular Probes Inc.) and NSCs were then observed using a confocal laser-scanning microscope (MRC1024, Nippon Bio-Rad Laboratories).

2.5. Detection of apoptosis

NSCs treated with diclofenac were cultured on Lab-Tek II Chamber Slides for 24 hr. The cells were then fixed with 4% paraformaldehyde in PBS for 10 min and rinsed. Chromatin staining was performed with Hoechst 33342 (Sigma-Aldrich) to detect nuclear condensation (a morphological change associated with apoptosis) and the cells were observed using a fluorescence microscope (IX 70, Olympus).

2.6. Western blotting analysis for detection of activated caspase

NSCs were treated with diclofenac at varying concentrations and incubated for 6 hr. The cells were then collected and homogenized in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 4 mM EGTA, 1% Triton X-100) containing a cocktail of protease inhibitors (Sigma-Aldrich). Cell extracts (30 μg of protein), prepared by centrifugation at 16,000 *g*, were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene difluoride membrane. The blots were blocked for 3 hr with 5% skim milk in Tris-buffered saline (pH 7.6) containing Tween 20 (0.1%).

We measured the levels of cleaved caspase-3, a key proteolytic enzyme of the caspase cascade that is involved in apoptosis, which is formed by the cleavage of procaspase-3 and is activated by caspase-9 to induce apoptosis. Western blots were probed with anti-cleaved caspase-3 polyclonal antibody at 4° overnight, treated with horseradish peroxidase-conjugated secondary antibody for 1 hr, and then detected using an ECL-plus kit.

2.7. Statistical analysis

Statistical comparisons were made using Scheffe's method after ANOVA. The results were considered significantly different at $P < 0.01$.

3. Results

3.1. Induction of cell death by the treatment with diclofenac but not other NSAIDs

On noncoated plates, undifferentiated NSCs formed a round bulk of neurosphere and were suspended in the

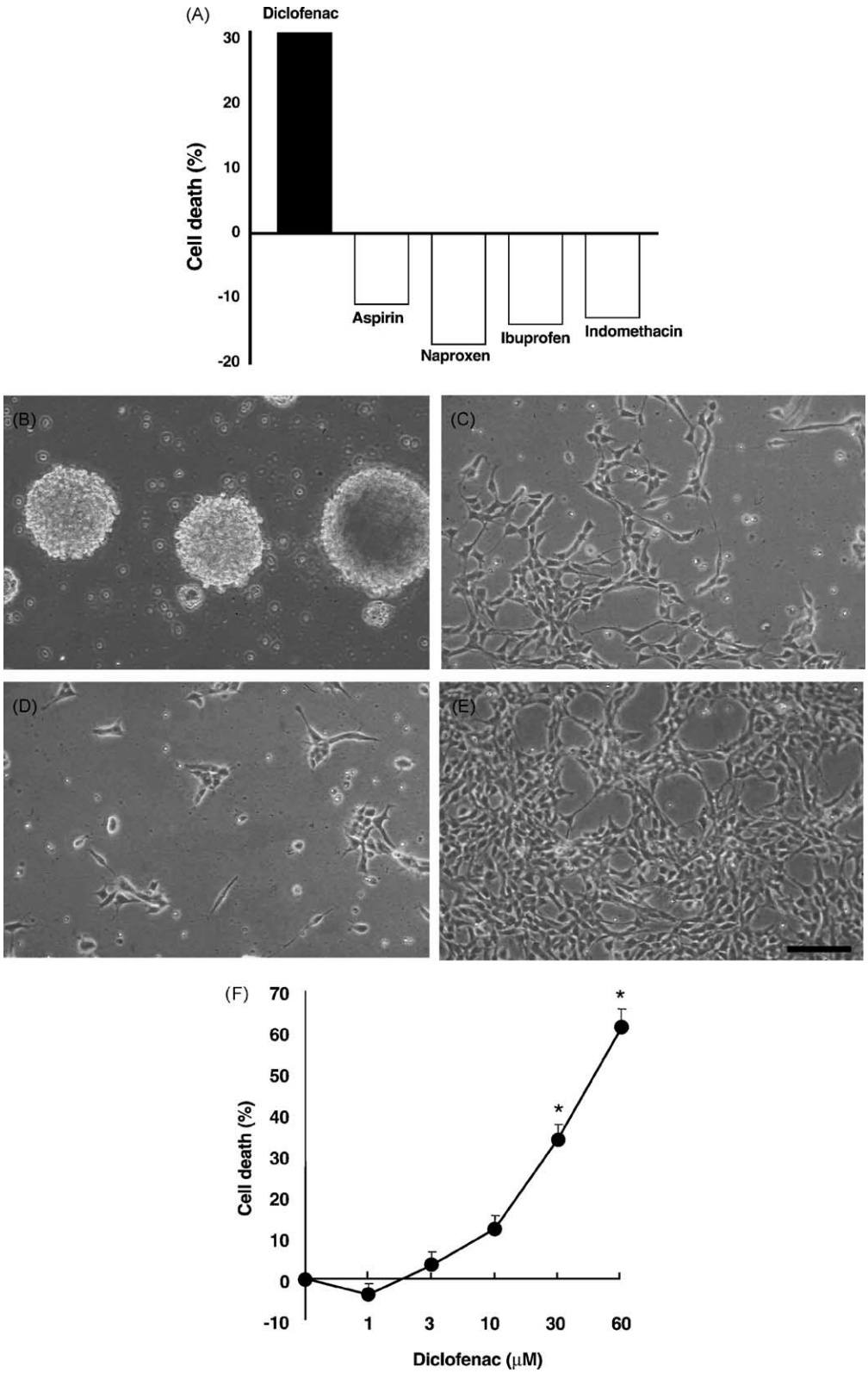

Fig. 1. (A) Effects of NSAIDs on the death of NSCs. NSCs were treated with diclofenac, aspirin, naproxen, indomethacin or (*S*)-ibuprofen (all at 10 μM) for 1 day, and the MTT assay was performed. Each value represents the percentage of dead cells compared with that of vehicle from three separate experiments. Compared with the other NSAIDs, only treatment with diclofenac caused cell death. (B) NSCs formed the neurosphere before differentiation. (C–E) Morphological changes of NSCs after incubation on poly-L-ornithine/laminin-coated plates, and simultaneous treatment with vehicle (C), 10 μM diclofenac (D) or 10 μM naproxen (E) for 2 days. NSCs extended their neurites and proliferated (C). Treatment with diclofenac (D) inhibited both proliferation and differentiation, while naproxen promoted cell proliferation and differentiation (E). Scale bar = 200 μm. (F) Concentration-dependent effect of diclofenac on the death of NSCs. Each value represents the percentage of dead cells compared with that of vehicle. Data represents mean ± SEM from six separate experiments. $^{*}P < 0.01$ vs. vehicle.

medium (Fig. 1B). When cultured on ornithine/laminin-coated plates, NSCs adhered to the plate, and then proliferated and differentiated (Fig. 1C).

NSCs on ornithine/laminin-coated plates were treated with diclofenac, naproxen, indomethacin, aspirin or (*S*)-ibuprofen (all at 10 μM) for 2 days. Treatment with diclofenac inhibited the growth and differentiation of NSCs (Fig. 1D). In contrast, naproxen promoted the proliferation and differentiation of NSCs (Fig. 1E). Other NSAIDs showed the same results as naproxen (data not shown). The ability of NSAIDs to cause the death of NSCs was evaluated using the MTT assay. Only diclofenac-induced cell death, while the other drugs inhibited the death of NSCs (Fig. 1A).

To investigate whether the effect of diclofenac was concentration-dependent, NSCs were treated with diclofenac (1, 3, 10, 30 or 60 μM) for 1 day, and the MTT assay was then performed. The induction of cell death was concentration-dependent and the effect was not saturated at a concentration of up to 60 μM (Fig. 1F).

3.2. Inhibitory effect of diclofenac on the differentiation of NSCs into neurons

To investigate the effect of diclofenac on the differentiation of NSCs into neurons, we observed the expression of MAP2 (a neuron-specific marker) by confocal laser microscopy (Fig. 2). NSCs treated with vehicle (Fig. 2A) or naproxen (30 μM, Fig. 2B) proliferated and differentiated into neurons with extended neurites. However, treatment with diclofenac (10 or 30 μM) dramatically inhibited the neurite outgrowth and differentiation of NSCs into neurons (Fig. 2C and D). It is noteworthy that some of the diclofenac-treated cells failed to express MAP2.

3.3. Apoptotic death by diclofenac via the activation of the caspase cascade

To determine whether diclofenac induces apoptosis of NSCs, we stained the nuclei of diclofenac-treated NSCs with Hoechst 33342. In contrast to vehicle-treated cells (Fig. 3A),

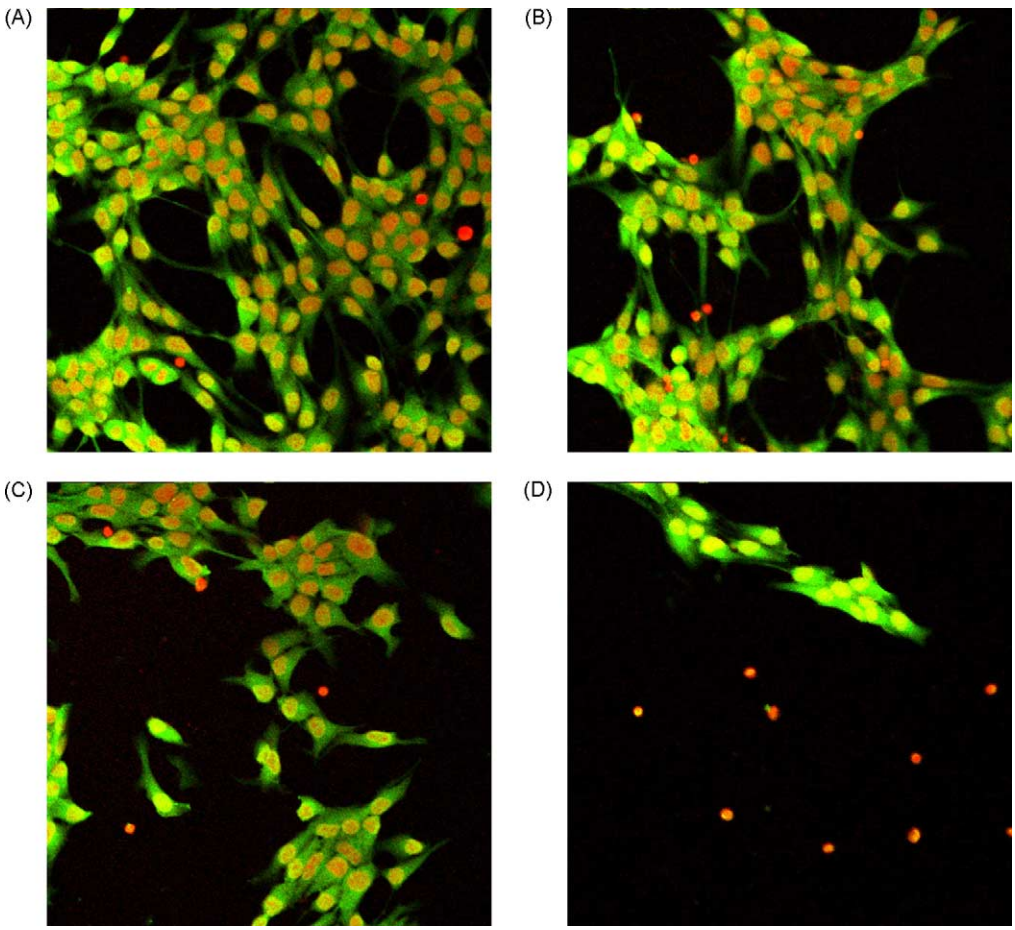

Fig. 2. Inhibition of the differentiation of NSCs into neurons by diclofenac. Undifferentiated cells were treated with diclofenac (10, 30 μM), naproxen (30 μM) or vehicle and cultured on poly-L-ornithine/laminin-coated plates for 2 days. The fixed cells were treated with anti-MAP2 polyclonal antibody, followed by Alexa Fluor 488 goat anti-rabbit IgG antibody. Nuclear staining was performed with SYTOX™ Orange. The confocal laser-scanning microscope images indicate MAP2 (a neuron-specific marker) and nuclei by green and red, respectively. Each photograph indicates: (A) vehicle; (B) naproxen 10 μM; (C) diclofenac 10 μM; and (D) diclofenac 30 μM.

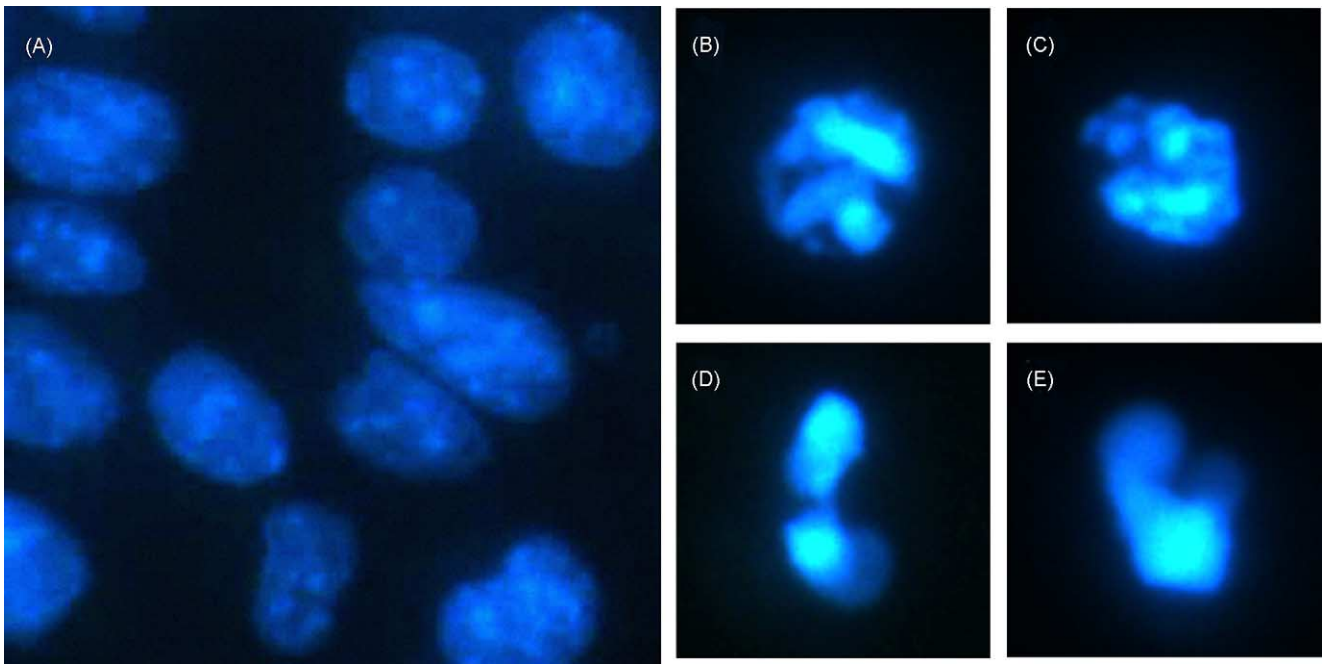

Fig. 3. The effect of diclofenac on nuclear condensation (A–E) of NSCs. Cells were treated with diclofenac (60 μM) or vehicle for 24 hr, and then chromatin staining was performed with Hoechst 33342. Compared to vehicle (A), diclofenac-induced nuclear condensation (B–E).

NSCs treated with diclofenac (60 μM) for 24 hr showed nuclear condensation, which is considered to be a morphological change associated with apoptosis (Figs. 3B–E).

Since it is well known that the caspase cascade is activated during apoptosis, we examined the expression of cleaved (activated) caspase-3. Western blot analysis showed that the activation of caspase-3 was increased by treatment with diclofenac in a concentration-dependent manner (Fig. 4). These results indicate that diclofenac induces apoptosis of NSCs *via* the activation of the caspase cascade.

4. Discussion

In current clinical practice, NSAIDs play essential roles as anti-inflammatory, analgesic and antipyretic drugs. In addition, diclofenac, one of NSAIDs, is widely used for the control of renal colic and the prophylaxis of the recurrent urinary calculi [11,12]. However, it is well known that NSAIDs can cause serious side effects, such as gastrointestinal damage, platelet dysfunction and convulsions. Yet, there is very little information concerning the effects of NSAIDs on the development of the central nervous system.

In the present study, we showed that diclofenac, but not other NSAIDs, induced the death of NSCs, and that diclofenac also inhibited the proliferation of NSCs and their differentiation into neurons. This is the first study to report that the effect of diclofenac on the proliferation and differentiation of NSCs differs from other NSAIDs. NSCs, which differentiate into neurons, astrocytes and oligodendrocytes, are found in the developing mammalian brain and are considered to be a major source of neurons. The ability of diclofenac to induce cell death and inhibit the differentiation of NSCs suggests that the drug could disturb embryonic development and have a teratogenic effect on the central nervous system. Actually, it has been reported

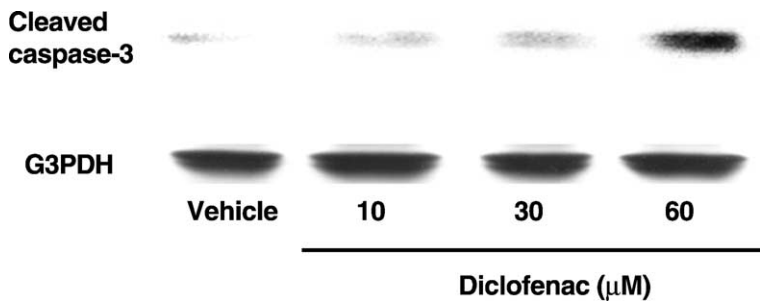

Fig. 4. The effect of diclofenac on caspase-3 activity. After cells were treated with diclofenac (10, 30 or 60 μM) or vehicle for 6 hr, Western blot analysis was performed using anti-cleaved (activated) caspase-3 antibody. Anti-G3PDH antibody was used to evaluate equivalent loading.

that diclofenac crosses the placenta [13], directly inhibits embryonic development, and exerts teratogenic effects on embryos in whole animals [14,15]. These reports are consistent with the results of the present study. It has been reported that NSCs are present in the adult brain and have the potential to become functional neurons. Therefore, diclofenac may interfere with neurogenesis, not only in the developing brain, but also in the adult brain.

In this study, we used micromole (μM) order concentration of NSAIDs for the investigation of the proliferation and differentiation of NSCs, and diclofenac showed the cell death more than 3 μM. It has been reported that blood concentrations reach to 1–20 μM in clinical use of several NSAIDs [16,17], similar conditions that we used in this study may occur in the central nervous system *in vivo*.

It has recently been reported that several NSAIDs, such as ibuprofen and naproxen, have neuroprotective effects against Alzheimer's disease due either to the scavenging of free radicals [18] or to a decrease in the production of amyloid β-proteins [19,20]. In the present study, we showed that some NSAIDs can promote the proliferation and differentiation of NSCs. Therefore, our data could explain one of the novel neuroprotective mechanisms by which NSAIDs help to prevent Alzheimer's disease. While several NSAIDs are now undergoing clinical trials to evaluate their efficacy for the treatment of Alzheimer's disease, our results indicate that clinicians should be cautious in the use of diclofenac for such therapies.

The induction of cell death by diclofenac was due to apoptosis and was accompanied by the activation of the caspase cascade. We clearly showed the involvement of the activation of caspase-3, the key proteolytic enzyme of the caspase cascade, in the apoptosis induced by diclofenac. In contrast, other NSAIDs, such as ibuprofen, naproxen, aspirin and indomethacin, did not activate caspase-3. It remains unclear why the effects of diclofenac on cell growth and apoptosis differed from those of the other NSAIDs. However, several possible mechanisms may involve the inhibitory effect of diclofenac on the arachidonic acid cascade. This cascade has two main reaction type mechanisms regulated by many enzymes, such as three known cyclooxygenases (COX-1, -2, and -3) and four main lipoxygenases (LOX-5, -12, -15-A and -B), which regulate synthesis of lipid mediators, such as prostaglandins and leukotriens. One possible mechanism of diclofenac on induction of cell death is a selectivity of COX-2 inhibition. It is known that the selectivity of COX-2 inhibition by diclofenac is comparatively higher than those of other NSAIDs. Inhibition of COX-2 may involve the mechanism.

Interestingly, COX-3 has been identified as COX-1 variant [21]. This new COX isozyme mRNA is expressed in the brain and heart, and contributes to pain and fever responses [22]. Diclofenac is the more potent inhibitor of COX-3 than other NSAIDs and preferentially inhibits COX-3 over COX-1 and COX-2. Taken together these findings, it is considered that the effect of diclofenac in our study may be relevant to its COX-3 inhibition which differs from other NSAIDs.

The other possible mechanism is the inhibition of LOX pathway by diclofenac. It is well known that most NSAIDs inhibit only COX and thereby shunt arachidonic acid to LOX pathway, resulting to decrease in prostaglandin synthesis in contrast increase in leukotriene synthesis. However, diclofenac inhibits both COX and LOX pathways, which results decreased levels of both prostaglandins and leukotrienes [23,24]. Because leukotrienes are thought to induce cell-survival signaling [25], the reduction of leukotriene levels by diclofenac might be responsible for the death of NSCs that was observed in our system. Arachidonic acid itself is reported to induce apoptosis of various cell lines [26,27]. However, it is also reported that diclofenac diminishes arachidonic acid release and stimulates its uptake into triglycerides by re-esterification without the effect on phospholipase A_2, resulting to reduce the intracellular level of free arachidonic acid [28]. Therefore, the levels of arachidonic acid in NSCs treated with diclofenac might be low in our experimental conditions. Further investigations are required to clarify the mechanisms of the diclofenac-induced cell death through arachidonic acid cascade.

In addition, there is the possible mechanism independent on the arachidonic acid cascade, which is through nuclear receptors such as PPAR-γ. It has been reported that diclofenac binds and antagonizes PPAR-γ at therapeutic concentrations similar to the concentrations we used, and decreases adipose cell differentiation and inhibits cancer cell proliferation [29]. On the other hand, it has also been reported that other NSAIDs bind and activate PPAR-γ, and induce adipocyte differentiation [30]. Although it is unclear the role of PPAR-γ in NSCs, these reports may support our data in the present study.

In conclusion, diclofenac but not other common NSAIDs inhibited the differentiation of NSCs and induced cell death. The death of the cells was due to apoptosis and was accompanied by the activation of the caspase cascade. The clinical use of diclofenac should therefore be approached with caution.

Acknowledgments

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