Etodolac, a selective cyclooxygenase-2 inhibitor, induces apoptosis by activating caspases in human malignant rhabdoid tumor cells (FRTK-1)

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Abstract. Malignant rhabdoid tumor (MRT) is a rare and highly aggressive tumor presenting in the kidney and soft tissue in childhood. However, effective treatment for MRT has not been established. We investigated the antitumor effect of etodolac, a selective cyclooxygenase-2 inhibitor, on MRT cells *in vitro* using the MRT cell line FRTK-1. Etodolac induced apoptosis of FRTK-1 cells through activation of caspase-8, -9 and -3. Moreover, several caspase inhibitors completely or partially inhibited etodolac-induced apoptosis. Our data indicated that etodolac had an antitumor effect on MRT cells and holds promise as a novel therapeutic strategy for MRT.

Introduction

Malignant rhabdoid tumor (MRT) is a very rare renal and soft tissue tumor that occurs in childhood, especially in infancy. Frequently MRT is highly aggressive, resistant to multi-agent chemotherapy, and fatal in metastasis (1). However, effective treatment for MRT has not been established, and more effective antitumor therapy for MRT must be developed in order to improve its aggressive biological behavior.

Cyclooxygenase (COX), also known as prostaglandin H2 synthase or prostaglandin endoperoxide synthase, is a key enzyme in the conversion of arachidonic acid to prostanoids (2). COX-2 is one of two COX types, the other being COX-1. COX-2 is undetectable in most normal tissues, but can be induced in various cell types by pro-inflammatory agents, growth factors and carcinogens (3). Overexpression of COX-2 in various malignancies, including carcinomas, lymphomas and some sarcomas, has been reported (4-11). COX-2 activation promotes tumor growth through production of prostaglandin E2, which blocks apoptosis or activates vascular endothelial growth factor and angiogenesis (12). There have been several studies on the induction of apoptosis in various carcinoma cells by selective COX-2 inhibitors and on the antitumor effects of some selective COX-2 inhibitors for soft tissue sarcoma cells (rhabdomyosarcoma and malignant fibrous histiocytoma) (13,14). However, drug-induced apoptosis such as that in carcinoma cells has not been reported in soft tissue sarcomas. Thus, the pathways of selective COX-2 inhibitor-induced apoptosis also have not been recognized in soft tissue sarcomas. The present study revealed that etodolac, a selective COX-2 inhibitor, induced apoptosis of FRTK-1, an MRT cell line, through caspase activation.

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Materials and methods

Reagent. Etodolac, a selective COX-2 inhibitor, was provided by Nippon Shinyaku Co. (Kyoto, Japan). Stock solution of etodolac was prepared in 99.5% ethanol and stored at -80˚C. Caspase inhibitors (Z-VAD-FMK, broad caspase inhibitor; Ac-IETD-CHO, caspase-8 inhibitor; Ac-LEHD-CHO, caspase-9 inhibitor; Ac-DMQD-CHO, caspase-3 inhibitor) were obtained from Peptide Institute, Inc. (Osaka, Japan) and were dissolved in dimethyl sulfoxide (DMSO) as stock solutions.

Cell line. The FRTK-1 cell line was established in our laboratory from a surgical specimen of renal MRT in an 18-month-old boy. The FRTK-1 cells showed overexpression of COX-2, as demonstrated by immunohistochemistry, Western blotting and reverse transcription-polymerase chain reaction analysis (15). FRTK-1 cells were grown in RPMI-1640 medium (Sigma R8758, St. Louis, MO, USA) supplemented with 15% heat-inactivated fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS, USA), 50 units/ml penicillin G and 50 μg/ml streptomycin. The cells were inoculated into 25-cm^2 tissue culture flasks (Iwaki Glass, Tokyo, Japan) and incubated at 37˚C in a humidified atmosphere with 5% CO_2.

Measurement of etodolac-induced cell death in vitro. Cell viability was assessed using a cell proliferation reagent, WST-1 (Roche Diagnostics, Mannheim, Germany) (16). FRTK-1 cells were seeded in 96-well plates (BD Falcon 353072, BD Biosciences, Franklin Lakes, NJ, USA) at a dose of 1.0x10^4

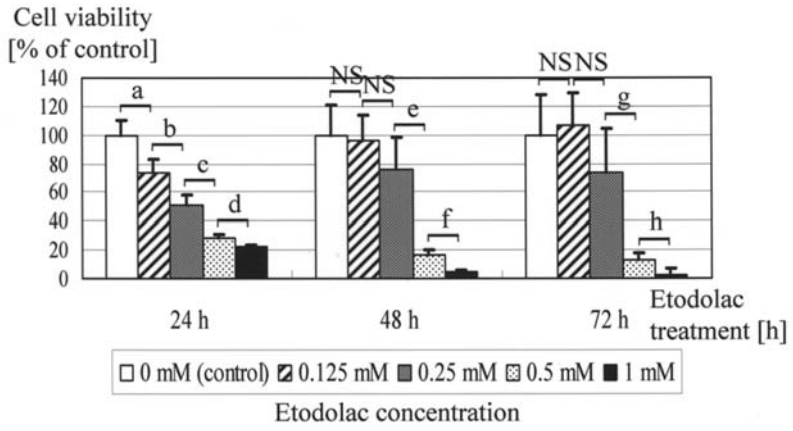

Figure 1. Effect of etodolac treatment on FRTK-1 cells. Etodolac significantly reduces cell viability in a dose-dependent manner (n=5). p-values were calculated based on Student's t-test (a, p=0.0062; b, p=0.0067; c, p=0.0004; d, p=0.0007; e, p=0.0009; f, p=0.0001; g, p=0.0050; h, p=0.0063; NS, not significant).

cells/well and incubated for 24 h at 37˚C in 100 μl of phenol-red-free RPMI-1640 medium (Sigma R7509) supplemented with 10% FCS, 50 units/ml penicillin G, 50 μg/ml streptomycin and 200 mM L-glutamine. FRTK-1 cells were treated by adding etodolac, and then dissolved and diluted to the desired concentration by 1 μl of 99.5% ethanol with 49 μl of culture medium (final concentration of ethanol: ≤0.67%), at final concentrations of 0 (control), 0.125, 0.25, 0.5 and 1 mM. At 24, 48 and 72 h after etodolac treatment, 15 μl of WST-1 reagent was added to each well and the plates were incubated for 2 h at 37˚C. The plates were then shaken for 1 min at room temperature, and the absorbance of 450-655 nm was measured using a multi-well ELISA reader (Microplate Reader, Model 550, Bio-Rad Laboratories, Hercules, CA, USA).

Morphological analysis and DNA fragmentation analysis of dead cells. FRTK-1 cells were seeded in 6-well plates (BD Falcon 353046) at a dose of $5.0x10^5$ cells/well and incubated for 24 h at 37˚C in 5 ml of culture medium. FRTK-1 cells were treated by adding etodolac, dissolved and diluted to the desired concentration by 50 μl of 99.5% ethanol (final concentration of ethanol: ≤0.99%), at a final concentration of 0 (control) or 1 mM. At 24, 48 and 72 h after the etodolac treatment, cells were removed using a rubber policeman. For morphological analysis of dead cells, 50 μl of the cell suspension was cytospun on silan-coated slides, which were stained by May-Giemsa staining and observed. Suspended cells were washed in ice-cold phosphate-buffered saline (PBS) twice and pelleted by centrifugation. To detect cell death, whether apoptotic or not, a DNA sample was extracted from each cell pellet using the Enhanced Apoptotic DNA ladder detection kit (BioVision, Mountain View, CA, USA) according to the manufacturer's protocol. Extracted DNA samples were electrophoresed through 1.8% agarose gels and stained with ethidium bromide.

Measurement of caspase-8, -9 and -3 activity. FRTK-1 cells were seeded in 6-well plates at a dose of $5.0x10^5$ cells/well and incubated for 24 h at 37˚C in 5 ml of culture medium. FRTK-1 cells were treated by adding 0 (control) or 1 mM etodolac, dissolved and diluted to the desired concentration by 50 μl of 99.5% ethanol (final concentration of ethanol: ≤0.99%). At 24 and 48 h after etodolac treatment, cells were removed with a rubber policeman. Suspended cells were washed in ice-cold PBS twice and pelleted by centrifugation. To detect the activity of caspase-8, -9 or -3, samples were extracted from cell pellets and measured by the APOPCYTO Caspase-8, -9 or -3 colorimetric assay kit (MBL, Nagoya, Japan), according to the manufacturer's protocol. The 96-well plates were then shaken for 1 min at room temperature, and absorbance at 405 nm was measured using a multi-well ELISA reader. The activities of the caspases were corrected by protein concentrations.

Blockage of apoptosis by various caspase inhibitors. Cell viability was assessed by the WST-1 method. FRTK-1 cells were seeded in 96-well plates at a dose of $1.0x10^4$ cells/well and incubated for 24 h at 37˚C in 100 μl of phenol-red-free medium. After 2 h of preincubation with 0 (control) or 100 μM caspase inhibitors (Z-VAD-FMK, Ac-IETD-CHO, Ac-LEHD-CHO or Ac-DMQD-CHO), dissolved and diluted to the desired concentration by 0.75 μl of DMSO with 39.25 μl of culture medium (final concentration of DMSO: ≤0.5%), FRTK-1 cells were treated by adding etodolac, dissolved and diluted to the desired concentration by 1.5 μl of 99.5% ethanol with 8.5 μl of culture medium (final concentration of ethanol: ≤1%), at a final concentration of 0 (control) or 1 mM. At 24 and 48 h after etodolac treatment, 15 μl of the WST-1 reagent was added to each well and the plates were incubated for 2 h at 37˚C. The plates were then shaken for 1 min at room temperature, and the absorbance of 450-655 nm was measured using a multi-well ELISA reader.

Data analysis. Data were summarized as means ± SD. Statistical analysis was performed using the unpaired Student's t-test. $p<0.05$ was considered statistically significant. All data were representative from two independent experiments.

Results

Etodolac-induced cell death in vitro. FRTK-1 cells inhibited growth in a dose-dependent manner (Fig. 1). With 0.125 or 0.25 mM of etodolac treatment, the viability of FRTK-1 cells

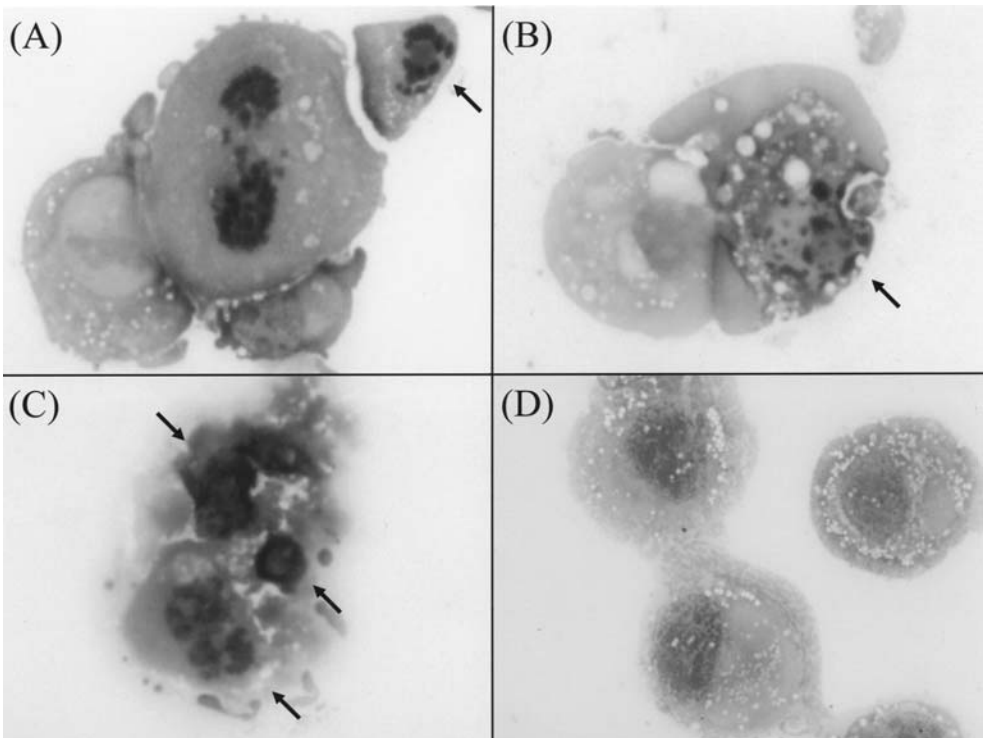

Figure 2. Morphology of FRTK-1 cells after etodolac treatment for 24 h (A), 48 h (B) and 72 h (C). Nuclear fragmentations are observed (arrows), in contrast to viable cells without etodolac treatment (72 h) (D). (May-Giemsa staining, x400)

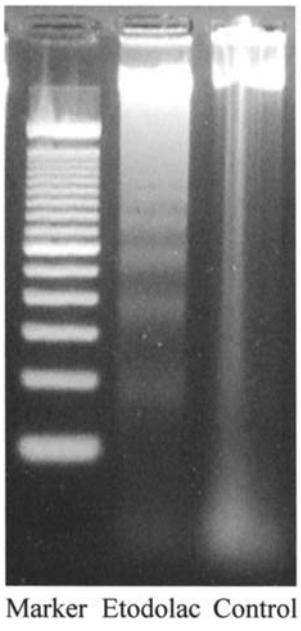

Figure 3. Evaluation of apoptosis using agarose gel electrophoresis. Lane 1, Marker, 100-bp DNA ladder; lane 2, Etodolac treatment (72 h); lane 3, Control (without etodolac treatment, 72 h).

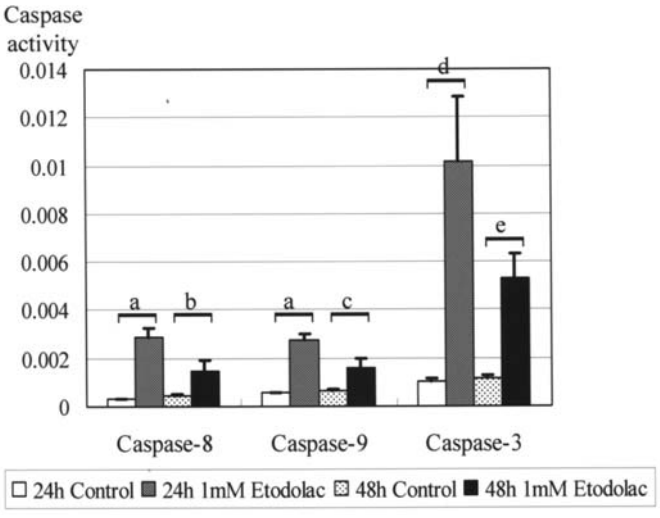

Figure 4. Activities of caspase-8, -9 and -3 with etodolac treatment (24 and 48 h) (n=3). p-values were calculated based on Student's t-test (a, p=0.0006; b, p=0.0424; c, p=0.0173; d, p=0.0091; e, p=0.0047).

was inhibited at 24 h, but recovered at 48 and 72 h. There was no statistically significant difference in cell viability between the etodolac treatment group and the control group at 48 and 72 h; however, with 0.5 or 1 mM of etodolac, cell viability was inhibited at 24 h and not recovered at 48 or 72 h.

Morphological analysis and DNA fragmentation analysis of dead cells. May-Giemsa staining showed nuclear fragmentation in the dead cells after 24 h of etodolac treatment (Fig. 2). Agarose gel electrophoresis of extracted DNA samples from the dead cells revealed a DNA ladder, indicating apoptosis (Fig. 3).

Activation of caspase-8, -9 and -3 with etodolac treatment. FRTK-1 cells treated with etodolac showed activation of

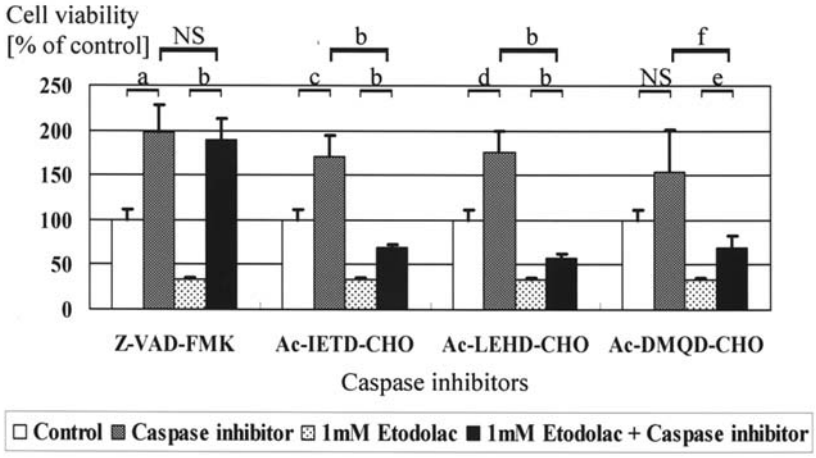

Figure 5. Effects of several caspase inhibitors on etodolac treatment (48 h) (n=5). p-values were calculated based on Student's t-test. (a, p=0.0003; b, p<0.0001; c, p=0.0005; d, p=0.0003; e, p=0.0004; f, p=0.0083; NS, not significant)

caspase-8, -9 and -3 at 24 or 48 h compared with the control group, which was not treated with etodolac (Fig. 4).

Inhibition of apoptosis by caspase inhibitors. Z-VAD-FMK, a broad caspase inhibitor, almost completely inhibited etodolac-induced apoptosis of FRTK-1 cells. Each of the caspase inhibitors, Ac-LEHD-CHO (a caspase-8 inhibitor), Ac-IETD-CHO (a caspase-9 inhibitor) and Ac-DMQD-CHO (a caspase-3 inhibitor), incompletely inhibited etodolac-induced apoptosis (Fig. 5).

Discussion

The present study revealed that etodolac, a selective COX-2 inhibitor, induced death in FRTK-1 cells (MRT cell line), and this death was demonstrated to be apoptotic by the results of a morphological examination (apoptotic body) and agarose gel electrophoresis (DNA ladder).

The apoptotic event was shown to occur in FRTK-1 cells through the activation of caspase-8, -9 and -3, as these caspases were activated in the FRTK-1 cells treated with etodolac. In addition, etodolac-induced apoptosis was completely inhibited by Z-VAD-FMK, a broad caspase inhibitor. However, each caspase inhibitor (caspase-8 inhibitor Ac-IETD-CHO, caspase-9 inhibitor Ac-LEHD-CHO, and caspase-3 inhibitor Ac-DMQD-CHO) showed incomplete inhibition of etodolac-induced apoptosis. These results suggest that the caspase cascade of etodolac-induced apoptosis of FRTK-1 cells involves various caspase pathways.

There have been several studies on the molecular mechanisms or factors involved in selective COX-2 inhibitor-induced apoptosis; down-regulation in bcl-2 protein expression (17), augmentation of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis (18), augmentation of Fas-mediated apoptosis (19), cytochrome c-dependent pathway (20), blocking of Akt activation (21), and increase in arachidonic acid and ceramide (22). In addition, apoptotic pathways in the selective COX-2 inhibitor-induced apoptosis of tumor cells depend on tumor cell types or selective COX-2 inhibitor types. However, the signaling pathways from the first signal initiated by etodolac to caspase activation have not been sufficiently elucidated. Therefore, it is necessary to investigate how caspase activation occurs.

Combined chemotherapy using selective COX-2 inhibitor and other antitumor drugs, such as carboplatin, is known to have synergistic effects in some tumors (23). There is cross-talk between epidermal growth factor receptor (EGFR) and COX-2 (24,25), and cooperative cell-growth in some carcinomas is shown to be inhibited by the combination treatment with an EGFR tyrosine kinase inhibitor, ZD1839, and a selective COX-2 inhibitor (26-29). In addition, the growth of MRT cells with EGFR expression demonstrates inhibition by a selective EGFR tyrosine kinase inhibitor, Gefitinib (ZD1839, Iressa), *in vitro* and *in vivo* (30). Therefore, the present results suggest that the combination of the EGFR tyrosine kinase inhibitor and a selective COX-2 inhibitor might be a promising effective therapy for MRT.

In conclusion, etodolac, a selective COX-2 inhibitor, induced apoptosis of FRTK-1 cells through the activation of caspase-8, -9 and -3. Although the selective COX-2 inhibitor-induced apoptosis of osteosarcoma cells has already been reported (31), the present study is the first, to our knowledge, to cover the apoptosis of MRT cells induced by the selective COX-2 inhibitor. This inhibitor might be effective for other aggressive sarcomas.

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