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Celecoxib inhibits cell growth and modulates the expression of matrix metalloproteinases in human osteosarcoma MG-63 cell line

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Abstract. – OBJECTIVE: The goal of this study was to determine the effect of celecoxib, a selective COX-2 inhibitor, on the growth inhibition of osteosarcoma and its potential anticancer mechanisms.

MATERIALS AND METHODS: Human osteosarcoma cell line MG-63 was used as a model. The inhibitory effect of celecoxib on cell proliferation was assessed by MTT assay. Flow cytometric analysis was used to detect the effects of celecoxib on cell cycle and apoptosis. Western blot analysis was used to detect the protein expression of RECK, matrix metalloproteinase (MMP)-2 and MMP-9 in celecoxib-treated MG-63 cells.

RESULTS: MTT assays showed that at a range of concentrations (0-80 μg/ml), celecoxib significantly inhibited the MG-63 cell proliferation in a time- and concentration-dependent manner. The half maximal inhibitory concentration (IC50) of celecoxib was 47.5 μg/ml for 24 h-treatment and 19.2 μg/ml for 48 h-treatment. Flow cytometric analysis demonstrated that treatment with 20 μg/ml celecoxib led to a significant cell cycle arrest at S-phase and an enhancement of apoptosis induction in MG-63 cells at 24 or 48h. Moreover, compared with 24 h-treatment, 48 h-treatment induced more S-phase arrest and apoptosis in MG-63 cells. Western blot analyses revealed that the expression of MMP-2 and MMP-9 was markedly down-regulated but RECK, an inhibitor of MMPs, was markedly up-regulated in MG-63 cells exposed to 20 μg/ml celecoxib for 24 or 48h. Furthermore, the effects of celecoxib on the expression of these molecules were more evident with the increase of treatment time.

CONCLUSIONS: Celecoxib inhibits the MG-63 cells proliferation through S-phase arrest and apoptosis induction. Celecoxib-induced down-regulation of MMP-2 and MMP-9 and up-regulation of RECK may contribute to the apoptosis induction and an alteration in local tumor microenvironment. These findings suggest that celecoxib may exert at least in part of its anticancer effects via up-regulation of RECK to inhibit the expression of MMP-2 and MMP-9.

Key Words:
Celecoxib, Osteosarcoma, Cell proliferation, Apoptosis, RECK, MMP-2, MMP-9.

Introduction

Osteosarcoma is a primary malignant cancer of bone with high malignancy grade, which arises primarily in children and adolescents, with a second peak in incidence in those over the age of 50[1-5]. During the past decades, the combination of surgical resection and chemotherapy has improved the 5-year survival rate. However, survival for patients with metastatic or relapsed osteosarcoma has remained virtually unchanged over the past 30 years, with an overall 5-year survival rate of about 20%[6,7]. Therefore, new therapies are needed.

Cyclooxygenases are enzymes required for the conversion of arachidonic acid into prostaglandins, which play a role in many physiologic and pathologic processes[8]. Cyclooxygenase-2 (COX-2), an inducible enzyme, is stimulated by a variety of cytokines, growth factors, and tumor promoters[9-11]. Studies[12,13] have demonstrated that COX-2 is overexpressed in many human malignancies, including osteosarcoma, where it produces high levels of prostaglandins and contributes to tumor growth. The mecha-

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nisms include the promotion of tumor angiogenesis, invasion, metastasis, and production of matrix metalloproteinases (MMPs) as well as inhibition of apoptosis[14]. The expression of COX-2 has been found significant higher in osteosarcomas of higher grade[13], which support the promising use of COX-2 inhibitors in the treatment of osteosarcomas.

Celecoxib is a newly synthesized COX inhibitor, which belongs to non-steroidal anti-inflammatory drug (NSAID)[15-18]. The clinical characteristics of celecoxib include high effectiveness, low toxicity, and less gastrointestinal reaction. In recent years, the use of celecoxib in cancer treatment has attracted more and more attention[19,20]. Studies[21-23] have shown that celecoxib can inhibit the proliferation, as well as induce differentiation, maturation and apoptosis of various cancer cells but has no obvious toxic and side effects on normal cells. However, the anticancer mechanisms of celecoxib against osteosarcoma still remain largely unknown. In this study, we use human MG-63 osteosarcoma cell line as a model to examine the effects of celecoxib on cell proliferation, apoptosis and cell cycle arrest. We also assessed the expression of MMP-2, MMP-9 and RECK modulated by celecoxib.

Materials and Methods

Antibodies and Reagents

Celecoxib, 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and propidium iodide (PI) were purchased from Sigma-Aldrich China Inc. (Shanghai, China). Rabbit anti-RECK, rabbit anti-MMP-2, rabbit anti-MMP-9 primary antibodies as well as horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Line and Culture

The osteosarcoma cell line MG-63 was obtained from ATCC (Manassas, VA, USA) and maintained in DMEM medium (Thermo Fisher Scientific, Nanjing, China) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO_2 incubator.

Cytotoxicity Tested by MTT Assay

The cytotoxicity of celecoxib was determined by MTT assay. The MG-63 cells at logarithmic phase were collected and seeded in 96-well culture plates at a density of 1×10^4 cells/well. The final volume in each well was 200 μl. After being cultured for 24 h, the medium was replaced with new DMEM medium containing vehicle DMSO (0 μg/ml celecoxib, control) or various concentrations of celecoxib (2.5, 5, 10, 20, 40 and 80 μg/ml). After incubation for 24, 48 or 72 h, 50 μl MTT was added to each well, followed by incubation for another 4 h. Then, 150 μl dimethil sulfoxide (DMSO) was added to each well and the 96-well plates were slightly vibrated for 10-15 min. OD values were measured at 570 nm using an ELX-800 full-automatic microplate reader (Omega Bio-Tek Inc., Norcross, GA, USA). Inhibition ratio (%) = [(OD570 of control group – OD570 of treatment group) / OD570 of control group]. Three independent experiments were performed in triplicate.

DAPI Staining for Apoptotic Cells

The MG-63 cells at logarithmic phase were plated on sterile coverslips placed in a 6-well plate and incubated overnight at 37°C before treatment with or without 10 or 20 μg/ml cerecoxib for 24 and 48 h. The cells were washed with pre-cooled phasphate buffered saline (PBS) for 2-3 times, and fixed with 4% triformol for 30 min. Then, the cells were stained with DAPI (1 μg/ml) for 15 min. After staining, the coverslips were air-dried and sealed, and the cells were examined and photographed using a fluorescence microscope with excitation wavelength of 350 nm and emission wavelength of 460 nm.

Cell Cycle and Apoptosis Detected by Flow Cytometry

The MG-63 cells at logarithmic phase were inoculated in 6-well plates (1×10^6 cells/well) and incubated overnight at 37°C before treatment with 20 μg/ml celecoxib for 0, 24 and 48 h, respectively. The cells were digested by trypsin and washed once with pre-cooled PBS. Then the cells were fixed with 1 ml pre-cooled 70% ethanol at 4°C overnight. Before analysis, 3 ml PBS was added and the cells were centrifuged at 1500 r/min for 5 min. Then the cells were stained with PI (50 ug/ml) for 30 min at room temperature in darkness. After staining, the cells were immediately analyzed using flow cytometry machine (BD Biosciences, Franklin Lakes, NJ, USA) to detect DNA content and cell cycle distribution. The extent of apoptosis was determined by measuring the DNA content of the cells below a sub-G1 peak. In flow cytometry,

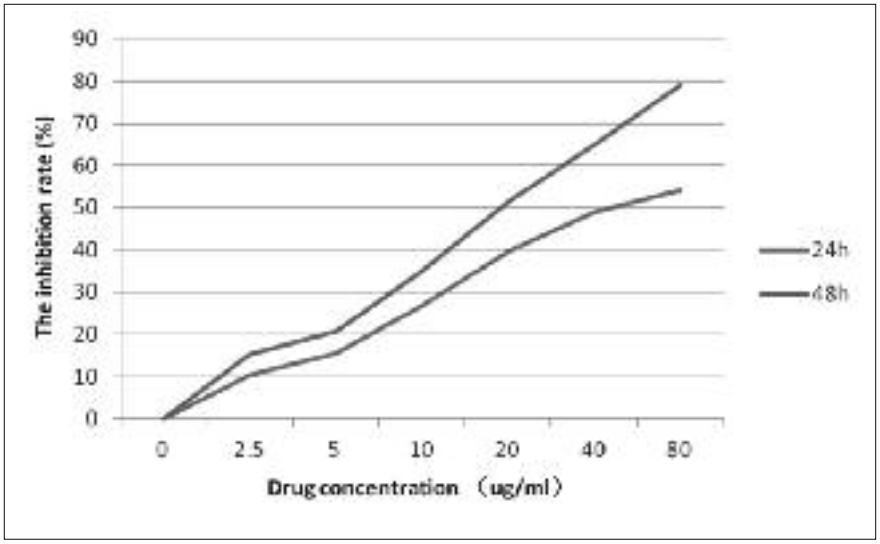

Figure 1. Celecoxib treatment inhibited MG-63 cell growth. MG-63 cells were treated with vehicle (as a control) or with 2.5, 5, 10, 20, 40 and 80 μg/ml celecoxib for 24 and 48 h. Cell viability was assessed by MTT assay. Cell inhibition rate was calculated as indicated in Materials and Methods.

the peak just before G1 was called Sub-G1 phase, and cell apoptotic rate is represented as the propor.

Western Blot Analysis

The MG-63 cells were exposed to 20 μg/ml cerecoxib for 0, 24 and 48 h before harvest. Protein concentrations were determined using bicinchoninic acid (BCA) assay. 20 μg protein extracts were resolved on SDS-polyacrylamide gel and then transferred to PVDF membrane. The membranes were blocked with 5% skimmed milk at room temperature for 2 h and then probed with specific primary antibodies against MMP-2, MP-9 and RECK at 4°C overnight. After being washed three times, the membranes were further incubated with appropriate secondary antibodies coupled to HRP, and developed using enhanced chemiluminescence detection reagents.

Statistical Analysis

The SPSS22.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Measurement data and enumeration data in our study were presented as mean ± standard deviation ($\bar{x} \pm s$) and ratio, respectively. $p < 0.05$ was considered as statistically significant.

Results

Celecoxib Inhibited MG-63 Cell Proliferation

To evaluate the growth inhibitory effects of celecoxib on osteosarcoma, human MG-63 cells were treated with or without various concentrations of celecoxib (2.5, 5, 10, 20, 40 and 80 μg/ml) for 24 and 48 h. Cell viability was assessed by using MTT assay. As shown in Figure 1, celecoxib significantly inhibited the cell growth in a time- and dose-dependent manner. The inhibition rates of cells were elevated from 11.6% (2.5 μg/ml celecoxib) to 56.1% (80 μg/ml celecoxib) after 24 h exposure and from 16.1% (2.5 μg/ml celecoxib) to 79.2 % (80 μg/ml celecoxib) after 48 h exposure. Compared with 24 h-treatment, 48h-treatment with celecoxib significantly increased the inhibition rates of cells at all given concentrations (2.5-80 μg/ml ($p < 0.05$). The IC50 of celecoxib was 47.5 μg/ml for 24 h-treatment and 19.2 μg/ml for 48 h-treatment.

Celecoxib Induced Morphological Apoptosis in MG-63 cell

To determine whether celecoxib induces apoptosis, the MG-63 cells were treated without or with 10 or 20 μg/ml celecoxib for 24 and 48 h, and apoptotic nuclei were then visualized using the DNA intercalating dye DAPI. The apoptotic nuclei were characteristic of nuclear fragmentation and condensation with bright fluorescence. We observed that few MG-63 cells underwent apoptosis under normal culture condition (untreated). However, treatment with celecoxib significantly increased the apoptotic changes in MG-63 cells, and as the treatment time increased, the apoptotic changes became more obvious (Figure 2).

Celecoxib Induced S-phase Arrest in MG-63 Cell Cycle

To elucidate the molecular mechanism underlying the anticancer activity of celecoxib, we

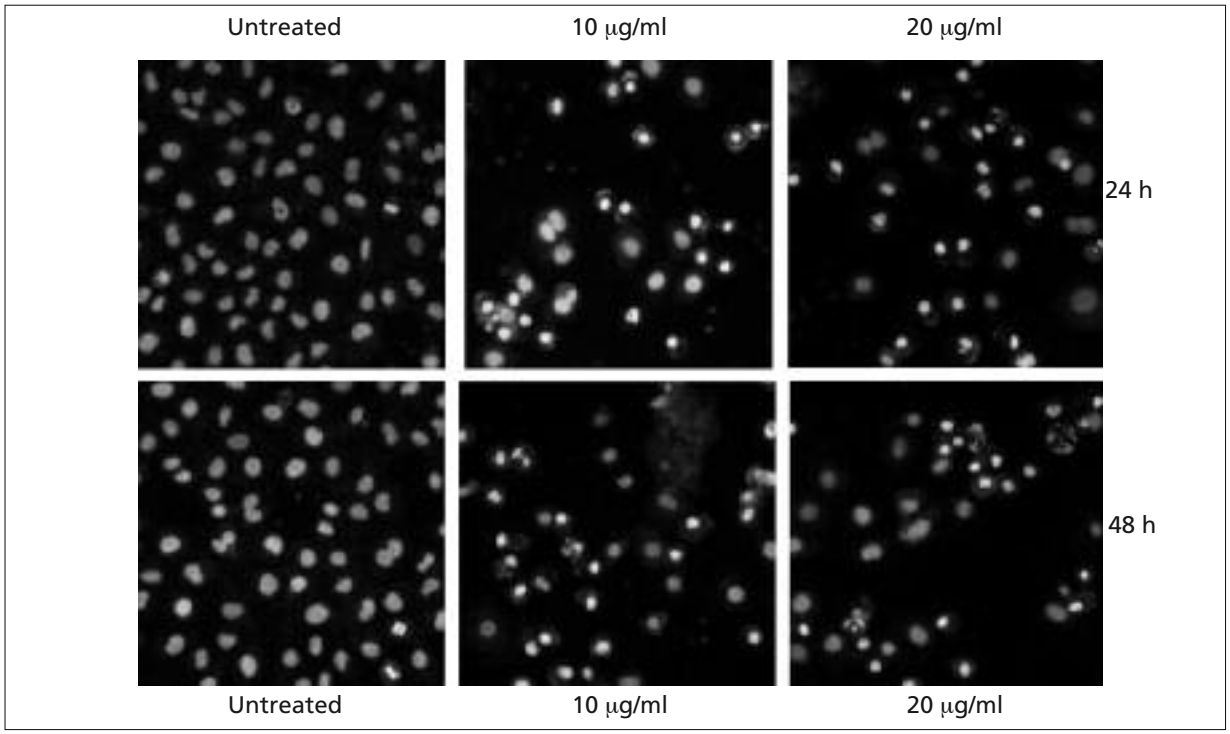

Figure 2. Effects of celecoxib treatment on MG-63 cell apoptotic morphology. MG-60 cells were treated with or without 10 or 20 μg/ml celecoxib for 24 and 48 h. The cell were then fixed and stained with DAPI. The apoptotic nuclei were visualized and photographed using a fluorescence microscope with a excitation wavelength of 350nm and a emission wavelength of 460 nm.

treated the MG-63 cells with 20 μg/ml celecoxib for 0, 24 and 48 h, and the cell cycle distribution (G0/G1, G2/M and S) was determined by flow cytometry. As shown in Figure 3, compared with control group (0h), the percentage of MG-63 cells at S phase were significantly increased in both 24h- and 48h-treatment groups ($p < 0.05$), whereas those at G0/G1 phase were declined with the increase of treatment time. The percentage of cells at G2/M phase had almost no change ($p > 0.05$). Our results indicate that celecoxib blocks the cell cycle at S phase.

Celecoxib Induced Apoptosis in MG-63 Cells by Flow Cytometric Analysis

Using DAPI staining method, we know that celecoxib could induce apoptotic morphology in MG-63 cells. To better quantitate the celecoxib-induced apoptosis, we treated the MG-63 cells with 20 μg/ml celecoxib and performed a flow cytometric analysis. As shown in Figure 4, celecoxib induced apoptosis in a time-dependent manner. Apoptosis rate was significantly elevated from 6.4% (24h) to 16.3% (48h) ($p < 0.05$). Compared with that in untreated group (0h, 0.4%), the apoptosis rate was significantly increased in both 24 h- and 48 h-treatment groups ($p < 0.01$).

Effects of Celecoxib Treatment on the Protein Expression of RECK, MMP-2 and MMP-9 in MG-63 cells

Previous studies have shown that matrix metalloproteinases (MMPs) including MMP2 and MMP9 are involved in tumor invasion and metastasis. In order to better understand the anticancer mechanism of celecoxib, we tested the effects of celecoxib on the expression of MMP2 and MP9 as well as their inhibitor, RECK. As shown in Figure 5, celecoxib significantly inhibited the expression of MMP-2 and MP-9 but increased the expression of RECK after treatment for 24 h or 48 h ($p < 0.05$). 48 h-treatment resulted in more obvious effects than 24 h-treatment ($p < 0.05$).

Discussion

Cycloxygenase-2 (COX-2) is normally expressed in some tissues such as brain, kidney and bone, but can be induced in pathological states

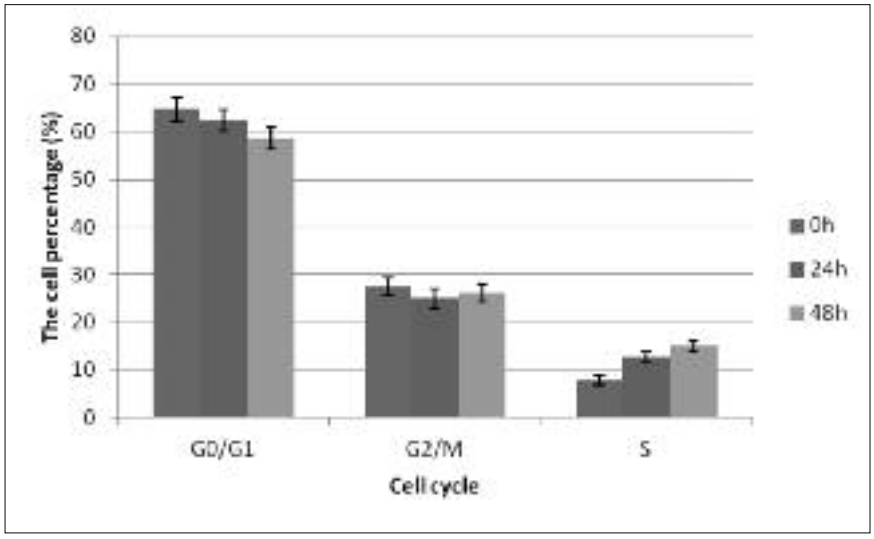

Figure 3. Effects of celecoxib treatment on the cell cycle distribution of MG-63 cells. MG-60 cells were treated with or without 20 μg/ml celecoxib for 24 and 48 h. The cells were then fixed with 70% ethanol and stained with PI. The cell cycle distribution (G0/G1, G2/M and S) was determined by flow cytometry. Compared with control group (0h), $^{*}p < 0.05$.

such as inflammation and tumor. COX-2 promotes tumorigenesis, cell proliferation, angiogenesis and immunosuppression as well as prevents apoptosis[24]. COX-2 overexpression has been reported in a variety of human tumors from premalignant to advanced stage, and often correlates with aggressive disease and poor prognosis[25]. Previous studies have shown that in human osteosarcoma cells, overexpression of COX-2 enhances the invasiveness[26] whereas down-regulation of COX-2 with antisense oligonucleotides inhibits the invasiveness of cells[27]. Dickens et al[28] reported a trend toward higher COX-2 expression in metastatic sarcomas than non-metastatic sarcomas, including in osteosarcoma, rhabdomyosarcoma, and Ewing sarcoma. Masi et al[13] demonstrated a significant higher expression of COX-2 in osteosarcomas of higher grade. *In vitro* and *in vivo* experiments have demonstrated that celecoxib, a selective inhibitor of COX-2, can exert its anticancer effect through inhibition of cell proliferation, induction of apoptosis, as well as inhibition of angiogenesis, tumor growth, invasion and metastasis[29-32]. In the present study, we confirmed that celecoxib induces apoptosis in human osteosarcoma MG-63 cells. Our results together with previous findings suggest a potential use of COX-2 selective inhibitors in the treatment of osteosarcoma.

Matrix metalloproteinases (MMPs) are a class of structurally related proteolytic enzymes whose activities depend on zinc and calcium ions. Abnormal expression of MMPs was found in many tumor tissues including osteosarcoma. Overexpres-

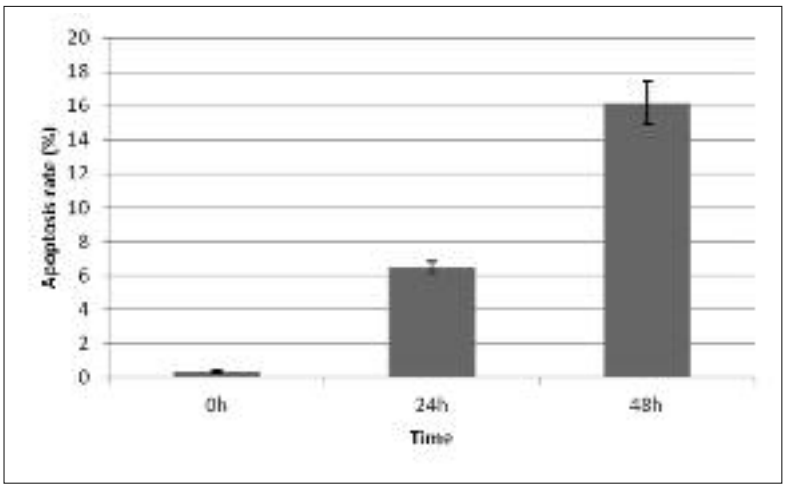

Figure 4. Celecoxib induced apoptosis in MG-63 cells. MG-63 cell were treated with or without 20 μg/ml celecoxib for 24 and 48 h. The apoptotic cells were determined by flow cytometric measurement of cellular DNA content. Compared with control group (0h), $^{*}p < 0.01$. Compared with 24 h-treatment, $^{\#}p < 0.05$.

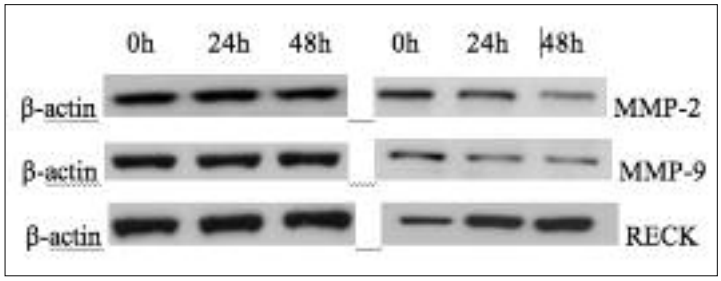

Figure 5. Celecoxib modulated the protein expression of MP-2, MP-9 and RECK in MG-63 cells. Cells were exposed to 20 μg/ml celecoxib for different time points as indicated, and then the total cellular lysates were prepared. The expression of MMP-2, MMP-9 and RECK were detected using Western blot analysis. β-actin was also detected as a loading control.

sion of MMPs has been shown to destroy the structure of the extracellular matrix (ECM) and basement membrane (BM), and promote the invasion and metastasis of cancer cells[33-36]. Studies have suggested that MMPs also contribute to tumor progression by promoting angiogenesis, inhibiting apoptosis and enhancing immunosuppression[37]. Among all MMPs, MMP-2 and MMP-9 are regarded as the most closely associated with the invasion and metastasis of tumor[38-40]. Recent meta-analyses have showed that osteosarcoma patients with high MMP-2 or MMP-9 expression were significantly associated with increased risk of mortality when compared to their counterparts with low or undetectable expression of MMP-2 or MMP-9[41-42]. Ma et al[43] reported that knockdown of relaxin in osteosarcoma MG-63 cells decreases cell proliferation and invasion via inhibiting MMP-9 expression. RECK is a MMP inhibitor found in recent years, which can inhibit MMP-2 and MMP-9 on post-transcriptional level to block the formation of blood vessels and metastasis of tumor. RECK is expressed in most normal tissues and cell lines but is weakly expressed or absent in tumor tissues and cell lines[44-46]. Studies have shown that compared with para-carcinoma tissues, RECK expression in tumor tissues was significantly decreased. In the present study, we observed that expression of MMP-2, MMP-9 was significantly decreased but RECK expression was significantly elevated in celecoxib-treated MG-63 cells. Our results suggest that celecoxib may exert its anticancer effect through down-regulation of the RECK expression.

Conclusions

Our present study demonstrates that celecoxib is able to inhibit cell proliferation, and induce apoptosis of human osteosarcoma MG-63 cells. Down-regulation of MMP-2, MMP-9 and up-regulation of their inhibitor, RECK, were also observed, which may contribute to apoptosis induction and an alteration in the local microenvironment. Our results suggest that celecoxib may exert at least in part of its anticancer effects via up-regulation of RECK to inhibit the expression of MMP-2 and MMP-9.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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