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Etodolac induces apoptosis and inhibits cell adhesion to bone marrow stromal cells in human myeloma cells

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Abstract

Objectives: Cyclooxygenase-2 (COX-2) is reported to regulate apoptosis and to be an important cellular target for therapy.
Methods: We examined whether etodolac, meloxicam, and thalidomide inhibited proliferation and induced apoptosis in myeloma cell lines (RPMI 8226 and MC/CAR cells).
Results: Etodolac induced apoptosis more strongly compared with thalidomide or meloxicam. Etodolac induced down-regulation of Bcl-2 protein and mRNA, activation of Caspase-9, -7 and -3, cIAP-1 and Survivin, and loss of mitochondrial membrane potential in a dose-dependent manner. In addition, when myeloma cells were coincubated with 50 μM etodolac on bone marrow stromal cells (BMSCs), myeloma cell adhesion to BMSCs was significantly inhibited compared with thalidomide or meloxicam coincubation, and the adhesion molecules VLA-4, LFA-1 (CD11a), CXCX4, and CD44 were suppressed on myeloma cells treated with etodolac. Moreover, 50–100 μM racemate of etodolac significantly inhibited the proliferation of myeloma cells compared to 100 μM *R*-etodolac or *S*-etodolac.
Conclusions: Etodolac induced loss of mitochondrial membrane potential and apoptosis via a COX-2-independent pathway, suppressed the expression of adhesion molecules, and inhibited myeloma cell adhesion to BMSCs compared with thalidomide or meloxicam. The activities of etodolac potentially extend to the treatment of patients with myeloma resistant to standard chemotherapy, including thalidomide.

Keywords: Etodolac; Myeloma; Apoptosis; Bcl-2; Cell adhesion

1. Introduction

Myeloma is a B-cell neoplasm characterized by clonal expansion of plasma cells in the hematopoietic bone marrow [1] that is associated with angiogenesis [2]. Despite the use of high-dose chemotherapy with autologous hematopoietic stem-cell transplantation, few patients with multiple myeloma (MM) are cured. Recent studies demonstrated anti-MM activity of thalidomide and its analogues based upon anti-angiogenic activity [3], direct induction of apoptosis or growth arrest [4], inhibition of interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF) secretion-induced MM cell adhesion to bone marrow stromal cells [5], inhibition of tumor necrosis factor α (TNF-α) signaling [4], and anti-MM cell immunity mediated by patients' natural killer cells [6]. Thalidomide is also reported to possess moderate non-selective or weak cyclooxygenase-2 (COX-2) inhibitory activity [7] and suppress lipopolysaccharide (LPS)-mediated induction of COX-2 and prostaglandin E_2 (PGE_2) synthesis, which support its anti-angiogenic action [8].

Cyclooxygenase (COX), a key enzyme required for PG synthesis, is transcribed from two distinct genes [9]. Two isoforms have been identified: a constitutively expressed form, known as COX-1, and an inducible form, referred to as COX-

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2, which is induced by mitogens [10], cytokines [10], and growth factors [11], and induces PG-related inflammation [12] and cell growth [13]. However, COX-2 overexpression has been reported in cancers of the colon [14,15], pancreas [10], breast [11], lung [10,16], and mucous membrane of the head and neck [9,13,16]. A substantial amount of evidence has accumulated suggesting that COX-2 plays an important role in promoting tumor progression in various organs. Therefore, COX-2 might be a molecular target for cancer therapy and, in recent years, the anti-proliferative and pro-apoptotic effects of selective COX-2 inhibitors have been reported for various cancers [1,17–20]. The anti-proliferative effects of many COX-2 inhibitors have been reported to involve not only COX-2-dependent [21,22], but also COX-2-independent mechanisms in leukemia and other cancers [23–25].

Bcl-2 belongs to a family of proteins regulating apoptosis and plays an important role in the modulation of drug resistance. It is reported that the expression level of Bcl-2 is elevated in rat intestinal epithelial cells overexpressing COX-2 [26] and in human colon cancers treated with PGE_2 [27]. We investigated the interaction between COX-2 inhibitors, including thalidomide, and apoptosis-regulating proteins.

In this report, we chose two COX-2 inhibitors, etodolac and meloxicam, which have the same effects on COX-2 inhibition. Generally, it has been reported that many COX-2 inhibitors have structures that exploit binding within the COX-2 side-pocket (via sulfonyl, sulfone, or sulfonamide groups) to achieve selectivity, resulting in inhibition of COX-2 effects [28]. However, the molecular mechanisms underlying the anti-neoplastic effects of etodolac, which has no sulfonyl, sulfone or sulfonamide groups, remain unclear. We investigated the apoptotic pathway induced by etodolac, and showed that etodolac-induced apoptosis was mediated through COX-2-independent and Caspase-9-dependent mitochondrial pathway, and was more effective compared with thalidomide or meloxicam in myeloma cells. Moreover, we compared the anti-proliferative effects of etodolac with the stereoisomers of etodolac, *R*- and *S*-etodolac, in myeloma cells, and showed that etodolac induced apoptosis more effectively than both *R*- and *S*-etodolac. These findings support the need for additional investigation into the use of etodolac as a therapeutic agent against drug-resistant myeloma cells. Finally, myeloma cells express various adhesion molecules, such as CD44, VLA-4, ICAM-1, LFA-1 (CD11a), and CXCR4 [29,30]. Cellular adhesion molecules have been reported to be related to the homing of myeloma cells to the bone marrow, the production of growth factors, and the recirculation of these tumor cells in the advanced stages of disease. We investigated the changes in expression of the adhesion molecules CD44, LFA-1, VLA-4, and CXCR4 on myeloma cells treated with etodolac. In addition, we investigated the inhibition of myeloma cell adhesion to bone marrow stromal cells (BMSCs) after treatment with thalidomide, etodolac, or meloxicam.

2. Materials and methods

2.1. Reagents

Thalidomide was purchased from Sigma Chemical Company (St. Louis, MO, USA). Highly selective COX-2 inhibitors, racemic etodolac (etodolac), and stereoisomers of etodolac (*R*- and *S*-etodolac) were provided by Nippon Shinyaku Co. Ltd. (Kyoto, Japan). The purity of the racemate and stereoisomers of etodolac is high, and they contain few impurities. A highly selective COX-2 inhibitor, meloxicam, was provided by Boehringer Ingelheim (Ingelheim, Germany). These drugs were dissolved in dimethyl sulfoxide (DMSO) (Sigma), and diluted in culture medium immediately before use. The final concentration of DMSO in all experiments was less than 0.01%, and all treatment conditions were compared with vehicle controls. 3,3′-Dihexyloxacarbocyanine iodide (DiOC6) was purchased from Molecular Probes (Eugene, OR, USA).

2.2. Cell lines and bone marrow stromal cells

Human myeloma-derived cell lines, RPMI 8226 and MC/CAR, were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). RPMI 8226 cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml streptomycin, and 200 U/ml penicillin (GIBCO-BRL, Gaithersburg, MD, USA). MC/CAR cells were cultured in RPMI 1640 containing 20% heat-inactivated FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 200 U/ml penicillin. All cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C. Human BMSCs were prepared from BM samples from donors for allogenic BM transplantation, after obtaining informed consent. Mononuclear cells were separated by Ficoll-Hypaque (Sigma) density-gradient centrifugation, and seeded into 96-well plates at 2×10^4 cells/well in 1 ml of Dulbecco's modified Eagle medium (DMEM) (GIBCO-BRL) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS in humidified 5% CO_2 at 37 °C. Cultures were fed weekly, replacing half of the growth medium containing half of the non-adherent cells with fresh complete medium. BMSCs were maintained until the cells were at least 80% confluent.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

RPMI 8226 and MC/CAR cells were cultured in 2 ml of complete medium containing 1×10^6 cells in the presence of thalidomide, etodolac, or meloxicam at 50 and 100 μM, and incubated at 37 °C. Total RNA was extracted after 0, 12 and 16 h of incubation using an RNeasy system (Quiagen, Tokyo, Japan), and 2 μg of RNA was reverse-transcribed using a first-strand cDNA synthesis kit (Roche, Indianapolis, IN, USA). PCR was carried out using a DNA thermal cycler (model PTC