Mefenamic Acid Induces Apoptosis in Oral Malignant Burkitt's lymphoma Through Caspase-3 and -9 Pathways Followed by Down-Regulation of Cox-2 and Overexpression of p27Kip-1

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Abstract

Mefenamic acid (MEF) is a non-steroidal anti-inflammatory drug (NSAID) which it is most often used for treating pain of some inflammation diseases in the short term as well as mild to moderate pain. Interestingly, mefenamic acid is known to have anti proliferative activity against various human cancers.

In the present study, induction of apoptosis in oral malignant Burkitt's lymphoma (Raji) cell through caspase-3 and -9 pathways was examined. The pure laboratory experimental with post-test only control group design was carried out in this study. The colorimetric assay caspase-3 and -9 were used to know the apoptosis induction proofed by increasing the proteolytic activity. Double staining (AO-EB) was confirmed to detect cell apoptosis. To know the level protein of Cox-2 in the treated cells was analized by Western blotting. The doses of mefenamic acid were set as 0, 5, 10, 20 and 40 μg/mL.

Results revealed the proteolytic activity of caspase-3 and -9 was markedly increased in cell treated with MEF at doses 10-40 μg/mL. Induction of apoptosis was also increased in double staining analysis at the same doses. However, down regulation of Cox-2 and overexpression of p27Kip-1 were detected in cells treatment.

In conclusion, MEF induces apoptosis in oral malignant Burkitt's lymphoma through increased the proteolytic activity of caspase-3 and -9 followed by down regulation of Cox-2 and up regulation of p27Kip-1 targeting this molecule could represent a promising new therapeutic approach for this type of cancer.

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Introduction

Mefenamic acid (MEF) is most often used for treating pain of some inflammation diseases in the short term (seven days or less), as well as mild to moderate pain including headache, dental pain, postoperative and postpartum pain.[1] Interestingly, mefenamic acid is known to have anti proliferative activity against various human cancers including colon cancer,[2] liver,[3] prostat[4] and hemolytic anemia.[5] Administration of MEF in cancer patients can increase sensitivity to chemotherapy drugs CDDP and 5-FU and increase their inhibitory activity in drug-resistant human cancer cells. Thus, the use of mefenamic acid can provide effective clinical outcomes by overcoming the resistance of chemotherapy drugs and their antineoplastic potential against several types of human cancer.[6] It was reported MEF was shown to inhibit the stimulated calcium uptake in cancer cells, suggesting that MEF gives a signal for apoptosis.[7] Apoptosis is a form of programmed cell death that is regulated by the Bcl-2 family and caspase family of proteins. The caspase cascade responsible for executing cell death following cytochrome *c* release is well

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described, however the distinct roles of caspase -3, -7 and -9 during this process are not completely defined.[8]

Inflammation has been identified as an important component during the process of proliferation, progression and dissemination.[9] Inflammation induced and maintained by cyclooxygenase 2 (COX-2) enzyme activities and COX-2 inhibition can be used as targets for cancer treatment. Among drugs with high COX-2 inhibitory activity, non-steroidal anti-inflammatory drugs (NSAIDs) play an important role,[10] and mefenamic acid is one of the most potent drugs.[11] Mefenamic acid is a phenamate derivative that is not only a potent inhibitor of aldoketoreductase (AKR), an enzyme that regulates the concentration of androgens, estrogens and progestins, but also catalyzes the reduction of ketosteroids.[12] NSAID inhibition of this enzyme is the target mechanism of the antineoplastic effect. Several anti-inflammatory drugs have also been studied experimentally, but the results obtained are variable and it is difficult to determine which substance has the greatest antineoplastic effect. Mefenamic acid has previously been reported as a promising antineoplastic drug in several types of human cancer cells, but the drug has never been examined on oral Burkitt's lymphoma cells.[11]

Caspases are a family of conserved cysteine proteases that play an essential role in apoptosis. Caspases will cleave a range of substrates, including downstream caspases, nuclear proteins, plasma membrane proteins and mitochondrial proteins, ultimately leading to cell death.[12] In mammalian, caspases can be subdivided into three functional groups: initiator caspases (caspase 2, 8, 9 and 10), executioner caspases (caspase 3, 6 and 7), and inflammatory caspases (caspase 1, 4, 5, 11 and 12). Initiator caspases initiate the apoptosis signal while the executioner caspases carry out the mass proteolysis that leads to apoptosis. Inflammatory caspases do not function in apoptosis but are rather involved in inflammatory cytokine signaling and other types of cell death such as pyroptosis.[13]

Burkitt's lymphoma (BL) is one of the most aggressive malignancies of lymphoid origins and accounts for 3-5% of all lymphomas. BL is a high grade B-cell neoplasm and usually found in the pediatric population. BL represents 40% of childhood non Hodgkin lymphoma (NHL).[14] The highest incidence is found in the endemic form of equatorial regions of Africa and Papua-New Guinea where it accounts for 50-70% of all pediatric malignancies.[15] It was reported that Epstein-Barr virus (EBV) infection, malaria, immunodeficiency, spontaneous and somatic mutation have been implicated in its etiology.[16] In the present study, mefenamic acid induces apoptosis in oral malignant burkitt's lymphoma through caspase-3 and -9 pathways was evaluated.

Materials and methods

Cell and cell culture

The oral malignant burkitt's lymphoma (Raji) cell line (ATCC CCL-86) was obtained from Department of Paracytology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. The cell line was cultured in Dulbecco's modified Eagle medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum, and 100 μg/ml streptomycin, 100 U/ml penicillin (Moregate BioTech, Bulimba, Australia). The cultures were incubated in a humidified atmosphere of 95% air and 5% $C0_2$ at 37 ^{0}C.[17]

Preparation of MEF various doses

MEF (Pfizer, Indonesia) with a dose of 500 mg was dissolved in 500 mL DMEM 10% FCS (= 1 mg/mL), and it was used as a stock solution. Furthermore, the stock solution was diluted to make doses of 5, 10, 20 and 40 μg/mL. All of these solutions were sterilized with a 0.45 μm diameter sterile syringe filter (Sigma-Aldrich, USA).

Activity of apoptosis using caspase-3 and -9

Apoptotic induction was analyzed using colorimetric assays (caspase-3 and -9; R&D Biotechne Syst, USA) according to the manufacturer's directions for 48 hours. Briefly, equal amounts of cell substrate were prepared from Raji cells treated with various doses of mefenamic acid (0, 5, 10, 20 and 40 μg/mL) and incubated with caspase-3 (DVED-pNA) or caspase-9 (LEHD-pNA) substrates in buffer solution. for 2 hours at room temperature. The absorbance was measured at a wavelength of 405 nm using a microplate reader (BioRad, USA). Each test performed triplicate.

Apoptosis induction using double-staining (AO-EB) assay

Acridine orange (AO) and ethidium bromide (EB) double staining were delivered in this study. DNA-binding dyes AO and EB (Sigma-Aldrich, USA) were used for the morphological detection of apoptotic cells. AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA). EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. After treatment with different doses of MEF for 48 h, the cells were detached, washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 10 min. The stained cells were observed by a fluorescence microscope (Zeiss, Germany) at 100 × magnifications. The cells were divided into three categories as follows: viable cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation). In each experiment more than 100 cells/sample were counted.

Western blot analysis

The Raji cells treated with various doses of MEF in Falcon tissue culture (Ø 10 cm) for 48 h were prepared and harvested. Samples containing equal amounts of protein (70 g) were electrophoresis on a SDS-polyacrylamide gel and transferred to a polyvynilidene fluoride (PVDF) membrane (BioRad, Hercules, CA, USA). The filters were blocked in TBS containing 5% non-fat milk powder at 37^0C for 1 h, and then incubated with a 1:500 dilution of primary antibodies against the Cox-2 protein (Cox 229, monoclonal antibody, Invitrogen, USA) and the p27Kip-1 protein (clone 1B4, mouse monoclonal antibody; Novocastra Laboratories, New Castle, UK). For detection of HRP-conjugated antibodies were used the enhanced chemiluminescent (ECL) plus kit (Amersham Pharmacia Biotech, UK). Anti µ-tubulin monoclonal antibody (Zymed laboratories, San Francisco, USA) was used for normalization of the western blot analysis.

Statistical analysis

Statistical differences between the means for the different groups were evaluated with Stat View 4.5 (Abacus Concepts, Berkeley, CA) using one-way ANOVA and *t-test*. The significance level was set at 5% for each analysis.

Results

Proteolytic activity of caspase-3 and -9

To determine the induction of apoptosis through the proteolytic activity of caspase-3 and -9, a colorimetric assay was used. Based on the study on caspase-3, it was known that there was an increase in proteolytic activity at a dose of 10 to 40 µg/mL by 1.60 to 2.10 fold increased. Meanwhile, on caspase-9 were detected at 2.80 to 5.70 fold increased (Figure 1).

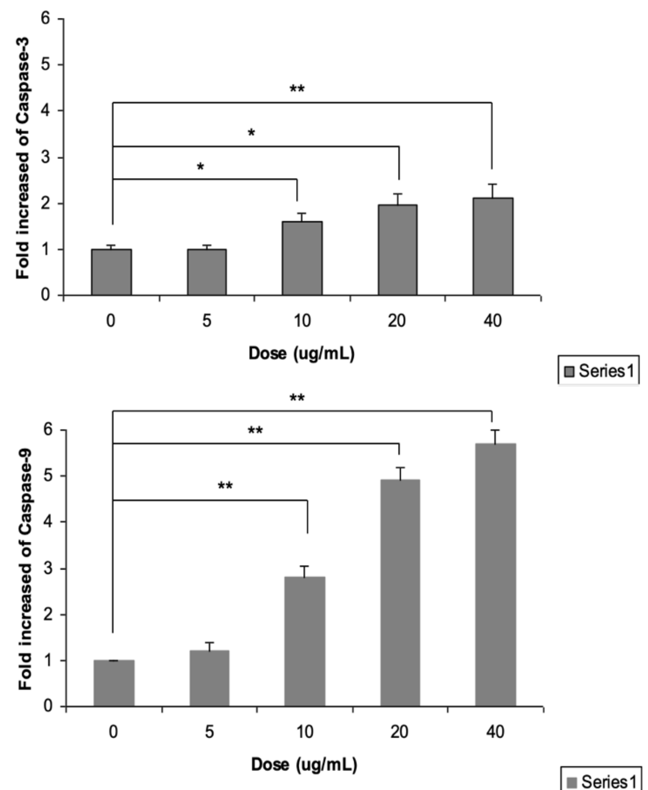

Figure 1. Colorimetric assay caspase-3 and-9 for apoptosis induction of Raji cells treated by various doses of MEF for 48 hours. (*, $P < 0.05$ and**, $P < 0.01$ when compared with that of control by one-way Anova).

Double staining analysis

Based on double staining analysis using Acridine Orange – Ethidium Bromide, it appears that there was an induction of cell apoptosis according to the increase in the dose of the MEF. Apoptosis in Raji cells was detected begun at a dose of 10 to µ40 µg/mL. The percentage of apoptosis in raji cells at a dose of 10-40 µg/mL was found to be 31.50 – 62.25% compared with that of control cells (Figure 2).

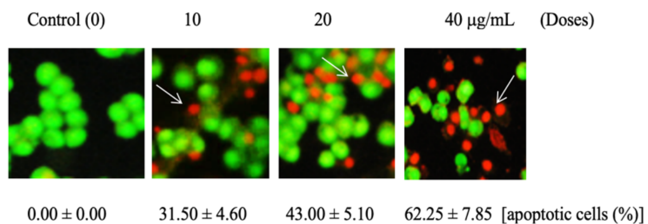

Figure 2. Double staining (AO-EB) assay. Cell apoptosis induction treated by various doses of MEF in Raji cell.

Western blotting analysis

Western blotting analysis was carried-out to determine the protein level of raji cells treated with various doses of MEF with molecular targets Cox-2 and p27Kip1. As shown in Figure 3, the expression of Cox-2 protein was decreased with increasing dose of the tested MEF. On the other hand, p27Kip1 protein expression was increased. As an internal control, μ-tubulin protein was confirmed which was detected to have relatively the same protein level for each test cell (Figure 3).

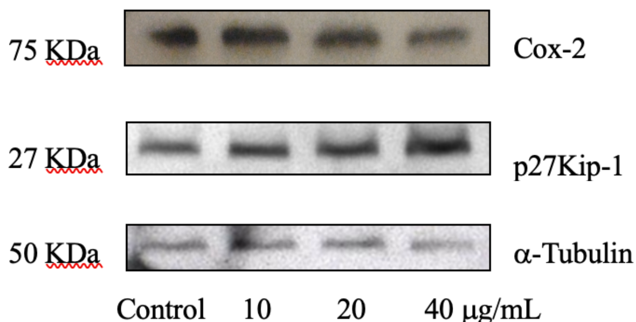

Figure 3. Protein level for each test cell.

Discussion

Cancer cells are normal cells that undergo mutations, genetic changes and grow uncontrollably. Cancer cell growth is continues, uncontrolled, uncoordinated, and it does not follow the regulation of normal cell growth, so that it can expand, spread, and penetrate into deeper tissues and causes damage to surrounding tissues.[19] Normal cell mutations can occur due to activation of proto-oncogenes or inactivation of tumor suppressor genes that can arise cancer. One of the mechanisms to kill cancer cells is the induction of apoptosis. Apoptosis is an intracellular programmed cell death mechanism by activating the caspases pathway. Apoptosis has two pathway mechanisms including intrinsic and extrinsic pathways.[20] Both mechanisms are very important to prevent the proliferation of cancer cells and serve as one of the control checkpoints in the cell cycle.

In the present study, we used caspase-3 and caspase-9 colorimetric assays to determine the apoptosis induction of MEF at various doses against oral malignant Burkitt's lymphoma cells. An increase in activation of proteolytic activity in cell treated with various doses of MEF strongly suggests that apoptosis occurred in those cultures. Increased the proteolytic activity of caspase-3 and-9 were detected with increasing MEF dose. Apoptotic induction through the intrinsic pathway (caspase-9) was shown in cell treated with doses 10-40 μg/mL of MEF. This result may be starting from a dose of 10 μg/mL has been able to activate pro-apoptotic protein in the mitochondrial membrane to release Apaf-1 which will activate pro-caspase-9 to become caspase-9. Finally, caspase-9 and caspase-3 will activate cell apotosis. A recent study reported that activated caspase-8 can directly cleave and activate the executioner caspases, such as caspase-3 via extrinsic pathways and caspase-9 through intrinsic pathway. Alternatively, it can cleave one of the Bcl-2 family members (such as Bid) to induce the release of mitochondrial cytochrome-c, which also leads to activation of caspase-3 via formation of apoptosome consisting of Apaf-1 and caspase-9. Furthermore, apoptosome formation and caspase-3 as an executer caspase will activate the cell apoptosis.[21] In the current study, increased activation of caspase-3 and -9 in Burkitt's lymphoma (Raji) cells treated with MEF (10-40 μg/mL) revealed that apoptosis occurred through both the intrinsic and extrinsic pathways. However, the intrinsic pathway or chemical-induced apoptosis has a higher proteolytic activity compared with the extrinsic pathway (receptor-induced apoptosis). This data indicate that MEF is more effective in increasing caspase-9 activity. In addition, the most important data obtained from this study was MEF can induce apoptosis of oral malignant Burkitt's lymphoma cells through two apoptotic pathways, intrinsic and extrinsic pathways. This data was also supported by the results of a double staining analysis which showed the occurrence of apoptosis in oral malignant Burkitt's lymphoma cells as indicated by reddish-orange colored cells.

The presence of an orange-red color in Raji cells treated with various doses of MEF indicates the occurrence of cell death. The AO-EB dye can react with the products of dead cells. In contrast to control cells were not experienced cell dead, the color remained light green even though they reacted with AO-EB agents. This study are consistent with Woo *et al.*,[21] who reported that MEF can induce apoptosis in human liver cancer cells through caspase-3 to cleave PARP-1 pathways. Other researcher reported that MEF has anticancer potential by inhibiting platelet-derived growth factor and also has the ability to reduce cancer cell proliferation, progression, angiogenesis, apoptosis, and invasiveness.[22] MEF is one of the NSAIDs and it was reported to be able to inhibit the Cox-2 enzyme.[23] Cox-2 is frequently expressed in cancer and is involved in the synthesis of prostaglandin E(2) which can promote tumor growth by binding. its receptors and activating signaling pathways which regulate cell proliferation, migration, apoptosis, and angiogenesis.[24] In the present study, Cox-2 protein expression decreased with increasing MEF dose, whereas p27Kip-1 expression increased. These data indicate that MEF has the potential to inhibit Cox-2 protein which was a trigger for cancer cell growth. In contrast, MEF can increase the expression of p27Kip-1 which functions as a tumor suppresser protein. These data can be used as the basis for molecular targets for treatment of malignant Burkitt's lymphoma via Cox-2 and p27Kip-1 molecules.

Conclusions

MEF induces apoptosis in oral malignant Burkitt's lymphoma through increased the proteolytic activity of caspase-3 and -9 followed by down regulation of Cox-2 and up regulation of p27Kip-1 targeting this molecule could represent a promising new therapeutic approach for this type of cancer.

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Declaration of Interest

The authors report no conflict of interest.

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