DHMEQ, a novel NF-κB inhibitor, induces apoptosis and cell-cycle arrest in human hepatoma cells

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Abstract. Several reports have indicated that nuclear factor-κB (NF-κB) is constitutively activated in a variety of cancer cells including hepatoma cells and plays a key role in their growth and survival. Dehydroxymethylepoxyquinomicin (DHMEQ) derived from the structure of an antibiotic epoxyquinomicin C is a novel NF-κB inhibitor. In the present study, we evaluated the effect of DHMEQ on the NF-κB activity in human hepatoma cells, Huh-7, HepG2 and Hep3B, and the anti-tumor effect of DHMEQ on these cells in vitro and in vivo. DHMEQ inhibited the steady-state transcriptional activity of NF-κB in all hepatoma cells. DHMEQ blocked the constitutive DNA-binding activity and TNF-α-mediated nuclear translocation of NF-κB in Huh-7 cells. DHMEQ (5-20 μg/ml) dose-dependently reduced the viable cell number of all hepatoma cells. DHMEQ (20 μg/ml) induced apoptosis in all hepatoma cells, especially in Hep3B cells, and cell-cycle arrest in Huh-7 and HepG2 cells. These effects were accompanied by downregulation of proteins involved in anti-apoptosis (Bcl-xL, XIAP or c-IAP2) and cell-cycle progression (cyclin D1), and induction of proteins involved in pro-apoptosis (Bax) and cell-cycle retardation (p21Waf1/Cip1), although the degree of changes by DHMEQ was different in each hepatoma cell type. Moreover, intraperitoneal administration of DHMEQ (8 mg/kg) significantly repressed the growth of Huh-7 tumor subcutaneously transplanted into BALB/c nu/nu athymic mice. Our results suggest that DHMEQ could qualify as a candidate for a new chemotherapeutic agent against human hepatoma.

Introduction

Hepatocellular carcinoma (HCC), hepatoma, is one of the most common malignancies worldwide. It is estimated that half a million cases occur annually worldwide (1). Several strategies have been implemented for the treatment of patients with HCC, such as surgical resection, percutaneous ethanol injection, radiofrequency ablation, transcatheter arterial embolization, and liver transplantation. However, reduced liver function derived from underlying liver cirrhosis has a profound effect on all treatment decisions and interferes with the use of these therapeutic modalities for HCC except liver transplantations (2). In addition, multicentric tumor recurrence rates are very high, even in patients with HCC who receive curative treatment. Therefore, HCC is a tumor with high lethality (1,3), and a novel approach for the treatment of HCC is needed.

The nuclear factor kappa B (NF-κB), a transcription factor family, consists of several structurally-related proteins such as RelA (p65), RelB, c-Rel, p50/p105 and p52/p100, which form homo- or heterodimers with each other and regulate the expression of a number of genes (4,5). NF-κB plays a well-known function in the regulation of immune responses and inflammation, but growing evidence supports a major role in oncogenesis. NF-κB regulates the expression of genes involved in many processes that play a key role in the development and progression of cancer such as proliferation, migration and apoptosis (6-8). Aberrant or constitutive NF-κB activation has been detected in many human malignancies including HCC (6-9).

Dehydroxymethylepoxyquinomicin (DHMEQ) is a novel NF-κB inhibitor produced by Dr Kazuo Umezawa (Keio...
University, Japan), based on the structure of epoxyquinomicin C originally isolated from Amycolatopsis (10,11). DHMEQ has been reported to repress renal inflammation in rats and osteoclastogenesis in cultured bone marrow cells through inhibiting NF-κB activity (12,13). In addition, DHMEQ have shown anti-tumor activity against several cancer cells including prostatic cancer (14,15), thyroid cancer (16), malignant myeloma (17,18), breast cancer (19) and adult T-cell leukemia (ATL) cells (20,21), in which NF-κB is constitutively activated and contributes to the growth and survival of these cells. The present study was set up to determine the effects of DHMEQ in human hepatoma cells both in vitro and in vivo and to additionally elucidate the molecular mechanisms underlying the action of this agent.

Materials and methods

**Cell culture and viability assay**. The human hepatoma cell lines, Huh-7, HepG2 and Hep3B were maintained in RPMI containing 10% fetal bovine serum (FBS). DHMEQ was a generous gift from Professor Kazuo Umezawa (Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan). It was dissolved in DMSO to prepare a 10 μg/μl solution and subsequently diluted in culture medium to a final DMSO concentration of ≤0.2%. To analyze cell viability, 3x10⁴ cells were placed into 96-well multiplates. One day later, the medium was replaced with fresh medium containing varying concentrations of DHMEQ or vehicle (0.2% DMSO) alone, and the cells were incubated for 48 h. Following removal of the medium and dead cells, viable cells were counted with Particle counter Z1 (Beckman Coulter, Inc., Fullerton, CA).

**Reporter gene transfection assay**. The pNFκB-luc (Stratagene, La Jolla, CA) containing four copies of the binding sequence of NFκB and firefly luciferase gene and pRL-CMV-luc (Promega, Madison, WI) containing the cytomegalovirus immediate early enhancer/promoter and expressing renilla luciferase gene were used in the assay. Cells were grown in 24-well multiplates in triplicate one day before transfection. In the next step, 1 μg of pNFκB-luc and 10 ng of pRL-CMV-luc were transfected into the cells using Lipofectin (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. After 6-h incubation, the medium was replaced with fresh medium containing varying concentrations of DHMEQ or vehicle (0.2% DMSO) alone, and the cells were incubated for 24 h. Luciferase activity in the cells was then determined by a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega).

**Electrophoretic mobility shift assay (EMSA)**. Huh-7 cells were incubated with 20 μg/ml of DHMEQ for 1-24 h. The nuclear extract was prepared as described previously (22). As a positive control, the nuclear extract from HUT-78 cells containing NF-κB (23) was used. EMSA was performed using an EMSA kit (Panomics, Inc., Redwood City, CA) according to the manufacturer's protocol. Briefly, the same amount of protein from each nuclear extract was incubated with a biotin-tagged NF-κB probe; 5'-AGTTGAAGGGACTTTCCCGAACGC-3' for 30 min at 15°C. The reaction mixture was electrophoresed using a 5% polyacrylamide gel containing 25 mM Tris-borate and 0.25 mM EDTA at 4°C and transferred to nylon membrane (Hybond-N+; Amersham Biosciences AB, Upsala, Sweden). The blots were incubated with a detection agent in the kit and visualized with SuperSignal® West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL).

**Western blotting**. The following antibodies were used in the experiments; rabbit polyclonal anti-human Bcl-xl, rabbit polyclonal anti-human XIAP, mouse monoclonal anti-human cyclin D1, mouse monoclonal anti-human p21Waf1/Cip1 (Cell Signaling Technology, Inc. Beverly, MA), rabbit polyclonal anti-human c-IAP2, mouse monoclonal anti-human Bax (B-9), rabbit polyclonal anti-human PTTG (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), mouse monoclonal anti-human β-actin (Sigma-Aldrich, Inc.). Cells were incubated with 20 μg/ml DHMEQ or vehicle (0.2% DMSO) alone for 24 h. Then cells were lysed by addition of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml of aprotinin, 1% NP-40, 0.5% sodium deoxycholate) for 10 min at 4°C, and insoluble material was removed by centrifugation at 14,000 rpm for 30 min at 4°C. The same amount of protein from each lysate (20 μg /well) was subjected to 15% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes which were then blocked for 1.5 h using 5% non-fat dried milk in PBS containing 0.1% Tween-20 (PBS-T), washed with PBS-T and incubated at 4°C overnight in the presence of each primary antibody. The membranes were washed with PBS-T and incubated with sheep anti-mouse IgG or donkey anti-rabbit IgG coupled with horseradish peroxidase (Amersham Biosciences AB). The enhanced chemiluminescence system (SuperSignal® West Pico Chemiluminescent Substrate; Pierce Chemical Co.) was used for detection.

**Immunofluorescent histochemistry**. Huh-7 cells were seeded onto 11-mm glass coverslips in 24-well plates at 2x10⁵ cells/well. The next day, the medium was replaced with serum-free medium, and the cells were pretreated with 20 μg/ml of DHMEQ or vehicle (0.2% DMSO) alone for 2 h then stimulated with 200 U/ml of TNF-α for 60 min. The cells were fixed with 4% paraformaldehyde in PBS for 10 min at 4°C, immersed in -20°C methanol for 10 min, and incubated in blocking buffer (5% normal horse serum in PBS) for 1 h. The cells were incubated with polyclonal rabbit anti-human p65 (RelA) (Biogenesis, Poole, UK) for 1 h at room temperature, washed three times in PBS, incubated with rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h, washed in PBS, and mounted in Vectashield® Mounting Medium (Vector Laboratories Inc., Burlingame, CA). Immunofluorescence for rhodamine was analyzed by Olympus BX50 microscope (Tokyo, Japan) and images were digitally captured using a Nikon DXM 1200 digital camera (Tokyo, Japan).

**Immunoprecipitation**. Following stimulation of the cells with vehicle (0.2% DMSO) alone for 24 h. Cells were washed in PBS and permeabilized with 70% ethanol at 4ºC at least overnight. After centrifugation,
cell pellets were resuspended in 1 ml PBS, treated with 0.25 mg/ml Ribonuclease A (Sigma-Aldrich, Inc., St. Louis, MO, USA) at 37ºC for 30 min, and stained with 50 μg/ml propidium iodide (Sigma-Aldrich, Inc.) for 30 min on ice. The DNA content in each cell nucleus was determined by an Epics XL flow cytometer (Beckman Coulter, Miami, FL).

In vivo study. All of the procedures involving animals and their care in this study were approved by the Ethics Committee of Nagasaki University in accordance with institutional and Japanese government guidelines for animal experiments. Four-week-old male BALB/c nu/nu athymic mice were obtained from Charles River Japan, Inc. Huh-7 cells (3x10^6) were implanted subcutaneously into the left thigh. Tumor volume was calculated according to the formula \(a^2 \times b \times 0.5\), where \(a\) and \(b\) are the smallest and largest diameters, respectively. When the tumor volume reached 50 mm^3, mice were randomly assigned into two groups, and received intraperitoneal injection of 8 mg/kg DHMEQ or vehicle alone every other day for 18 days. Tumor size and body weight of mice were monitored at least every 4 days for 5 weeks.

Statistical analysis. The statistical analysis was performed using Student’s t-test. Unless otherwise indicated, average values were expressed as mean values with SD. \(P<0.05\) was considered as statistically significant.

Results

DHMEQ inhibits the constitutive NF-κB activity in human hepatoma cells. Effect of DHMEQ on the transcriptional activity of NF-κB in human hepatoma cells was determined by transient transfection assay using luciferase reporter plasmid, pNFκB-Luc, which contains four repeats of the binding sequence of NF-κB. DHMEQ dose-dependently repressed the transcriptional activity of NF-κB in Huh-7, HepG2, and Hep3B cells (Fig. 1A). These results suggest that DHMEQ inhibited the steady-state transcriptional activity of NF-κB in these cells. Next, NF-κB binding activity to the κB DNA site was analyzed by EMSA (Fig. 1B). Nuclear extracts from unstimulated Huh-7 cells and positive control cells formed two shifted bands, fast migrating and slow migrating. Addition of 100 times molar excess of unlabeled competitor DNA completely abrogated both bands, indicating that these bands corresponded to NF-κB-DNA complexes. Time-course study showed that 20 μg/ml of DHMEQ treatment diminished the NF-κB binding activity in Huh-7 cells in a time-dependent manner. These results suggested that NF-κB
was constitutively activated in human hepatoma cells and DHMEQ inhibited its activity.

DHMEQ inhibits the TNF-α-mediated nuclear translocation of p65. We studied the effect of DHMEQ on the TNF-α-mediated nuclear translocation of p65, a component of NF-κB, in Huh-7 cells by immunofluorescence microscopy. After 60-min stimulation with 200 U/ml of TNF-α, p65 translocated from cytoplasm to nucleus. In contrast, pretreatment with 20 μg/ml of DHMEQ inhibited the TNF-α-mediated nuclear localization of p65 (Fig. 2).

DHMEQ induces apoptosis and cell-cycle arrest in human hepatoma cells. To elucidate the effect of DHMEQ on the viability of hepatoma cells, HuH-7, HepG2 and Hep3B were incubated with varying concentrations of DHMEQ for 48 h. The viable cell number was decreased in all hepatoma cells by DHMEQ in a dose-dependent manner (Fig. 3A). Similarly, DHMEQ at a concentration of 20 μg/ml decreased the viable cell number in a time-dependent manner (Fig. 3B). To examine whether DHMEQ induced apoptosis or cell-cycle arrest in hepatoma cells, we analyzed the contents of DNA in those cells using flow cytometry after propidium iodide staining. In all hepatoma cells, especially in Hep3B, DHMEQ increased the number of cells in the subG1 phase of cell-cycle, representing apoptotic cells (Fig. 4). In addition, Huh-7 and HepG2 cells treated with DHMEQ showed a decrease in the number of cells in the S-phase and an increase in the number of cells in the G0/G1 phase. These results suggest that DHMEQ reduced the viable cell number through inducing apoptosis and cell-cycle arrest at G0/G1 phase in hepatoma cells.

DHMEQ downregulates the expression of proteins involved in anti-apoptosis and cell-cycle progression. To elucidate the mechanism of cytotoxic effect of DHMEQ on human hepatoma cells, we examined the effect of DHMEQ on the expression of apoptosis-related proteins, including Bcl-xL, XIAP, c-IAP2, Bax by Western blotting. As shown in Fig. 5, Bcl-xL expression was downregulated by DHMEQ in Hep3B but not in Huh-7 and HepG2 cells. XIAP expression was downregulated by DHMEQ in Huh-7 and HepG2 but not in Hep3B cells. c-IAP2 expression was downregulated by DHMEQ in Huh-7 and HepG2.
Hep3B but not in HepG2 cells. On the contrary, DHMEQ slightly stimulated Bax expression in all hepatoma cells. We next examined the effect of DHMEQ on the expression of cell-cycle regulating proteins, including cyclin D1, p21Waf1/Cip1 and pituitary tumor transforming gene (PTTG). DHMEQ repressed the cyclin D1 expression in Huh-7 and HepG2 but not in Hep3B cells. In contrast, the expression of p21Waf1/Cip1 which inhibits G0/G1 to S-phase transition (24) was upregulated by DHMEQ in all hepatoma cells. The expression of PTTG, a regulator of cell division (25), was downregulated by DHMEQ in all hepatoma cells.

Anti-tumor effect of DHMEQ in vivo. Huh-7 cells were subcutaneously implanted and tumors were established in athymic mice because Huh-7 cells were more efficiently transplantable than the other cells. After the tumor volume reached 50 mm³, a solution of DHMEQ (8 mg/kg) was injected into peritoneal space every other day for 18 days, and tumor size was monitored. Injection of DHMEQ significantly repressed the tumor growth compared with vehicle-injection (Fig. 6). DHMEQ treatment at the dosage used was well tolerated, and did not lead to weight loss or increase of serum transaminase attributable to toxicity (data not shown).

Discussion

In the present study, reporter assay using pNFκB-Luc revealed that DHMEQ dose-dependently inhibited the steady-state transcriptional activity of NF-κB in all hepatoma cells as reported in other cancer cells (14-21). The precise mechanism of inhibition of NF-κB activity by DHMEQ is still unclear, however, it has been reported that DHMEQ directly inhibits the nuclear translocation of NF-κB rather than the degradation of IκBα which retains NF-κB in cytoplasm (11,26). EMSA assay showed that nuclear extracts from unstimulated Huh-7 cells formed NF-κB-DNA complexes which appeared as two shifted bands, and these complex formations were abolished by DHMEQ. We did not perform the super-shift assay to determine what components of NF-κB were included in these complexes. However, we have already reported using anti-p50 and p65 antibodies that fast migrating or slow migrating complex in Huh-7 cells corresponded to the p50/p50 homodimer or p65/p50 heterodimer, respectively (27). In addition, immunohistochemical study (Fig. 2) showed that DHMEQ inhibited the TNF-α-mediated nuclear translocation of p65 in Huh-7 cells. Taken together, it is possible that DHMEQ effectively inhibits constitutive and TNF-α-mediated nuclear translocation of NF-κB containing p65 in hepatoma cells.

In our study, the treatment of 20 μg/ml of DHMEQ for 48 h reduced the viable cell number to almost one third of control in all hepatoma cells. In addition, intraperitoneal injection of 8 mg/kg of DHMEQ significantly repressed the growth of Huh-7 hepatoma inoculated subcutaneously in athymic mice. Since the used concentrations of DHMEQ in vitro and in vivo were similar to the previous studies in prostatic cancer (14,15), thyroid cancer (16), multiple myeloma (17,18), breast cancer (19), and ATL cells (20,21), it is likely that the susceptibility of human hepatoma cells to DHMEQ is equivalent to other cancer cells. Previous studies have concluded that DHMEQ reduced the viable cell number through inducing apoptosis (14-21). In fact, 20 μg/ml of DHMEQ induced apoptosis in all hepatoma cells, especially in Hep3B cells. However, DHMEQ also reduced the number of cells in S-phase and increased that in G0/G1 phase in Huh-7 and HepG2 cells, suggesting that DHMEQ not only induced apoptosis but also inhibited the G0/G1 to S cell-cycle transition.
progression in these cells. This was supported by the results from Western blotting (Fig. 5), in which the expression of cyclin D1, a regulator of G0/G1 to S progression, was downregulated by DHMEQ in Huh-7 and HepG2 cells. Our observation was consistent with the recent report that DHMEQ induced cell-cycle arrest at G0/G1 phase in ATL cells accompanying a decrease of cyclin D1 expression (21).

NF-κB such as a p65/p50 heterodimer regulates the expression of many genes involved in anti-apoptosis and cell-cycle progression (4-8). Of these, Bcl-xL, CIAP1,2, XIAP, FLIP, TRAF1,2 and cyclin D1 are well known NF-κB-target genes (4-8.28). In previous studies, DHMEQ repressed the expression of Bcl-xL, CIAP1,2, XIAP, FLIP and cyclin D1 in several cancer cell types (14-21), by which DHMEQ could promote apoptosis and block cell-cycle progression in cancer cells. However, the effects of DHMEQ on the expression of these genes were different in the cancer cells used. For instance, DHMEQ downregulated the expression of Bcl-xL in thyroid cancer cells, in ATL, and U266 myeloma cells (16,17), but not in prostatic cancer cells and 12PE myeloma cells (14,17). Similar phenomenon was observed in our study. DHMEQ did not equally downregulate the expression of Bcl-xL, c-IAP2, XIAP and cyclin D1 in three hepatoma cell types. Since these genes are regulated not only by NF-κB but also by other transcriptional factors, the dependence of the gene expression on NF-κB may be different in each hepatoma cell type. In this study, in addition to cyclin D1, the expression of p21Waf1/Cip1 and PTTG was modulated by DHMEQ. Although the expression of these genes is not directly regulated by NF-κB, altered expression of these proteins may, at least in part, mediate the anti-tumor effect of DHMEQ in hepatoma cells.

NF-κB is thought to a molecular target in the treatment of cancer (6,29,30). A phase I clinical study of PS341, a proteasome inhibitor, which represses the NF-κB activity through stabilizing IκB protein and induces apoptosis in cultured cancer cells including hepatoma cells (31,32) is ongoing in patients with advanced cancer (33). In the present study, we have demonstrated that DHMEQ exhibited anti-tumor activity against human hepatoma cells through inhibiting NF-κB activity as reported in other cancer cells. Therefore, DHMEQ is also a promising candidate as a therapeutic agent in patients with advanced cancer including HCC.

References


