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The Proteasome Inhibitors Epoxomicin and MG262 Suppress Urokinase-Type Plasminogen Activator Expression by Human Oral Squamous Carcinoma Cells (HSC-3)

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We examined the effects of proteasome inhibitors, epoxomicin and MG262, on urokinase-type plasminogen activator (uPA) mRNA expression and uPA production in HSC-3 cells, a human oral squamous carcinoma cell line. Epoxomicin and MG262 suppressed uPA mRNA expression and uPA production in a dose-dependent manner. A time course study demonstrated a marked decrease in uPA mRNA expression from as early as 6 h after initiation of exposure to epoxomicin (50 nM) or MG262 (50 nM), and the suppressive effects were similar or a little stronger at 12 and 24 h. Epoxomicin and MG262 also decreased the transcriptional activity of nuclear factor (NF)-κB-dependent promoter. The inhibitory effect of MG262 on the constitutive NF-κB activity was stronger than that of epoxomicin. Because transcription of uPA gene is known to depend on NF-κB activity, the suppression of uPA gene expression by these proteasome inhibitors is conceivably mediated by inhibition of constitutive NF-κB activity. Furthermore, epoxomicin and MG262 reduced the invasive activity of HSC-3 cells. The suppressive effect of MG262 on the invasive activity was stronger than that of epoxomicin. The decrease in invasive activity by these proteasome inhibitors is at least partly mediated by the suppression of uPA production.

Key words : uPA, epoxomicin, MG262, NF-κB, oral squamous carcinoma cells

Introduction

Degradation of extracellular matrix around tumor cells is a crucial process in tumor invasion. Several types of proteases produced by tumor cells play an important role in this process. Regulation of the production and activity of those proteases is considered to have much influence on tumor invasion.

Urokinase-type plasminogen activator (uPA)[1-3] is a serine protease that is considered to play a key role in tissue degradation and cell migration under physiological and pathological conditions, including tumor invasion and metastasis. It catalyzes the conversion of inactive zymogen plasminogen to its active form plasmin. Plasmin can directly promote tumor invasion by cleaving matrix proteins such as laminin, type-IV collagen, and fibronectin or indirectly by activating several types of pro-matrix metalloproteinases and pro-

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uPA. uPA binds to its cell-surface receptor (uPAR), activating plasminogen much more efficiently than the fluid-phase enzyme[4]. The enzymatic activity of uPA is counter-balanced by plasminogen activator inhibitor (PAI)-1 and PAI-2, among which PAI-1 is thought to be the major physiologic inhibitor of uPA[5]. Increased expression of uPA has been reported in various malignancies including prostate[6,7], breast[8], colon[9], and lung[10] cancers. Generally, increased expression of uPA seems to be associated with augmented invasive-metastatic potential and poor prognosis[11~13].

Degradation of intracellular proteins mediated by ubiquitin-proteasome system is important in regulation of cellular function. Several key regulatory proteins involved in cell proliferation and differentiation are regulated by proteasome-mediated proteolysis, resulting in the activation or inhibition of specific cell signaling pathways. In recent years proteasome inhibitors have emerged as promising anticancer agents[14~16]. A boronate proteasome inhibitor, velcade (also known as PS-341/bortezomib)[16~19] has been shown to inhibit the proliferation and/or induce apoptosis in various tumor cells.

uPA gene expression has been shown to be regulated by nuclear factor (NF)-κB signaling pathways[20, 21]. NF-κB is a transcription factor that regulates the transcription of various genes related to inflammation, immune response, and tumorigenesis[22,23]. Because ubiquitin-proteasome system[24,25] is involved in the activation of NF-κB, modulation of proteasome function is considered to affect the NF-κB activity.

In the present study, we examined the effects of a epoxyketone proteasome inhibitor, epoxomicin[26] and a boronate proteasome inhibitor, MG262[27] on uPA mRNA expression and uPA production by HSC-3 cells[28], a human oral squamous carcinoma cell line. The effects of these proteasome inhibitors on constitutive NF-κB activity and invasive activity of HSC-3 cells were also examined.

Materials and Methods

1. Chemicals

Epoxomicin was purchased from Alexis (San Diego, CA, USA) and MG262 was from Biomol Research Laboratories (Plymouth Meeting, PA, USA). These reagents were dissolved in 100% ethanol before use. [α-^{32}P]dCTP (specific radioactivity 110 TBq/mmol) was obtained from Tokyo Biomedicals, Inc. (Tokyo, Japan).

2. Cell culture

HSC-3 cells were provided by Dr. S. Ohida (Tsurumi University, Yokohama, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% newborn calf serum (Gibco-BRL, Grand Island, NY, USA). Cultures were incubated at 37℃ in a humidified atmosphere of 95% air/5% CO_2. For treatment of cells at 70% confluence, the culture medium was replaced with serum-free DMEM for 24 h, and then the cells were exposed to each test substance in fresh serum-free DMEM. Cells were grown in T75 flasks (Nalge Nunc International, Rochester, NY, USA) or 90 mm dishes (Nalge Nunc International) for RNA experiments, while cells were grown in 6-well plates (Nalge Nunc International) to measure uPA secretion by ELISA and in 60 mm dishes (Nalge Nunc International) for transient transfection.

3. RNA extraction and Northern blot analysis

The DNA fragment used as a human uPA cDNA probe for RNA analysis was generated by RT-PCR

using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) and two oligonucleotide primers (5'-ACATTCACTGGTGCAACTGC-3' and 5'-CAAGCGTGTCAGCGCTGTAG-3'). The resulting DNA fragment (672 bp) was cloned into pSTBlue-1 vector (Novagen, Madison, WI, USA), and its identity was verified by dideoxy sequencing. The recombinant plasmid was digested with *Bam*H I and *Sal* I to isolate the uPA cDNA.

Total RNA was extracted from HSC-3 cells by the guanidinium thiocyanate-phenol-chloroform method[29]. Then the RNA (20 μg) was electrophoresed on 1.2% agarose-2.2% formaldehyde gel and was transferred to a nylon membrane (Hybond N^+; Amersham Biosciences, Buckinghamshire, UK). Blots were hybridized with the human uPA cDNA probe, which was labeled with ^{32}P by the random primer method using [α-^{32}P]dCTP and the Megaprime DNA labeling kit (Amersham Biosciences). Hybridization was performed at 42℃ for 48 h in 50% formamide, 5×Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS) and 5×standard saline phosphate ethylenediaminetetraacetic acid (SSPE). Then the membranes were washed with 2×SSPE-0.1% SDS and 0.2×SSPE-0.1% SDS at 65℃, and were exposed to a Kodak K-Screen (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The screens were scanned with a Molecular Imager FX (Bio-Rad Laboratories, Inc.) and the resulting images were analyzed using Quantity One 4.1.1 software (Bio-Rad Laboratories, Inc.). Blots were rehybridized with β-actin cDNA labeled with ^{32}P to provide a reference signal for normalization.

4. Measurement of uPA concentration

uPA secretion into the culture medium by HSC-3 cells was measured by ELISA using Zymutest uPA Antigen (Hyphen Biomed, Andresy, France). A 100 μL of culture medium was two fold diluted with F-Sample Diluent provided in the kit, and then two-site immunoassay was performed according to the manufacturer's recommendations. This ELISA has homogeneous reactivity to the various forms of uPA including single-chain uPA and high molecular weight uPA (receptor-bound uPA and uPA comlexed with PAI-1, 2).

5. Transient transfection and luciferase assay

HSC-3 cells were transiently transfected with 5 μg of the luciferase reporter plasmid (pNF κ B(2)-Luc ; Panomics, Inc., Redwood City, CA, USA) and 0.2 μg of the *Renilla* luciferase control plasmid (pRL-TK ; Toyo Ink Mfg. Co., Ltd, Tokyo, Japan) using Gene Porter 2 tansfection reagent (Gene Therapy Systems, SanDiego, CA, USA). After transfection, cells were incubated with test substances in fresh serum-free DMEM for 48 h. Cell lysates were prepared, after which *Firefly* and *Renilla* luciferase activities were measured by using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). *Firefly* luciferase activity was normalized for the corresponding *Renilla* luciferase activity.

6. Invasion assay

Invasion assay was performed by using a CytoSelect 24-Well Cell Invasion Assay (Laminin, Colorimetric Format) (Cell Biolabs, Inc., San Diego, CA, USA). The chamber of the assay system possessed polycarbonate membrane inserts (8 μm pore size), the upper surface of which had been coated with a uniform layer of dried murine laminin I matrix. Suspensions of HSC-3 cells were prepared in serum-free DMEM supplemented with test substances. The cell suspension (1×10^5 cells) was transfused to each insert of the chamber of the cell invasion assay system and incubated for

16 h. After removal of non-invasive cells, invaded cells were stained. Dye bound to the cells was extracted and quantified colorimetrically.

7. Statistical analysis

Data are presented as the mean±S.D. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test with Statview 5.0 software (Abacus, Berkeley, CA, USA). A P value of less than 0.05 was considered statistically significant.

Results

1. Effects of epoxomicin and MG262 on uPA mRNA expression and uPA production.

Effects of epoxomicin and MG262 on uPA mRNA expression by HSC-3 cells were examined by Northern blot analysis. HSC-3 cells were found to express uPA mRNA (2.3 kb) constitutively. As shown in Fig. 1, epoxomicin and MG262 down-regulated uPA mRNA expression in a dose-dependent manner.

In order to determine whether these proteasome inhibitors not only decreased uPA mRNA expression but also uPA secretion by HSC-3 cells, uPA concentration in the conditioned medium was measured by ELISA. As shown in Fig. 2, epoxomicin and MG262 decreased uPA secretion in a dose-depemdent manner. Marked suppression was observed at higher concentrations (50 and 100 nM) of epoxomicin and MG262, with the uPA secretion declining to less than 20% of that in vehicle-treated control cells.

Time courses of the effects of epoxomicin and MG262 on uPA mRNA expression was also determined by using HSC-3 cells treated with 50 nM epoxomicin or 50 nM MG262 (Fig. 3). A

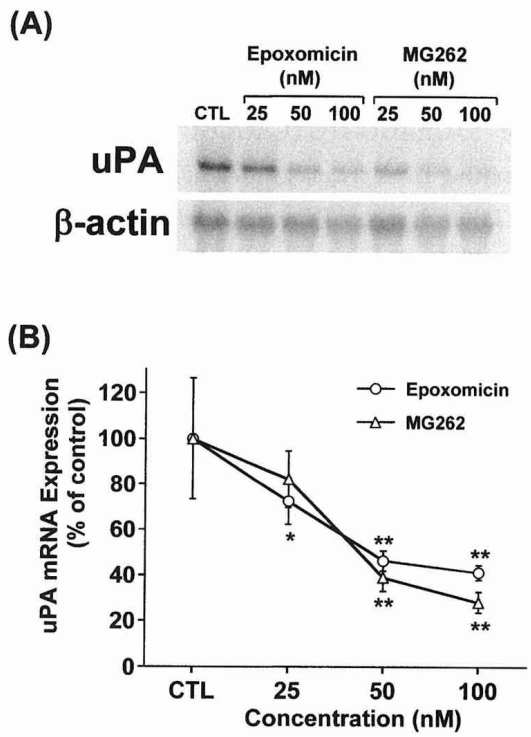

Fig. 1. Dose-response effects of epoxomicin and MG262 on uPA mRNA expression by HSC-3 cells.
HSC-3 cells were incubated with various concentrations of epoxomicin or MG262 in serum-free DMEM for 24 h. (A) Total RNA (20 μg) was subjected to Northern blot analysis for uPA and β-actin mRNAs. (B) uPA mRNA was quantified by densitometric analysis of the Northern blots, and its level was normalized for that of β-actin mRNA. Data are expressed as the mean±S.D. of triplicate determinations. *: $p<0.05$ and **: $p<0.01$ compared with vehicle control (CTL).

marked suppression of uPA mRNA expression was observed as early as 6 h after the initiation of treatment with epoxomicin (50 nM) or MG262 (50 nM), and the suppressive effects were similar or a little stronger at 12 and 24 h.

2. Effects of epoxomicin and MG262 on transcriptional activity of the NF-κB-dependent promoter.

Transcription of uPA gene is known to depend

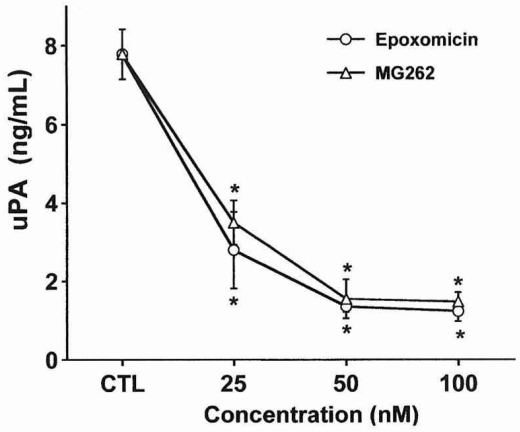

Fig. 2. Effects of epoxomicin and MG262 on uPA secretion by HSC-3 cells.
HSC-3 cells were incubated with various concentrations of epoxomicin or MG262 in serum-free DMEM for 24 h. Then the immunoreactive uPA concentration in the medium was measured by ELISA. Data are expressed as the mean±S.D. of triplicate determinations. *: $p<0.01$ compared with vehicle control (CTL).

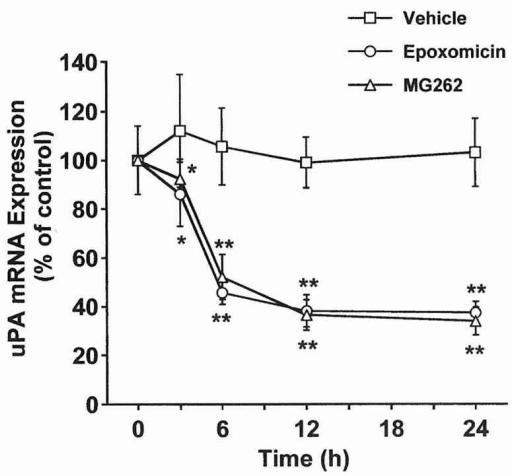

Fig. 3. Time course of the effects of epoxomicin and MG262 on uPA mRNA expression by HSC-3 cells.
HSC-3 cells were incubated with vehicle alone, epoxomicin (50 nM) or MG262 (50 nM) in serum-free DMEM for the indicated times. Total RNA was subjected to Northern blot analysis. uPA mRNA was quantified by densitometric analysis of Northern blots, and its level was normalized for that of β-actin mRNA. Data are expressed as the mean±S.D. of triplicate determinations. * : $p<0.05$ and **: $p<0.01$ compared with vehicle control at each time point.

on NF-κB activity[20,21]. Therefore, effects of epoxomicin and MG262 on NF-κB activity were examined to elucidate the mechanism by which these proteasome inhibitors down-regulated uPA gene expression. A luciferase reporter plasmid containing 6 repeats of the NF-κB binding site upstream of a TATA box promoter was transiently transfected into HSC-3 cells. As shown in Fig. 4, transcriptional activity of the NF-κB-dependent promoter was significantly decreased by these proteasome inhibitors, and suppressive effect of MG262 on the transcriptional activity appeared to be relatively greater compared with that of epoxomicin. Consequently, the suppression of uPA gene expression by these proteasome inhibitors is conceivably mediated by inhibition of constitutive NF-κB activity.

3. Effects of epoxomicin and MG262 on invasive activity

It is known that there is a strong correlation between uPA expression and invasive potential in certain malignacies[3,30].

Because HSC-3 cells are known to exhibit highly invasive property[31,32], they are considered suitable for the study on regulation of invasion.

To assess the effects of epoxomicin and MG262 on invasive activity, HSC-3 cells were subjected to invasion assay by using the assay chamber equipped with inserts bearing laminin matrix layer. As shown in Fig. 5, significant decreases in invasive activity were observed in the cells treated with epoxomicin (100 nM) or MG262 (50

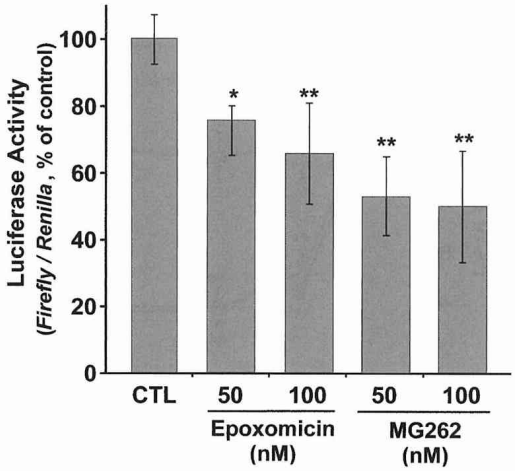

Fig. 4. Effects of epoxomicin and MG262 on transcriptional activity of the NF-κB-dependent promoter in HSC-3 cells.
HSC-3 cells were transiently transfected with 5 μg of luciferase reporter plasmid that contains NF-κB-dependent promoter (NFκB(2)-Luc) and 0.2 μg of *Renilla* luciferase control plasmid (pRL-TK). After transfection, cells were incubated with indicated concentrations of epoxomicin or MG262 in serum-free DMEM for 48 h. Cell lysates were prepared and both *Firefly* and *Renilla* luciferase activity were measured. *Firefly* luciferase activity was normalized for the corresponding *Renilla* luciferase activity. Data are expressed as the mean±S.D. of triplicate determinations. *: $p<0.05$ and **: $p<0.01$ compared with vehicle control (CTL).

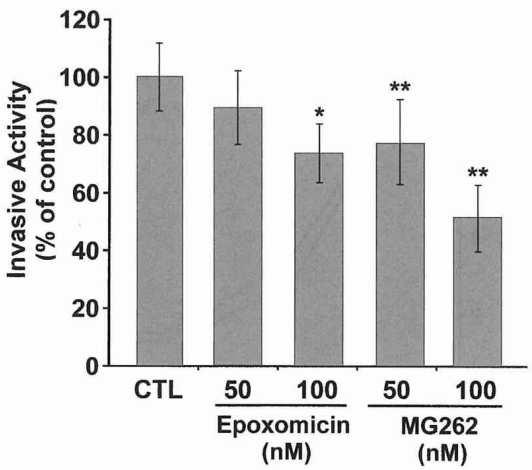

Fig. 5. Effects of epoxomicin and MG262 on invasive activity of HSC-3 cells.
Suspensions of HSC-3 cells were prepared in serum-free DMEM supplemented with indicated concentrations of epoxomicin or MG262. The cell suspension (1×10^5 cells) was added to each insert of the chamber of laminin cell invasion assay system and incubated for 16 h. After removal of non-invasive cells, invaded cells were stained. Dye bound to the cells was extracted and quantified colorimetrically. Data are expressed as the mean±S.D. of quadruplicate determinations. *: $p<0.05$ and **: $p<0.01$ compared with vehicle control (CTL).

or 100 nM). 100 nM MG262 reduced the invasive activity by 48% compared with vehicle control.

Discussion

There has been growing evidence that uPA plays crucial role in tumor invasion and metastasis[3,30]. Plasminogen activasion system governed by uPA appears to be one of the attractive target for cancer treatment. In this study, we demonstrated that both epoxomicin and MG262 suppressed uPA mRNA expression and uPA production by HSC-3 cells (Fig. 1～3). To our knowledge, this is the first report on the regulation of uPA gene expression by proteasome inhibitors. Amiloride[33], dexamethasone[34], *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone[20], and oxamflatin[35] has been reported to down-regulate uPA gene expression.

uPA gene expression has been shown to be regulated by NF-κB signaling pathways[20,21]. It has been found that NF-κB binding sites are located in uPA promoter region[20,36]. NF-κB is a transcription factor that regulates the transcription of various genes related to inflammation, immune response, and tumorigenesis[22,23]. NF-κB is a member of the Rel family of proteins and is typically a heterodimer

composed of p50 and p65 (RelA) subunits. Activation of NF-κB is regulated by inhibitor protein termed IκB. In quiescent cells, NF-κB resides in the cytosol in latent form, bound to IκB. In response to various stimuli, IκB is phosphorylated by IκB kinase. The phosphorylated IκB is ubiquitinated and then undergoes degradation by proteasome[24,25]. Subsequently, NF-κB translocates into the nucleus and binds to a specific DNA sequence (NF-κB binbing site) in the promoter of each target gene leading to stimulation of its transcription. Several studies have shown that certain proteasome inhibitors can prevent the degradation of IκB resulting in inhibition of NF-κB activation[25,37,38]. Velcade, a potent proteasome inhibitor, has been shown to exert antitumor effects such as antiproliferative effect, proapoptotic effect, and antiangiogenic effect along with inhibitory effect on NF-κB activation[16~19].

We demonstrated that epoxomicin and MG262 reduced the constitutive NF-κB activity of HSC-3 cells (Fig. 4). Consequently, the suppression of uPA gene expression by these proteasome inhibitors is conceivably mediated by inhibition of constitutive NF-κB activity. The inhibitory effect of MG262 on constitutive NF-κB activation was stronger compared with that of epoxomicin (Fig. 4). Though both of epoxomicin[26] and MG262[27] are known to be potent proteasome inhibitors, they are classified into distinct types of compound (epoxomicin : an epoxyketone derivative ; MG262 : a boronic acid derivative). The stability in cytoplasm and the mode of interaction with other intracellular molecules than proteasome complex might be different among the two proteasome inhibitors. MG262 might inhibit proteasome function more intensively than epoxomicin in HSC-3 cells.

The correlation between uPA expression and tumor cell invasion has been studied extensively[3,30]. Modulation of uPA production is assumed to affect the invasive activity of tumor cells. Invasive activity of HSC-3 cells was decreased by epoxomicin and MG262 (Fig. 5). The decrease in invasive activity by these proteasome inhibitors is conceivably mediated by the suppression of uPA production at least partly.

100 nM epoxomicin and 100 nM MG262 suppressed the invasive activity of HSC-3 cells more potently relative to 50 nM of each reagent (Fig. 5), in spite of little difference in the uPA secretion level between treatment with 50 nM and 100 nM of each proteasome inhibitor (Fig. 2). The reason for this apparent discrepancy remains unknown. It is possible to speculate that only at 100 nM these proteasome inhibitors might also down-regulate the expression of some other proteases that could induce extracellular matrix degradation.

Inhibition of the constitutive NF-κB activity can induce the suppression of the expression of the proteases related to extracellular matrix degradation leading to tumor cell invasion including uPA. The suppressive effect of MG262 on the invasive activity was moderately stronger relative to that of epoxomicin (Fig. 5). This result appears to reflect the stronger inhibitory effect of MG262 on the constitutive NF-κB activity.

Application of proteasome inhibitors may become a new strategy for preventing the plasmin-promoted invasion and metastasis of cancer.

Conclusion

Epoxomicin and MG262 suppressed uPA mRNA expression and uPA production by HSC-3 cells.

The suppression of uPA gene expression by these proteasome inhibitors is conceivably mediated by inhibition of constitutive NF-κB activity. Invasive activity of HSC-3 cells was also decreased by epoxomicin and MG262.

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