［原著］HPV E6 is an H_2O_2 responsive molecule associated with IL-8 production

HPV E6 is an H_2O_2 responsive molecule associated with IL-8 production

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ABSTRACT

Angiogenesis induced by inflammation occurs in infected sites and is crucial for the initiation of cancer progression. In cervical cancer, IL-8 (Interleukin-8) production is linked to cancer progression. At inflamed sites, neutrophils deploy a potent antimicrobial arsenal, including ROS. Here, we show that a ROS (H_2O_2) activates p38 MAPK to increase the expression of IL-8 in HeLa cells (HPV18-positive cells). We found that knockdown of HPV (Human Papillomavirus) E6 inhibited H_2O_2- and MG132-induced IL-8 production in HeLa cells. These findings elucidate an interesting signaling pathway by which inflammation can induce angiogenesis to cause cervical cancer progression. *Ryukyu Med. J., 29(3,4)23～30, 2010*

Key words: HPV, H_2O_2, IL-8

INTRODUCTION

Cancer progression is enhanced by angiogenesis factors, such as IL-8[1-4)]. HPV, the major risk factor for cervical cancer, is responsible for inducing angiogenesis[5-11)]. The E6 and E7 proteins inactivate and inhibit the expression of the p53 and RB tumor suppressor proteins, respectively. The expression of human papillomavirus type 16 E6 oncoproteins in primary foreskin keratinocytes is sufficient to alter the expression of angiogenic factor IL-8[5)]. Inflammation, another risk factor for cancer, is also responsible for angiogenesis by altering cytokine expression[12-14)]. At inflamed sites, neutrophils deploy a potent antimicrobial arsenal, including ROS. The three main ROS are superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radicals. Although ROS are extremely antimicrobial by virtue of their ability to kill microbial pathogens, in chronic inflammation, the continued production of ROS by neutrophils contributes to the expression of a variety of different inflammatory cytokines[15-18)].

The ubiquitin-proteasome pathway is inactivated by diverse stimuli, such as H_2O_2, alcohol and solar ultraviolet (UV) -A radiation. Proteasome inhibition participates in multiple cellular processes such as chemokine production and apoptosis[19,20,21)]. The critical role of proteasome inhibition in chemokine expression after H_2O_2 exposure has been recently demonstrated using proteasome inhibitor[22-24)]. Furthermore, we recently reported that H_2O_2 induced IL-8 production in HeLa cells (HPV18-positive squamous cell carcinoma of the cervix). However, the mechanism by which H_2O_2 mediates IL-8 production in HeLa cells remains to be determined[18)].

The mitogen-activated protein kinase (MAPK) p38 has been shown to be activated by oxidative stress, UV light radiation and proteasome inhibition[15,22,25)]. p38 MAPK is also implicated in the signaling pathway for IL-8 production[15)].

In investigating the mechanism by which H_2O_2 induces IL-8 production in HPV-positive cells, we have uncovered a signaling pathway, namely, one involving inhibition of proteasome activity and

activation of p38 MAPK, leading to a pathway involving HPV E6, which induces IL-8 production in HeLa cells.

MATERIALS and METHODS

Reagents

SB203580 (p38 MAPK inhibitor) and MG-132 were purchased from Calbiochem (La Jolla, CA, USA). SB203580 was dissolved in dimethyl sulfoxide (DMSO; Dako) to obtain a stock solution. The DMSO concentration was kept below 0.04% in all the cell cultures so that it had no detectable effect on cell growth or cell death.

Cell culture and treatment

The human cervical cancer cell line HeLa was routinely cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Sigma), 30units/ml penicillin and 30 μg/ml streptomycin at 37℃ under 5% CO_2. Serum starvation was achieved by incubation in DMEM medium containing 0.5% fetal bovine serum for at least 16 h prior to the direct addition of H_2O_2 to this culture medium. SB203580 (10 μM) was added to the culture medium 45 min before the direct addition of H_2O_2 (Wako).

SDS-PAGE and western blotting

Cell cultures (80-100% confluent) were treated, washed twice with ice-cold phosphate buffered saline and lysed in buffer containing 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche). Cells were lysed in lysis buffer by incubating for 20 min at 4℃. Following centrifugation at 15,000 rpm for 10 min, the pellet was suspended in 3×Laemmli sample buffer. Protein samples (20-40 μg) were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% resolving gel). Proteins were transferred electrophoretically onto nitrocellulose membranes (Pall Corporation), which were subsequently incubated for 2 h at room temperature with blocking buffer that contained 5% nonfat dry milk in TBS-T (20 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20). They were washed with TBS-T (0.1% Tween-20) and then incubated overnight at 4℃ in TBS-T buffer containing 5% BSA (Wako) with appropriate antibodies. The antibodies used in western blot assays are shown in Table 1.

The protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (GE Healthcare Bio-Sciences). The signal from the membrane was detected and imaged using a LAS-4000 imager (Fujifilm Corporation). The membranes were then stripped (30 min at 56℃) in stripping buffer containing 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl pH 6.7 and reprobed with β-actin and p38 as loading control. All western blots were performed at least three times for each experiment.

Real-time RT-PCR

Total RNAs were extracted from the harvested cells using Isogen (Nippon Gene). After removal of genomic DNA, approximately 1 μg of RNA was used to generate cDNA using Reverse Transcriptase M-MLV (RNase H) (TaKaRa) and random hexamers (Fermentas). Real-time PCR was performed with SYBR Green PCR master mixes (Applied Biosystem) on a 7000 real-time PCR system. Relative quantification was performed in a multiplex reaction using U1AsnRNP1

Table 1 **Antibodies used in western blot assays and their titers**

Primary antibody and titer	Corporation and batch no.	Secondary antibody and titer	Target protein and batch no.
Rabbit phospho-p38 MAP kinase antibody (1:500)	CellSignaling, 9211	Donkey Anti Rabbit IgG/HRP (1:5000)	43 kD, NA934
Rabbit p38 MAP kinase antibody (1:500)	Cell Signaling,9212	Donkey Anti Rabbit IgG/HRP (1:5000)	43 kD, NA934
Mouse p53 antibody (1:500)	Dako, M7001	Goat Anti Mouse IgG/HRP (1:5000)	53 kD, AP124P
Mouse β-actin antibody (1:50000)	SIGMA, A1978	Goat Anti Mouse IgG/HRP (1:5000)	42 kD, AP124P N.S

as an endogenous control. The PCR primers are listed in Table 2.

Table 2 **Primers of selected genes**

Gene name	Primers	
	Sense	Antisense
IL-8	ACTCCAAACCTTTCCACCC	AAACTTCTCCACAACCCTCTG
U1AsnRNP1	CAACGACAGCCGAGACATGTA	AGCCTCCATCAAATACCCATTC

Preparation of small interfering RNAs and transfection

Synthesized siRNA duplexes were obtained from Invitrogen. The siRNA sequences targeting HPV18 E6 corresponded to nucleotides (5′-CUA ACUAACACUGGGUUAUTT-3′, siRNA1-HPV18 E6) and (5′-AUAACCCAGUGUUAGUUAGTT-3′, siRNA2-HPV18 E6) of the coding region[26]. The sequences for the negative control (siRNA-scramble) were 5′-CCAUUCCGAUCCUGAUCCG TT-3′ and 5'-CGGAUCAGGAUCGGAAUGGTT-3'. Cells in exponential growth phase were plated in a 35 mm dish containing antibiotic-free medium at 30% confluence and were transfected with 20nM siRNA-HPV18 E6, siRNA-scramble and then transfected using Oligofectamine (Invitrogen) and Opti-MEM® I (Invitrogen), according to the manufacturer's protocol. Silencing was examined 24 h after transfection.

Determination of human IL-8 concentration

We determined concentrations of human IL-8 from HeLa cells using an ELISA kit (Bio Legend) according to the manufacturer's instructions.

Statistical analyses

All data are expressed as means ± S.D. We accumulated data for each condition from at least three independent experiments. We evaluated the significance using Student's t-test for comparisons between two mean values. We carried out multiple comparisons between more than three groups with ANOVA followed by the Tukey-Kramer test.

RESULTS

Proteasome inhibition induces IL-8 mRNA expression and p38 MAPK activation

To identify the mechanism by which H_2O_2 induces IL-8 mRNA expression in HeLa cells, we studied the effects of proteasome inhibition on IL-8 production in HeLa cells. Proteasome inhibition is regarded as an important event that leads to IL-8 production after H_2O_2 exposure in retinal epithelial cells[22,23]. MG132, a proteasome inhibitor, induces IL-8 mRNA expression in HeLa cells (Fig. 1A). Because p38 MAPK activation induced by proteasome inhibition is reported to cause IL-8 production[22,23], we evaluated whether p38 MAPK is activated by proteasome inhibition in HeLa cells. After treatment with MG132, the levels of phospho-p38 of HeLa cells increased but the level of p38 expression remained unchanged (Fig. 1B). These results suggest that p38-MAPK activation is a target for proteasome inhibition in HeLa cells.

p38 MAPK is a target for H_2O_2

Activation of p38 MAPK has been reported to be involved in IL-8 production induced by proteasome inhibition[22,23] ; therefore, we explored whether p38 MAPK is involved in H_2O_2-induced IL-8 signaling. We examined p38-MAPK activation by H_2O_2 over different time courses. H_2O_2 induced p38 MAPK phosphorylation in HeLa cells (Fig. 2A). The p38-MAPK inhibitor SB203580

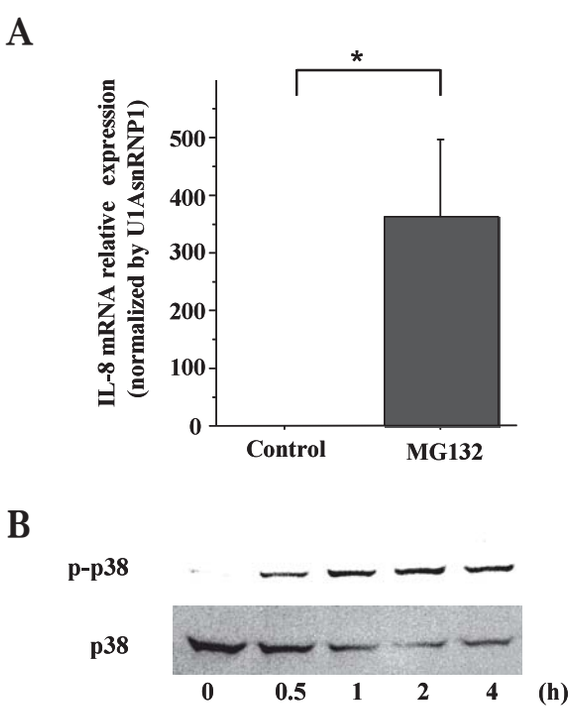


Fig. 1 **MG-132-induced expression of IL-8 in HeLa cells.** (A) Expression of IL-8 mRNA induced by treatment with 10 μM MG-132 for 6 h. (B) Time courses of phosphorylation of p38 induced by incubation with 10 μM MG-132. Results are shown as means ± S.D. of three independent experiments. *P < 0.05.

nearly abolished H_2O_2-induced IL8 expression (Fig. 2B). These findings suggest that p38-MAPK activation contributes to H_2O_2-evoked IL8 expression in HeLa cells.

HPV18 E6 mediates H_2O_2- and MG132-induced IL-8 production

Because the expression of HPV oncoproteins has been reported to increase IL-8 expression[5], we next explored the hypothesis that an HPV-encoded protein mediates H_2O_2- and MG132-induced IL-8 production. Only two HPV-encoded proteins, E6 and E7, are expressed in HPV-positive squamous cell carcinomas. To investigate the effects of E6 protein on H_2O_2- and MG132-induced IL-8 production, we performed RNA interference (RNAi) with small interfering RNAs (siRNAs) to silence the expression of the E6 proteins in HeLa cells. E6 protein cannot be detected by direct immunoblotting, so p53 expression serves as a surrogate for E6 expression. E6 siRNA selectively induced p53 expression in HeLa cells (Fig. 3A). Knockdown of HPV E6 substantially inhibited the IL-8 mRNA expression after H_2O_2 and MG132 treatment, whereas control siRNA did not affect IL-8 mRNA expression and secretion (Fig. 3B, C, D). These results suggest that HPV E6 protein is an important regulator of IL-8 production in HeLa cells.

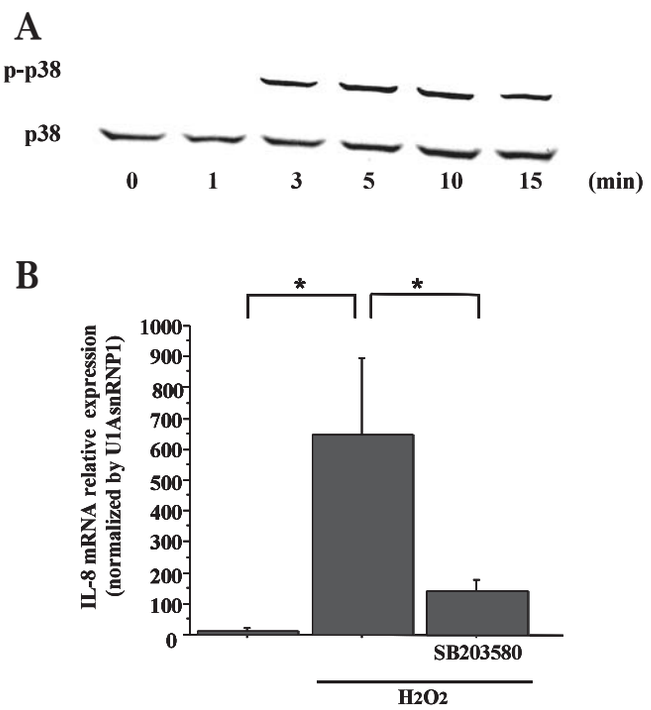
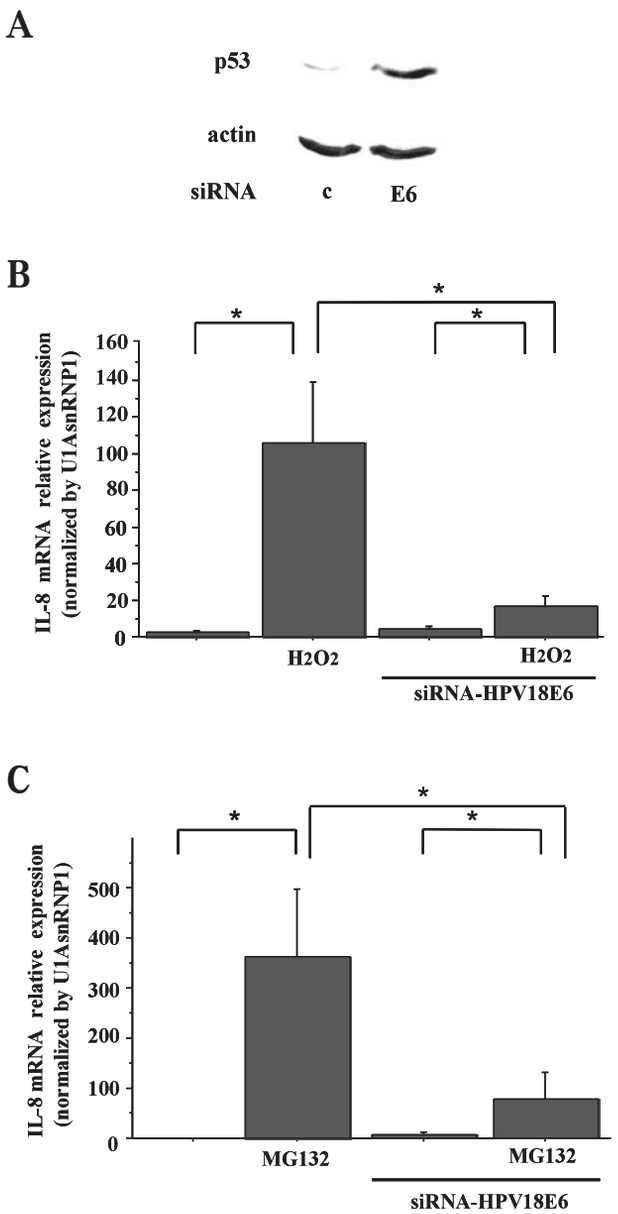


Fig. 2 **p38 MAPK is a downstream target by which H_2O_2 causes IL-8 production.** (A) Time courses of phosphorylation of p38 induced by incubation with 250 μM H_2O_2. (B) Inhibitory effects of p38 MAPK inhibitor SB203580 on H_2O_2 (250 μM) -induced expression of IL-8 mRNA. Results are shown as means ± S.D. of three independent experiments. *P < 0.05.



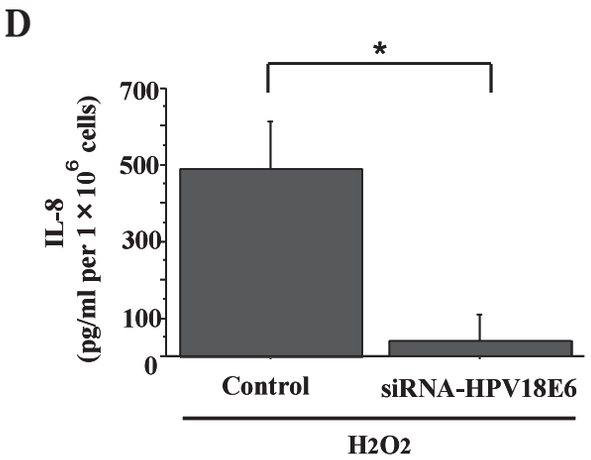


Fig. 3 **HPV18 E6 mediates H_2O_2- and MG132-induced IL-8 production.** (A) Western blots for p53 (a surrogate for E6 expression). Cells were transfected with the HPV18 E6 siRNA, and immunoblotting was performed with the antibody for p53 after 24 h. C, Scrambled control siRNA. (B) Effects of siRNA for HPV18 E6 on H_2O_2-induced IL-8 expression. (C) Effects of siRNA for HPV18 E6 on MG132-induced IL-8 production. (D) ELISA showing the effects of HPV18 E6-siRNA on IL-8 secretion after H_2O_2 exposure. Results are shown as means ± S.D. of three independent experiments. *P < 0.05.

DISCUSSION

H_2O_2 has previously been reported to inhibit proteasome in epithelial cells[22,23].We have demonstrated in the present study, as shown schematically in Fig. 4, that MG-132 and H_2O_2 caused expression of IL-8, the candidate of various cancer biomarker[27]. These findings suggest a pivotal role for proteasome inhibition in H_2O_2-induced IL-8 production in HeLa cells.

At inflamed sites, multiple cells including neutrophils, macrophages and epithelial cells express NADPH oxidase, which generates ROS [28,29]. Cervical cancer progression is probably mediated by neutrophils that have infiltrated into cervical epithelium. Neutrophils produce IL-8 which represents the most potent specific neutrophil chemoattractant and angiogenesis factor[3]. The degree of elevation of IL-8 abundance is correlated with both cancer progression and increased levels of infiltration of neutrophils in cervical epithelium[3]. Notably, 250 M H_2O_2 did not induce cell death in HeLa cells. Hence, we hypothesize that moderate ROS exposure may be a key signal for IL-8 production and initiation of cancer progression, whereas excessive ROS production may induce severe tissue damage and cell death.

Because the proteasome is involved in the regulation of gene expression and because it can be inactivated by H_2O_2 exposure[22,23,30-37], our results suggest that a specific target of H_2O_2 signaling leading to IL-8 production in HeLa cells might be proteasome inhibition. Our results are consistent with previous studies that showed that H_2O_2 inactivates the proteasome and induces IL-8 production in retinal pigment epithelial cells[22,23].

Our results show that H_2O_2-induced activation of p38 MAPK can cause IL-8 production. IL-8 expression is regulated by many signaling pathways and transcription factors, such as p38 MAPK[38,39], PI3K[40,41], nuclear factor-κB, activator protein (AP) -1[42,43]. H_2O_2 activated AP-1 but not nuclear factor-κB in A549 epithelial cells[46]. Moreover, it has been reported that the analysis of transcription factor activation revealed induction of activator protein-1 (c-Jun) activity by proteasome inhibition, whereas nuclear factor-κB (p50 and p65) was not activated[44,45]. These previous reports and our data provide a model (Fig. 4) in which H_2O_2 induces the AP-1 pathway, which can cause IL-8 production in HeLa cells (Fig. 4). Silencing of HPV E6 protein did not inhibit the activation of p38 MAPK (data not shown), indicating that HPV E6 acts downstream of pathways mediated by p38 MAPK.

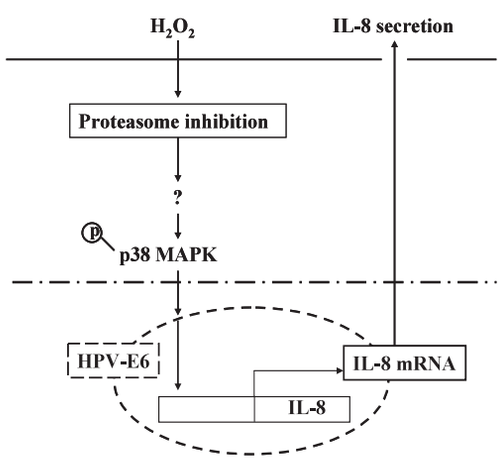


Fig. 4 **Proposed model of the newly identified IL-8 production pathway in the regulation of proteasome inhibition.** The model illustrates H_2O_2 induces IL-8 production and p38 MAPK activation through HPV18E6. HPV18E6 is involved in the downstream event of MAPK activation.

To the best of our knowledge, we have identified HPV E6 for the first time as a molecule involved downstream of H_2O_2 and proteasome inhibition, which increases IL-8 production. In conclusion, our findings on important functions of HPV E6 in IL-8 production may provide a platform for the development of therapeutic strategies against various cervical cancers with angiogenesis.

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