Down modulation of IL-18 expression by human papillomavirus type 16 E6 oncogene via binding to IL-18

Young-Sik Cho[a], Jeong-Woo Kang[a], MinChul Cho[a], Cheong-Weon Cho[a], ShinJe Lee[a], Yong-Kyung Choe[a], YongMan Kim[a], InPyo Choi[a], Sue-Nie Park[b], SooHyun Kim[c], Charles A. Dinarello[c], Do-Young Yoon[a,*]

[a] *Korea Research Institute of Bioscience and Biotechnology, Taejon 305-600, South Korea*
[b] *Korea Food and Drug Administration, Seoul 122-704, South Korea*
[c] *University of Colorado Health Sciences Center, Denver, CO 80262, USA*

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Abstract To understand modulation of a novel immune-related cytokine, interleukin-18, by human papillomavirus type (HPV) 16 oncogenes, HaCaT, normal keratinocyte cell line, and C-33A, HPV-negative cervical cancer cell line, were prepared to establish stable cell lines expressing E6, E6 mutant (E6m), E6E7, or E7 constitutively. Expressions of various HPV oncogene transcripts were identified by RT-PCR. Expression of HPV oncogene E6 was reversely correlated to the expression of interleukin-18, a novel pro-inflammatory cytokine. The expression of E6 in C-33A, independent of E6 splicing, resulted in decreased IL-18 expression and that of IL-18 was also significantly reduced in HaCaT cells expressing E6. The level of p53 was reduced in C-33A cells expressing E6 whereas not altered in HaCaT cells expressing E6, suggesting that E6 downregulated IL-18 expression via an independent pathway of p53 degradation in HaCaT cells which have a mutated p53 form. However, E7 did not affect IL-18 expression significantly in both C-33A and HaCaT cells. Cotransfection experiments showed that E6 oncogene did not inhibit the activities of IL-18 promoter P1 and P2, suggesting that E6 oncogene indirectly inhibited IL-18 expression. Taken together, E6, E6m and E6/E7 inhibited IL-18 expression with some variation, assuming that cells expressing E6 oncogene can evade immune surveillance by downregulating the expression of immune stimulating cytokine gene, IL-18, and inhibiting the cascade of downstream effects that follow activation of the IL-18 receptor.

Key words: Human papillomavirus E6 oncogene; Interleukin-18; Cervical cancer; Interferon γ

1. Introduction

Cervical cancer is one of the leading causes of female death from cancer worldwide with about 500 000 deaths per year [1]. A strong association between certain human papillomaviruses (HPVs) (HPV types 16 and 18) and cervical cancer has already been established [2]. HPVs have circular, double-stranded DNA genomes that are approximately 8 kb in size and encode eight genes. Two viral proteins, E6 and E7, are selectively retained and expressed in carcinoma cells and co-operated in immortalization of primary keratinocytes [3]. E6 binds to p53, promoting its degradation via ubiquitin mediated proteolysis [4] and increasing genetic instability [5] by over-riding cell cycle checkpoints for DNA repair. E7 binds to the retinoblastoma protein (Rb) [6], leading to dissociation of the E2F-1 transcription factor and activation of genes responsible for DNA synthesis and cell proliferation [7]. The host's inflammatory and cell mediated immune response serve important roles in determining whether HPV infections persist, regress or progress [8]. Keratinocytes contribute directly to the host response by secreting immunoregulatory cytokines such as interleukin (IL)-1, that recruit and activate leukocytes at the site of infection. Cervical carcinoma derived cell lines [9] and keratinocyte cell lines immortalized by HPV-16 demonstrate altered expression of several immunoregulatory cytokines, suggesting that HPV gene products perturb cytokine production or secretion [10–12]. Among them, levels of IL-6 and IL-8 are significantly higher in the cervicovaginal washing fluid from patients with cervical cancer than those in controls while the transcription of interferon γ (IFN-γ) gene is significantly reduced in both cervical intraepithelial neoplasia and cervical cancer tissue as compared to normal cervix [10]. Recently, IL-18 treatment protects mice against acute herpes simplex virus type 1 infection via both IFN-γ dependent and independent pathways [13]. However, natural infection is slow to produce an appropriate therapeutic immune response to these proteins, probably because HPV has adopted a strategy to prevent the effective presentation of viral antigens to the host immune system. Moreover, HPV infection is non-lytic so there is little release of viral antigen to professional antigen presenting cells [14]. An optimal therapeutic strategy for anogenital HPV infection would be, therefore, to accelerate the induction of a strong virus specific immune response by inducing local inflammation and the cytokines necessary to invoke HPV specific immunity.

IL-18, initially described as IFN-γ inducing factor (IGIF) [15], is a pro-inflammatory cytokine [16]. The cytokine IL-18 possesses pleiotropic biological properties such as activation of nuclear factor κB (NF-κB) [17], Fas ligand expression

*Corresponding author. Fax: (82)-42-860 4593.
E-mail address: dyyoon@mail.kribb.re.kr (Do-Young Yoon).

Abbreviations: IL, interleukin; HPV, human papillomavirus; E6m, E6 mutant; IFN-γ, interferon γ; RT-PCR, reverse transcription-polymerase chain reaction

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(FasL) [18], induction of both CC and CXC chemokines [19], enhancement of the production of IFN-γ and granulocyte macrophage-colony stimulating factor [20], and induction of $CD4^+$ cytolytic T cell response [21]. In addition, IL-18 enhances the expression of FasL on NK cells which attack specifically cells presenting Fas proteins on membrane but not control parental cells [22].

Recently, several proteins showing homologs to human and murine IL-18 binding protein (IL-18BP) were found from several viruses [23,24]. Molluscum contagiosum virus (MCV), a human poxvirus, causative agent of papular skin lesions, persists for a long period without signs of inflammation, similar to HPV infection. Interestingly, it has been reported that MCV has proteins with high affinity to human and murine IL-18 proteins and inhibited IL-18 mediated IFN-γ production [25].

Although there are many suggestions that IFN-γ and cell mediated immunity are important factors in determining the progression status of HPV related cervical lesion including cervical cancer [12,26,27], there is no direct evidence that high-risk HPV oncoprotein E6 can modulate the function of IL-18. In this experiment, to investigate in vitro whether the HPV type 16 E6 and E7 influence expression of IL-18, HaCaT and HPV-negative human cervical cancer cell line C-33A were stably transfected with E6, E6 mutant (E6m), E6/E7 or E7 gene. The downregulation of IL-18 molecule on protein and transcriptional levels was observed in transfectants with E6 gene but not in those with E7 gene. In addition, to investigate whether significant downregulation of IL-18 is dependent on direct binding by E6, the interaction between E6 and IL-18 was investigated in an in vitro binding assay and was also subjected to competition with p53 protein, which has been known to form a ternary complex with the E6 oncoprotein and the E6-associated protein (E6AP), leading to its degradation via the ubiquitin pathway. We also examined whether high-risk HPV 16 E6 oncoprotein could affect the IL-18 induced IFN-γ production in human PBMCs to elucidate the possible immune escape mechanisms of HPV infected cervical lesion including cervical cancer.

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal anti-human IL-18 antibody clone #18-8 (IgG_{2a}) was made and used as described [28]. Clone #18-8 recognizes both precursor and mature forms of IL-18. The following were purchased: anti-human IL-18R antibody, human IL-12p70 and hIL-18 (R&D system, Minneapolis, MN, USA), hIL-2 (BM, Mannheim, Germany), Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, amphotericin B and fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA), OptEIA human IFN-γ ELISA kit (PharMingen, San Diego, CA, USA), glutathione–Sepharose bead (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), Ni-nitrilotriacetic acid resin (Qiagen, West Sussex, UK), Immobilon-P membrane (Millipore, Bedford, MA, USA), goat polyclonal antibodies against C-19 and N-17 of HPV 16 E6 protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human p53 antibody (Ab-6, Oncogene, Cambridge, MA, USA), BCIP/NBT (Bio-Rad Laboratories, Hercules, CA, USA), Histopaque-1077, polymyxin B, RPMI 1640, phytohemagglutinin P (PHA), imidazole, phenylmethylsulfonyl fluoride (PMSF), aprotinin, FITC conjugated anti-mouse IgG, alkaline phosphatase conjugated anti-mouse IgG and horseradish peroxidase conjugated anti-mouse IgG (Sigma, St Louis, MO, USA). PCR reagents were from Promega (Madison, WI, USA) and Stratagene (La Jolla, CA, USA). IL-18BP was expressed and purified as described [29]. Other reagents were of analytical grade.

2.2. Cell lines

HPV-negative C-33A and HPV-positive cells such as SiHa, CaSki, and HeLa were purchased from American Type Culture Collection (Rockville, MD, USA). We also used a spontaneously immortalized epithelial cell line HaCaT as a control. The cell lines were maintained in DMEM supplemented with 100 U/ml penicillin–100 μg/ml streptomycin, and 10% heat-inactivated FBS at 37°C in a humidified incubator with 5% CO_2. SiHa and CaSki cell lines used in this experiment have been known to contain 1 or 2 and 60–600 copies of HPV 16 type, respectively [30,31]. HeLa cells have been reported to harbor 10–50 copies of HPV 18 type by which carcinoma is induced [32]. C-33A cells are cervical carcinoma derived from other causes than HPV infection [33]. Human PBMC used in these experiments were obtained from fresh whole blood of healthy volunteers after informed consent or from packed red cell purchased from Chung-Nam National Blood Bank (Taejon, South Korea). PBMC were isolated from buffy coat of heparinized whole blood or citrate–phosphate–dextrose treated packed red cells by centrifugation on a density gradient of Histopaque-1077, then washed three times with phosphate buffered saline (PBS). PBMC were suspended at a final concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 100 U/ml penicillin–100 μg/ml streptomycin, 25 ng/ml amphotericin B, 10% heat-inactivated FBS and 50 μM of 2-mercaptoethanol and cultured at 37°C in a humidified incubator with 5% CO_2.

2.3. Preparation of expression vectors for E6, E6m, E7, E6/E7

The CaSki cells were derived from a cervical carcinoma and contained integrated HPV 16 genomes. E6, E6/E7 and E7 were made by PCR amplification from total RNA isolated from CaSki cell lines using following primer pairs: 5′-GCG GCC GCC ACC ATG TTT CAG GAC CAC AG-3′ (sense) and 5′-CTG CGG CCG CGA TTA CAG CTG GGT TTT CTC T-3′ (antisense); 5′-GCG GCC GCC ACC ATG TTT CAG GAC CAC AG-3′ (sense) and 5′-AGG CGG CCG CGA TTA TGG TTT CTG AGA ACA-3′ (antisense); 5′-GCG GCC GCC ACC ATG GCA TGG CAT GGA GAT ACA CCT-3′ (sense) and 5′-AGG CGG CCG CGA TTA TGG TTT CTG AGA ACA-3′ (antisense). The PCR products were inserted into PCR®2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) or T vector, which had been prepared from pBluescript KS(+) (Invitrogen, Carlsbad, CA, USA) according to the method of Marchuk et al. [34] as an intermediate step for subcloning into corresponding expression vector. In brief, either E6 or E7 gene containing *Not*I was ligated into pOPI3 which was prepared by digesting pOPI3CAT™ (Stratagene, La Jolla, CA, USA) with *Not*I to remove CAT gene and dephosphorylated with calf intestinal alkaline phosphatase for preventing self-ligation. E6/E7 gene, which excised from PCR®2.1-TOPO vector by digestion with *Eco*RI, was ligated into pTARGET (Promega, Madison, WI, USA) in forward direction to construct pTARGET/E6E7. E6m was prepared according to the procedure by Shirasawa et al. [35]. Briefly, for expression of full length E6, E6m was amplified by sequential PCR using specifically designed primer sets such as sense primer, antisense primer and mutagenic primers containing two mismatches in splicing donor sites located within the E6 ORF. Two partial E6 inserts with overlapped regions were amplified by PCR using pOPI3/E6 as template and combined primer sets of either E6 sense primer (5′-GAA GAT CTC TAT GTT TCA GGA CCA CAG-3′) and antisense mutagenic primer (217–236) (5′-AGT CAT ATA GCT CGC GTC GC-3′) or (5′-GCG ACG CGA GCT ATA TGA CT-3′) sense mutagenic primer and E6 antisense primer (5′-TTA CAG CTG GGT TTT CTC T-3′). The resulting PCR products were mixed, denatured and annealed slowly after addition of E6 sense and antisense primers. Second PCR was performed for completing full length E6m that is not subjected to be splicing. PCR product was subcloned into PCR 2.1TOPO vector to construct TOPO/E6m. E6m, excised from *Bgl*II/*Sal*I, was inserted into mammalian expression vector pTARGET to express the full length E6. Their expressions in transfectants were identified by reverse transcription-polymerase chain reaction (RT-PCR) using specific primers due to the low levels of protein expression.

2.4. Establishment of stable transfectants

Cells grown in DMEM were prepared to establish stable cell line expressing oncogenes. Transfection was performed using SuperFect™ (Qiagen, Germany) according to the manufacturer's instructions. Briefly, C-33A and HaCaT cells were seeded in 6 cm dishes to reach

70% confluence at 24 h. Medium was then removed, and complex of 30 μl SuperFect and 5 μg DNA was directly transferred to the cells. After the plate was incubated for 2 h at 37°C and 5% CO_2, medium containing the remaining complexes was removed, and replaced with fresh cell growth medium. Cells were allowed to incubate for 48 h, followed by growing in selective DMEM medium containing G-418 (800 μg/ml) (Gibco BRL) for 2 months. G418 resistant colonies were pooled for each cell population.

2.5. RT-PCR

RT-PCR was carried out to identify expression of E6, E7 and E6/E7 from total RNA isolated from stable transfectants according to the manufacturer's instructions (Stratagene). In brief, the cDNA was synthesized from total RNA in 50 μl reaction volume containing 5 μl 10× first-strand buffer, 1 μl RNase block ribonuclease inhibitor (40 U/μl), 2 μl 100 mM dNTPs and 1 μl MMLV-RT (50 U/μl) and mixtures were gently incubated at 37°C for 1 h. The resulting cDNA was amplified using E6 and E7 specific primers as describe above under following PCR cycles (30 cycles: 1 min at 95°C, 1 min at 57°C and 1 min 30 s at 72°C). The PCR products were analyzed on 1% agarose gel. The primers for IL-18 were based on the published sequence of human IL-18 [20]: 5′-CAT GCC ATG GCT GCT GAA CCA GTA GAA GA-3′ (sense) and 5′-CGG GAT CCA ATA GCT AGT CTT CGT TTT G-3′ (antisense). The primer sequences for β-actin as an internal standard were 5′-GTG GGG CGC CCC AGG CAC CA-3′ (sense) and 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ (antisense).

2.6. Northern blot

To prepare Northern probe for IL-18, its cDNA was synthesized by RT-PCR using primers described above. IL-18 cDNA was labelled with random priming and used as a probe. Total cellular RNA was extracted from C-33A cells expressing E6 or E7 using the RNAzol B reagent (Tel-Test, Friendswood, TX, USA) according to the manufacturer's instructions. RNA samples (20 μg) were separated on 1.2% agarose/formaldehyde gels and transferred to nylon membranes. The filter was hybridized with radiolabelled IL-18 cDNA probe, washed and autoradiographed at −70°C.

2.7. Plasmid constructs and expressions of proteins for E6, IL-18 and p53

E6 gene was amplified by PCR from total RNA isolated from CaSki cell lines as described above. E6 insert from pBluescript KS(+)/E6 was inserted into *Bam*HI–*Sal*I site of PET28a (Novagen, Madison, WI, USA) as described [36]. IL-18 gene was synthesized using total RNA from normal keratinocyte cell line, HaCaT. The primers for mature hIL-18 were the following: 5′-CGC GGA TCC TAC TTT GGC AAG-3′ (sense), 5′-CCG GAA TTC AAT AGC TAG TCT TCG-3′ (antisense). After subcloning the IL-18 PCR products into T vector which had been prepared from pBluescript KS(+), and confirming IL-18 sequence, mature IL-18 was subcloned into *Bam*HI–*Sal*I site of pET28a. Construction of the p53 was carried out by RT-PCR using total RNA extracted from HaCaT and the following primer pairs with enzyme site italicized: 5′-GGA ATTC*CA-TATG*GA GGA GCCGCAGTCAGATCC-3′ (sense), 5′-CG*GGATC-C* TCA GTC TGAGTCAGGCCCTT-3′ (antisense). PCR product was digested with *Nde*I and *Bam*HI and cloned into the corresponding sites of plasmid pET3a (Novagen). Histidine fusion proteins were expressed in *Escherichia coli* BL21 (DE3) using pET28/E6,/IL-18 and purified with Ni-nitrilotriacetic acid resin as described [37]. The eluted proteins were identified by Western blot analysis using corresponding primary antibodies and alkaline phosphatase conjugated secondary antibodies (anti-goat antibody and anti-mouse antibody). BCIP/NBT was used as a substrate for color development. The protein concentration was determined by Bradford method. Then the purified proteins were aliquoted and stored at −70°C until use.

2.8. The effect of p53 on the binding between E6 and IL-18

E6 insert was excised from digestion of pBluescript KS(+) (Stratagene, La Jolla, CA, USA)/E6 with *Bam*HI/*Sal*I and subcloned into corresponding expression vectors pGEX4T-1 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) which had been prepared by digesting with *Bam*HI/*Sal*I. Transformation of pGEX/E6 into DH5α was performed to express GST-fused E6. pGEX/E6 was expressed in DH5α, prepared in PBS containing 0.5% Triton X-100 and then sonicated. The resulting lysates were centrifuged for 30 min at 12 000 rpm to remove the pellet containing cell debris. Supernatants were used as cell lysate for binding assay. Binding assays were performed by combining GST–E6 immobilized on GSH–Sepharose with increasing doses of lysates containing His-tagged IL-18 protein ranging from 5 to 800 μg in binding buffer, PBS containing 0.5% Triton X-100. The mixtures were rotated in microcentrifuge tubes at 4°C for 1 h. The beads were washed three times with binding buffer, and then boiled for 5 min in 50 μl of SDS gel loading buffer. The proteins solubilized in the sample buffer were subjected to electrophoresis on a 12% polyacrylamide gel, transferred to membrane and immunoblotted using an antibody that specifically recognizes IL-18. To investigate whether p53 inhibited the binding between E6 and IL-18, increasing doses of lysates containing p53 protein were incubated with binding mixtures of E6 and IL-18.

2.9. Immunoblotting analysis

Cells from 10 cm dishes were lysed in lysis buffer containing protease inhibitor aprotinin (10 μg/ml) and 0.5 mM PMSF. The protein concentration in the lysate was determined with Bradford assay, and equal amounts of protein (50 μg) from each lysate were electrophoresed on 12.5% polyacrylamide SDS gels. Proteins were transferred onto Immobilon-P membrane. p53 and IL-18 were detected with the monoclonal anti-p53 antibody and monoclonal anti-IL-18 antibody #18-8 (IgG_{2a}), respectively. Proteins were visualized by adding a substrate BCIP/NBT for alkaline phosphatase conjugated to secondary antibody.

2.10. Transient transfection and CAT assay

HaCaT and C-33A cells were cotransfected with pOPι3/E6 and IL-18 promoter pCAT-p1-2688 or pCAT-p2-2.3 plasmid [38] by using SuperFect™ (Qiagen, Germany) according to the manufacturer's instructions. After 6 h, cells were washed twice with PBS and fresh complete medium was added to the cells. About 24 h later, the cells were washed with ice-cold PBS, resuspended in 0.25 M Tris (pH 7.8), and subjected to three cycles of freezing and thawing. Cell lysates were heated for 10 min to inactivate CAT inhibitors and then assayed for CAT enzyme activity by TLC method [39]. As a control, cells were cotransfected with pCH110 plasmid (Pharmacia, Piscataway, NJ, USA) for β-galactosidase activity.

2.11. IFN-γ assay

Human PBMCs in RPMI medium were seeded into 96 well plates in 200 μl at final concentrations of 1×10^6/ml and 5×10^5/ml, respectively, and pretreated with 10 μg/ml polymyxin B to neutralize endotoxins. Human IL-18 was used as an inducing agent and PHA as a costimulator in PBMC. For recombinant E6 protein from *E. coli* was assessed whether it could inhibit the IFN-γ production by IL-18. The E6 protein or neutralizing antibody were mixed with IL-18, preincubated at room temperature for 30 min, and treated in PBMCs for 20 h, and the secreted IFN-γ was measured with an IFN-γ ELISA kit according to the manufacturer's instructions.

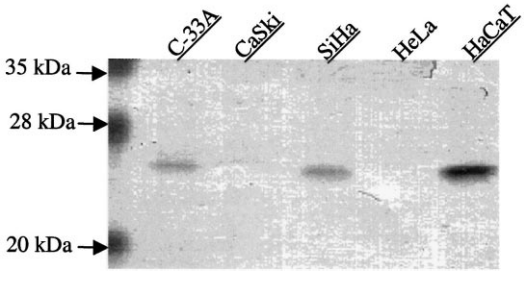

Fig. 1. Identification of IL-18 expression by Western blot analyses in cervical cancer cell lines. 50 μg of proteins extracted from each indicated cell lines were separated on SDS/12% polyacrylamide gel and electroblotted to an Immobilon-P membrane. The IL-18 was detected with mouse monoclonal anti-hIL-18 antibody #18-8 (IgG_{2a}). The bound anti-IL-18 antibody was detected by alkaline-phosphatase conjugated secondary antibody followed by NBT/BCIP substrate for development.

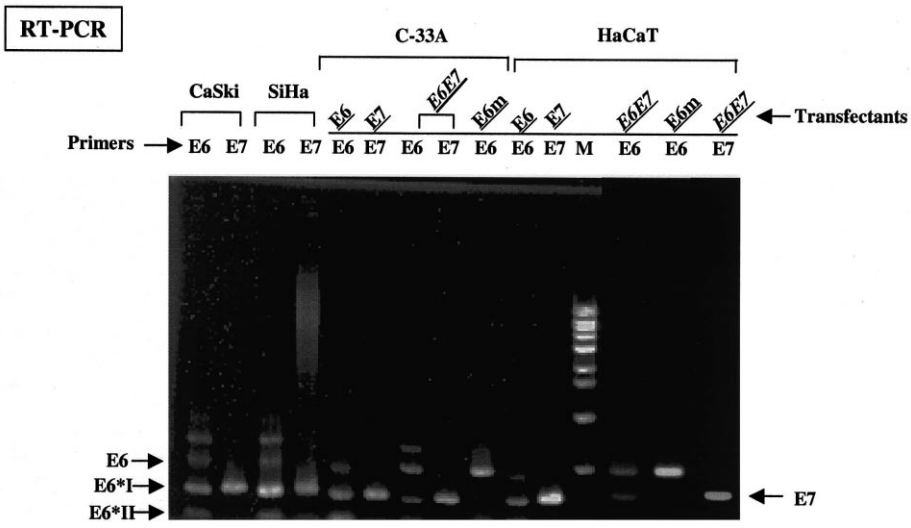

Fig. 2. The identification of E6 and E7 expression in C-33A and HaCaT cells stably transfected with E6, E6m, E6/E7 and E7. E6 and E7 expression in transfectants were detected by RT-PCR using total RNA and combined primer sets as described in Section 2. M: molecular weight marker as in Fig. 3.

2.12. Statistical analysis

ANOVA using Fisher's least significant difference was used. Data were expressed as the mean ± S.D.

3. Results

3.1. IL-18 expression was downregulated in cervical cancer cell lines HeLa and CaSki which represent for HPV type 18 and HPV type 16 infection, respectively

We preliminary surveyed the expression levels of IL-18 in cervical cancer cell lines and normal keratinocyte HaCaT cells. Western blot analyses showed that IL-18 expression level was strong in HaCaT and moderate in C-33A and SiHa harboring 1 or 2 copies of HPV 16while IL-18 protein was not detected in HeLa and CaSki harboring 10–50 copies of HPV type 18 and 60–600 copies of HPV type 16 genome, respectively (Fig. 1). Our data revealed that the expression of IL-18 was significantly downregulated in HPV-positive cervical cancer cell lines when compared to HaCaT and C-33A, suggesting that HPV infection may be correlated to the IL-18 expression.

3.2. Expression levels of both IL-18 and p53 were decreased in C-33A cells expressing E6 oncogene

In order to address the correlation between HPV infection and IL-18 expression level, representative oncogenes E6 and E7 of HPV were stably transfected into non-cervical cell line HaCaT, and HPV-negative cervical cancer cell line C-33A. As shown in Fig. 2, the expressions of E6, E6m, E7 and E6/E7 were confirmed in respective transfectants by RT-PCR. As previously reported [40], most of E6 transcripts were in the spliced form E6*I, and full length E6 and the other spliced form E6*II were detected in small amounts while E6m, bearing a point mutation in the splice donor, failed to produce the truncated E6. The expression pattern of E6/E7 was similar to that of E6 but the untruncated E6 level was higher than other truncated E6 species. It has been well known that E6 protein forms complexes with p53 [41,42] and can target p53 for degradation through the ubiquitin dependent pathway [1]. The functional activity of E6 expression in transfectants was de-

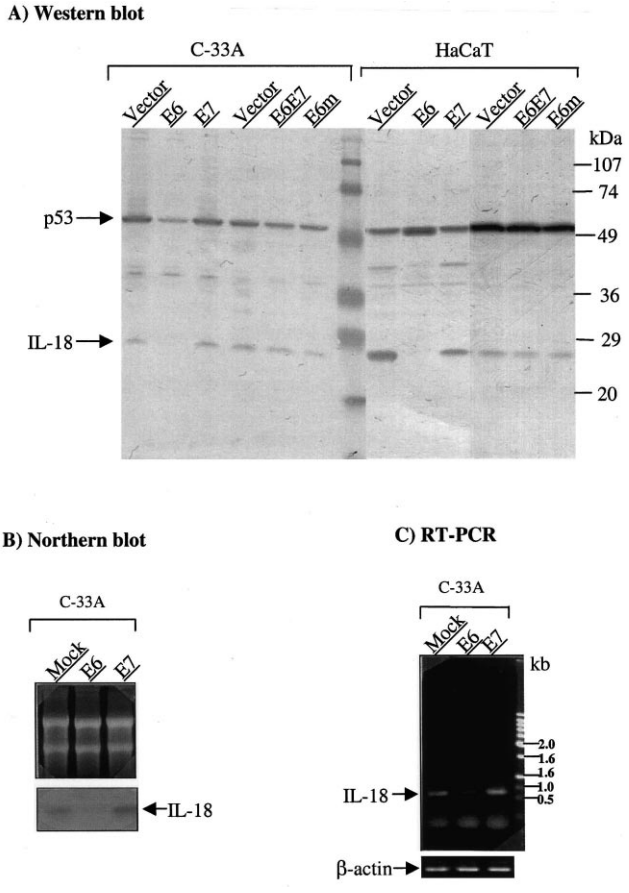

Fig. 3. Immunoblot analysis (A) of IL-18 and p53 in stable transfectants C-33A and HaCaT keratinocytes expressing E6 and E7 oncogenes, Northern blot (B) and RT-PCR (C) analyses of IL-18 mRNA level in C-33A cells expressing E6 or E7 oncogenes. Preparations of protein lysate and mRNA, electrophoresis, transfer, probing and RT-PCR were performed as described in Section 2. The IL-18 and p53 were detected with monoclonal anti-hIL-18 antibody #18-8 (IgG_{2a}) and mouse anti-human p53 antibody, respectively.

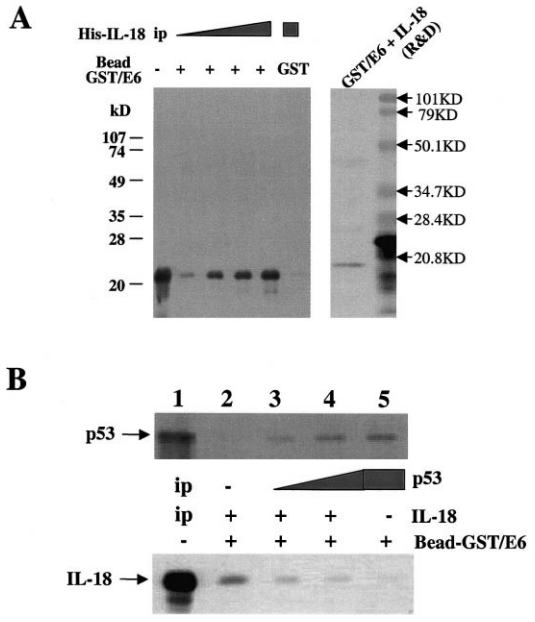

Fig. 4. Interaction of E6 with IL-18 in vitro binding assay and competitive inhibition of E6-IL-18 binding by p53 protein. A: The entire coding sequence of HPV 16 E6 was fused in frame with GST and resultant product GST–E6 was immobilized on GSH–Sepharose bead. Increasing lysates of bacterially expressed IL-18 were incubated with a purified GST–E6 fusion protein or with GST alone (control) bound to beads. IL-18 bound to E6 was analyzed by SDS–PAGE on a 12.5% gel followed by Western blot analysis using monoclonal anti-hIL-18 antibody #18-8 (IgG_{2a}). B: Constant amount of his-tagged IL-18 and increasing doses of p53 were incubated with a purified GST–E6 fusion protein bound to bead. IL-18 or p53 bound to E6 was analyzed by SDS–PAGE followed by Western blotting using monoclonal anti-IL-18 antibody #18-8 or mouse anti-human p53 antibody, respectively. 1, p53 (1/10 dilution); His IL-18 (1/10 dilution); 2, bead GST E6+His IL-18 (80 μl); 3, bead GST E6+His IL-18 (80 μl)+p53 200 μg; 4, bead GST E6+His IL-18 (80 μl)+p53 400 μg; 5, bead GST E6+p53 400 μg.

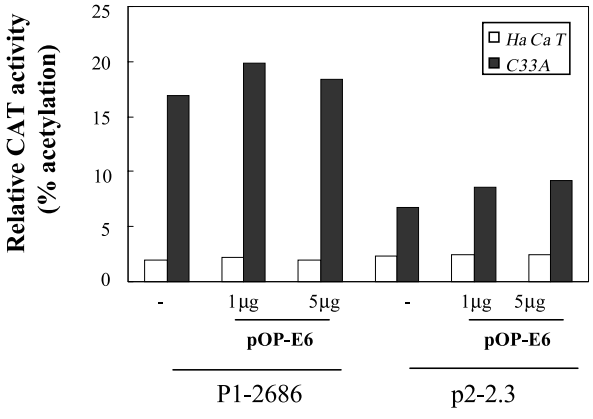

Fig. 5. Effects of E6 on p1 and p2 promoter activation of IL-18. HaCaT and C-33A cells were cotransfected with 1 μg or 5 μg of p1-2686 (p1 promoter) or p2-2.3 (p2 promoter) plasmid derived from IL-18 promoter and 1 μg of β-galactosidase expression vector (pCH110), together with increasing amounts (μg) of E6 expression vector or control vector. After 24 h, CAT activities normalized by β-galactosidase activity were determined. The values are shown as mean ± S.D. from three independent experiments with similar results.

termined by degradation of endogenous p53 protein (Fig. 3A). In order to investigate whether E6 could inhibit the expression of IL-18 mRNA, the levels of IL-18 mRNA were identified by Northern blot and RT-PCR, showing that IL-18 mRNA was decreased by E6 transfection (Fig. 3B,C). The p53 protein in C-33A cells expressing E6 was sensitive to degradation whereas E6 expressed in HaCaT cells did not affect p53 in terms of its degradation (Fig. 3A). As similar to downregulation of p53, C-33A cells expressing E6 decreased the IL-18 expression significantly, and transfectants with E6/E7 or E6m inhibited its expression a little. In case of HaCaT cells, similar results were observed with C-33A cells transfected with E6, E6/E7 and E6m with respect to the IL-18 expression level. However, irrespective of E6 expression, levels of p53 in HaCaT were significantly unaffected.

3.3. IL-18 was competitively bound to E6 with p53

In order to investigate whether IL-18 bound to E6, we tested the in vitro binding assay using recombinant GST-fused E6 and recombinant mature IL-18. As shown in Fig. 4, IL-18 was able to bind to E6 immobilized on GSH–Sepharose beads (Fig. 4A) and its binding was a little bit inhibited by addition of p53 (Fig. 4B), suggesting that IL-18 could bind to the p53 binding site of E6. In binding experiments for GST-fused E6, we used total cell lysates which contain either overexpressed p53 or IL-18 and a lots of other proteins. So, we added excess of proteins to bead-bound proteins for interaction.

3.4. IL-18 promoters were not inhibited by E6

In order to investigate how IL-18 expression was inhibited by E6, we cotransfected HaCaT and C-33A cells with pOPi3/E6 and IL-18 promoter pCAT-p1-2688 or pCAT-p2-2.3 plasmid. As shown in Fig. 5, E6 oncogene did not affect the activities of IL-18 promoter P1 and P2, suggesting that E6 downregulated IL-18 independent of the IL-18 promoter.

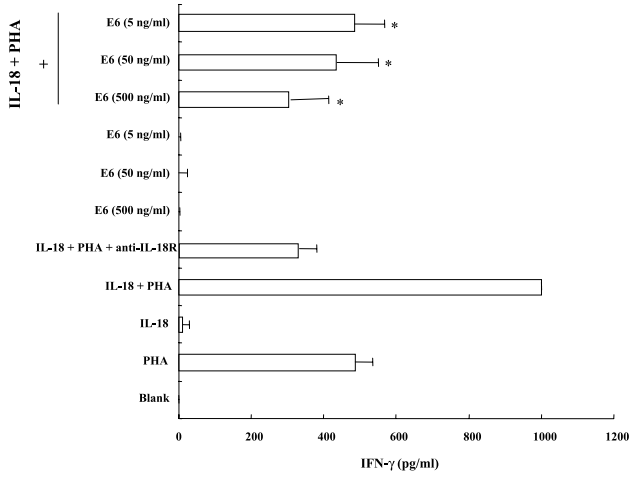

Fig. 6. The effect of HPV oncoprotein E6 on IL-18 induced IFN-γ production in PBMCs. Polymyxin B (10 μg/ml) was added into the cells to neutralize endotoxin. The concentrations of the reagents used were as follows; IL-18 (50 ng/ml), PHA (1 μg/ml), neutralizing anti-IL-18R antibody (0.5 μg/ml). The oncoproteins or neutralizing antibody were mixed with IL-18, preincubated at room temperature for 30 min, and treated into PBMCs for 20 h. This result represents one of five experiments. The values are shown as mean ± S.D. from triplicates. $P < 0.05$ compared to cells treated with both IL-18 and PHA.

3.5. HPV 16 E6 oncoprotein inhibited IL-18 induced IFN-γ production

Because E6 downregulated IL-18, we also investigated whether HPV 16 E6 oncoprotein could inhibit IL-18 induced IFN-γ production in PBMC from five different donors. The effect of IL-18 on IFN-γ production was confirmed by neutralizing anti-human IL-18R antibody treatment. IL-18 usually induces IFN-γ in the presence of mitogen or with combination of IL-12 treatment. The coincubation of E6 protein and IL-18 showed a significant decrease in IL-18 induced IFN-γ production in PHA treated PBMCs (Fig. 6).

4. Discussion

It has been reported that there is a reduction in the expression levels of type 1 cytokines in HPV induced cervical lesions [10,43]. We investigated the correlation between HPV infection and expression level of type 1 cytokine IL-18. Representative oncogenes E6 and E7 of HPV were stably transfected into HPV-negative cervical cancer cell line C-33A (Fig. 2). The HPV 16 polycistronic mRNA transcribed from E6 and E7 ORFs initiate from the p97 early promoter [39]. E6 gene encodes, in addition to the full length E6 open reading frame (ORF: 450 bp), truncated E6 ORFs (E6*I (300 bp) and E6*II (150 bp)) which are generated as a result of differential splicing [35,44]. In cervical carcinoma tissues, premalignant lesions, and cell lines derived from cervical cancer the E6*I encoding mRNA is a major transcript. Whenever we perform RT-PCR with RNAs extracted from CaSki, SiHa and C-33A transfectants expressing E6/E7, an additional band appears over the full length E6. As mentioned in Section 2, E6/E7 gene in HPV-containing cells such as CaSki and SiHa is expressed as a bicistron and processed into splicing, resulting in E6*I/E7 and E6*II/E7 as well as E6/E7. Therefore, by interaction of PCR primers specific for E6 with E7 sequence following E6, additional larger products are non-specifically generated under our RT-PCR condition. mRNA species for E6 can be designated on the basis of migration patterns which correspond to those for PCR products obtained from respective cDNAs under the same condition. Similar migration patterns were shown in a previous report [40]. C-33A cells transfected with E6 decreased significantly the IL-18 expression, and transfectants with E6/E7 or E6m inhibited its expression a little as well as downregulation of p53 (Fig. 3A). As previously reported [40], C-33A cells transfected by E6 showed that most of E6 transcripts were spliced form E6*I, which might be essential for degradation of mutant p53, tumor suppressor protein. E6 expression in C-33A leads to degradation of mutant p53 (Fig. 3A), while levels of p53 in HaCaT cells expressing E6 remains constant, indicating that mutant p53 present in HaCaT cells is insensitive to the E6 targeted degradation via the ubiquitin pathway. This result was also previously reported [45].

Similar to precursor IL-1β (proIL-1β), precursor IL-18 (proIL-18) does not contain a signal peptide required for the removal of the precursor amino acids with subsequent secretion. The activity of the precursor is exceedingly low, but after cleavage by caspase-1, the mature form is fully active. In addition to caspase-1, caspase-3, though being likely to play a greater role in cell death, cleaves mature or proIL-18 after aspartic acid 71 and 76, resulting in inactive peptides [46]. Thus, proIL-18 may serve as a negative regulator of Fas mediated cell death by acting as a sink for the enzymatic activity of caspase-1 and caspase-3. IL-18 administration to mice as an adjuvant provided enhanced NK cell activation [21] and IL-18 may possess host-defense functions independent of IFN-γ [47]. Pre- and early treatment with IL-18 reduced infection severity, prolonged life via increase of innate defense against tumors [48,49].

In this study, to elucidate whether IL-18 was affected by HPV oncogenes, stable cell lines expressing HPV 16 E6 or E7 in C-33A and HaCaT cells were established. Our data revealed that p53 and IL-18 were less expressed in HPV-positive cell lines CaSki and HeLa whereas HPV-negative cell lines HaCaT and C-33A cells expressed p53 and IL-18 at high levels. The expression of E6 in HaCaT and C-33A cell lines was reversely correlated to the expression of IL-18, assuming that HPV infection can evade immune surveillance by downregulating the expression of immune stimulating cytokine. Our experiment was focused to investigate how IL-18 was modulated by E6. In vitro binding experiments showed that his-tagged mature IL-18 bound to GST–E6 (Fig. 4), suggesting that once IL-18 bound to E6, IL-18 might be degraded by E6 via the ubiquitin pathway or any other pathways. The partial competitive inhibition of IL-18 bound to E6 by p53 protein at a high concentration suggests that either the binding domain on E6 protein may be shared by both p53 and IL-18 or structural changes in E6 responsible for IL-18 binding may be induced by p53 binding to E6. We also cotransfected C-33A and HaCaT with both IL-18 promoters and E6 to elucidate whether E6 oncogene could inhibit IL-18 promoters (Fig. 5). These cotransfection experiments showed that there was no effect of E6 on the activities of IL-18 promoter p1 and p2, suggesting that E6 oncogene downregulated IL-18 indirectly. The decrease of IL-18 levels by E6 and interaction of IL-18 with E6 may interfere with the local inflammatory process caused by the cascade of downstream effects that follow activation of the IL-18 receptor via binding to IL-18. Similarly, a family of proteins encoded by MCV is able to bind with high affinity to human and murine IL-18 molecules and inhibited IL-18 mediated IFN-γ production in a dose dependent manner [25]. IL-18 has been shown to protect mice against herpes simplex virus infection by IFN-γ dependent and independent pathways [13]. Although the role of IL-18 on HPV pathogenesis has not been determined yet, the binding of the IL-18 to E6 is not only beneficial to viral infection with respect to the critical role of IL-18 in defense against virus but also provides a mechanism for evasion of the immune system by HPV. Additional studies are under way to further elucidate the downregulatory mechanism of IL-18 by E6 and the role of IL-18 in the local immunity of cervical mucosa against HPV-related cervical lesions.

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References

[1] Scheffner, M., Munger, K., Byrne, J.C. and Howley, P.M. (1991) Proc. Natl. Acad. Sci. USA 88, 5523–5527.
[2] zur Hausen, H. (1999) Proc. Assoc. Am. Physicians 111, 581–587.
[3] Hawley-Nelson, P., Vousden, K.H., Hubbert, N.L., Lowy, D.R. and Schiller, J.T. (1989) EMBO J. 8, 3905–3910.

[4] Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J. and Howley, P.M. (1990) Cell 63, 1129–1136.
[5] White, A.E., Livanos, E.M. and Tlsty, T.D. (1994) Genes Dev. 8, 666–677.
[6] Munger, K., Werness, B.A., Dyson, N., Phelps, W.C., Harlow, E. and Howley, P.M. (1989) EMBO J. 8, 4099–4105.
[7] Zerfass, K., Levy, L.M., Cremonesi, C., Ciccolini, F., Jansen-Durr, P., Crawford, L., Ralston, R. and Tommasino, M. (1995) Gen. Virol. 76, 1815–1820.
[8] Frazer, I.H. (1996) Curr. Opin. Immunol. 8, 484–491.
[9] Dinarello, C.A. (1996) Blood 87, 2095–2147.
[10] Pao, C.C., Lin, C.Y., Yao, D.S. and Tseng, C.J. (1995) Biochem. Biophys. Res. Commun. 214, 1146–1151.
[11] Merrick, D.T., Winberg, G. and McDougall, J.K. (1996) Cell Growth Differ. 7, 1661–1669.
[12] Woodworth, C.D. and Simpson, S. (1993) Am. J. Pathol. 142, 1544–1555.
[13] Fujioka, N., Akazawa, R., Ohashi, K., Fujii, M., Ikeda, M. and Kurimoto, M. (1999) J. Virol. 73, 2401–2409.
[14] Frazer, I.H., Fernando, G.J., Fowler, N., Leggatt, G.R., Lambert, P.F., Liem, A., Malcolm, K. and Tindle, R.W. (1998) Eur. J. Immunol. 28, 2791–2800.
[15] Nakamura, K., Okamura, H., Wada, M., Nagata, K. and Tamura, T. (1989) Infect. Immun. 57, 590–595.
[16] Puren, A.J., Fantuzzi, G., Gu, Y., Su, M.S. and Dinarello, C.A. (1998) J. Clin. Invest. 101, 711–721.
[17] Matsumoto, S., Tsuji-Takayama, K., Aizawa, Y., Koide, K., Takeuchi, M., Ohta, T. and Kurimoto, M. (1997) Biochem. Biophys. Res. Commun. 234, 454–457.
[18] Dao, T., Ohashi, K., Kayano, T., Kurimoto, M. and Okamura, H. (1996) Cell. Immunol. 173, 230–235.
[19] Balashov, K.E., Rottman, J.B., Weiner, H.L. and Hancock, W.W. (1999) Proc. Natl. Acad. Sci. USA 96, 6873–6878.
[20] Ushio, S. et al. (1996) J. Immunol. 156, 4274–4279.
[21] Micallef, M.J., Tanimoto, T., Kohno, K., Ikeda, M. and Kurimoto, M. (1997) Cancer Res. 57, 4557–4563.
[22] Tsutsui, H., Matsui, K., Kawada, N., Hyodo, Y., Hayashi, N., Okamura, H., Higashino, K. and Nakanishi, K. (1997) J. Immunol. 159, 3961–3967.
[23] Novick, D., Kim, S.H., Fantuzzi, G., Reznikov, L.L., Dinarello, C.A. and Rubinstein, M. (1999) Immunity 10, 127–136.
[24] Smith, V.P., Bryant, N.A. and Alcami, A. (2000) J. Gen. Virol. 81, 1223–1230.
[25] Xiang, Y. and Moss, B. (1999) Proc. Natl. Acad. Sci. USA 96, 11537–11542.
[26] de Gruijl, T.D. et al. (1999) Eur. J. Cancer 35, 490–497.
[27] Stanley, M. (1998) Eur. J. Dermatol. 8, 8–22.
[28] Cho, Y.S. et al. (2001) J. Biochem. Mol. Biol. 34, 80–84.
[29] Kim, S.H., Eisenstein, M., Reznikov, L., Fantuzzi, G., Novick, D., Rubinstein, M. and Dinarello, C.A. (2000) Proc. Natl. Acad. Sci. USA 97, 1190–1195.
[30] Baker, C.C., Phelps, W.C., Lindgren, V., Braun, M.J., Gonda, M.A. and Howley, P.M. (1987) J. Virol. 61, 962–971.
[31] Meissner, J.D. (1999) J. Gen. Virol. 80, 1725–1733.
[32] Schwarz, E., Freese, U.K., Gissmann, L., Mayer, W., Roggenbuck, B., Stremlau, A. and zur Hausen, H. (1985) Nature 314, 111–114.
[33] Mythily, D.V., Krishna, S. and Tergaonkar, V. (1999) J. Gen. Virol. 80, 1707–1713.
[34] Marchuk, D., Drumm, M., Saulino, A. and Collins, F.S. (1990) Nucleic Acids Res. 19, 1154.
[35] Shirasawa, H., Jin, M.H., Shimizu, K., Akutsu, N., Shino, Y. and Simizu, B. (1994) Virology 203, 36–42.
[36] Cho, Y., Cho, C., Joung, O., Lee, K., Park, S. and Yoon, D. (2000) Antivir. Res. 47, 199–206.
[37] Yoon, D., Yoon, H., Kim, K., Lim, J., Oh, E., Choe, Y., Chung, Y., Dinarello, C.A. and Park. S. (1998) The Biochemical Society of Korea-Spring Meeting, p. 162.
[38] Kim, Y.M., Kang, H.S., Paik, S.G., Pyun, K.H., Anderson, K.L., Torbett, B.E. and Choi, I. (1999) J. Immunol. 163, 2000–2007.
[39] Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell. Biol. 2, 1044.
[40] Smotkin, D., Prokoph, H. and Wettstein, F.O. (1989) J. Virol. 63, 1441–1447.
[41] Storey, A. et al. (1998) Nature 393, 229–234.
[42] Werness, B.A., Levine, A.J. and Howley, P.M. (1990) Science 248, 76–79.
[43] Clerici, M. et al. (1997) J. Natl. Cancer Inst. 89, 245–250.
[44] Smotkin, D. and Wettstein, F.O. (1986) Proc. Natl. Acad. Sci. USA 83, 4680–4684.
[45] Magal, S.S., Jackman, A., Pei, X.F., Schlegel, R. and Sherman, L. (1998) Int. J. Cancer 75, 96–104.
[46] Akita, K. et al. (1997) J. Biol. Chem. 272, 26595–26606.
[47] Osaki, T., Peron, J.M., Cai, Q., Okamura, H., Robbins, P.D., Kurimoto, M., Lotze, M.T. and Tahara, H. (1998) J. Immunol. 160, 1742–1749.
[48] Kawakami, K., Qureshi, M.H., Zhang, T., Okamura, H., Kurimoto, M. and Saito, A. (1997) J. Immunol. 159, 5528–5534.
[49] Bohn, E., Sing, A., Zumbihl, R., Bielfeldt, C., Okamura, H., Kurimoto, M., Heesemann, J. and Autenrieth, I.B. (1998) J. Immunol. 160, 299–307.