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Imiquimod Suppresses Propagation of Herpes Simplex Virus 1 by Upregulation of Cystatin A via the Adenosine Receptor A_1 Pathway

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Imiquimod is recognized as an agonist for Toll-like receptor 7 (TLR7) in immunocompetent cells. TLR7, as well as TLR3 and TLR8, triggers the immune responses, such as the production of type I interferons (IFNs) and proinflammatory cytokines via recognition of viral nucleic acids in the infected cells. In this study, we proposed that imiquimod has an IFN-independent antiviral effect in nonimmune cells. Imiquimod, but not resiquimod, suppressed replication of human herpes simplex virus 1 (HSV-1) in FL cells. We analyzed alternation of gene expression by treatment with imiquimod using microarray analysis. Neither type I IFNs, nor TLRs, nor IFN-inducible antiviral genes were induced in imiquimod-treated FL cells. Cystatin A, a host cysteine protease inhibitor, was strongly upregulated by imiquimod and took a major part in the anti-HSV-1 activity deduced by the suppression experiment using its small interfering RNA. Upregulation of cystatin A was suggested to be mediated by antagonizing adenosine receptor A_1 and activating the protein kinase A pathway. Imiquimod, but not resiquimod, was shown to interact with adenosine receptor A_1. Imiquimod-induced anti-HSV-1 activity was observed in other cells, such as HeLa, SiHa, and CaSki cells, in a manner consistent with the cystatin A induction by imiquimod. These results indicated that imiquimod acted as an antagonist for adenosine receptor A_1 and induced a host antiviral protein, cystatin A. The process occurred independently of TLR7 and type I IFNs.

Imiquimod [R837; 1-(2-methylpropyl)-1*H*-imidazo[4,5-c]quinolin-4-amine] and resiquimod [R848; 4-amino-2-(ethoxymethyl)-α,α-dimethyl-1*H*-imidazo[4,5-c]quinoline-1-ethanol], which are immune response modifiers of the imidazoquinoline family, display profound antiviral and antitumoral activity *in vitro*, *in vivo*, and in clinical applications (22). Imiquimod preferentially activates Toll-like receptor 7 (TLR7), but its agonistic effect on TLR8 appears to be much weaker, whereas resiquimod efficiently activates both TLR7 and TLR8 (18, 30). They activate immune cells via the MyD88-dependent signaling pathway and exert its effect through induction of immune cell-mediated antiviral and antitumor responses (10, 15). TLR-mediated activation of MyD88 activates the transcription factor NF-κB, which upon activation migrates to the nucleus and upregulates transcription of various cytokines, especially alpha/beta interferon (IFN-α/β), tumor necrosis factor alpha (TNF-α), interleukin-2 (IL-2), IL-6, IL-8, and IL-12 (14, 20). There are differences in the induction of cytokines and apoptosis between imiquimod and resiquimod. These differences are considered to be determined primarily by the specificity of their ligands and their distribution in the cells. TLR7 is preferentially expressed in plasmacytoid dendritic cells (pDCs), B cells, and regulatory T cells, whereas TLR8 is expressed in monocytes, macrophages, monocyte-derived dendritic cells, myeloid dendritic cells, regulatory T cells, and neutrophils (9). However, there are reports that TLR7 and TLR8 are expressed in nonimmune cells such as epithelial cells (19, 42).

Imiquimod is recognized as showing indirect antiviral activity (21); it mediates antiviral activity through IFN and cytokine induction via TLR7 signaling from immune cells. Antiviral activity of imiquimod has been demonstrated against a variety of viruses, and its clinical efficacy has been demonstrated against genital warts, herpes genitalis, and molluscum contagiosum (21, 22). Imiquimod activates dendritic cells such as resident Langerhans cells in the skin, possibly resulting in prolonged protective Th1-skewed immunity against viral infections and malignant tumors (34, 35). In the animal experiment, imiquimod has been shown to induce CCL2-dependent recruitment of pDCs, and once these pDCs have been transformed into killer DCs, they are able to eliminate tumor cells directly (6). The mode of action of imiquimod against herpes simplex virus (HSV) is principally that of immune reinforcement effects mediated by the induced cytokines, including IFN-α, derived from immune cells (21).

Imiquimod additionally acts as an antagonist for adenosine receptors (31). Adenosine and ATP, which mainly originate from damaged cells, modulate intracellular concentration of cyclic AMP (cAMP) via the adenosine receptors, which are G-coupled seven transmembrane receptors (5, 25). Four subtypes of adenosine receptors have been identified, namely, A_1, A_{2A}, A_{2B}, and A_3. Adenosine receptor A_1 transduces a negative signal that downregulates protein kinase A (PKA) activity, followed by cAMP concentration. Conversely, adenosine receptor A_{2A} and A_{2B} transduce positive signals, which stimulate cAMP formation (7, 11). In immune cells, a variety of macrophage functions, including phagocytosis, antigen presentation, target cell killing, the production of NO, IL-6, IL-10, IL-12, and TNF-α, and the expression of major histocompatibility complex class II molecules, are modulated by adenosine receptor signaling (26, 29). Schön et al. reported that imiquimod acted as an antagonist for adenosine receptors A_{2A} and A_3 and significantly suppressed cAMP levels, which resulted in the suppression of the production of proinflammatory cytokines, such as TNF-α, IL-1, and IL-6 (31).

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In the present study, we showed that imiquimod, but not resiquimod, had a direct antiviral effect on human herpes simplex virus 1 (HSV-1) replication in nonimmune cells independent of the TLR signaling pathway and IFN production. We demonstrated that the TLR-independent anti-HSV-1 activity of imiquimod was caused by the induction of cystatin A, which is a host cysteine protease inhibitor, via the antagonizing adenosine receptor A_1 signaling.

MATERIALS AND METHODS

Reagents. Imiquimod and resiquimod were purchased from LKT Laboratories (St. Paul, MN) and Alexis (Plymouth Meeting, PA), respectively. Human recombinant cystatin A was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The following agonists were purchased from Tocris Bioscience (Ellioville, MO): adenosine receptor A_1 agonist, 2′-Me-CCPA (2-chloro-*N*-6-cyclopentyl-2′-methyladenosine); A_1 antagonist, DPCPX (8-cyclopentyl-1,3-dipropylxanthine); $A_{2A/B}$ agonist, CV 1808 (2-phenylaminoadenosine); A_{2A} agonist, CGS 21680 hydrochloride (4-[2-[[6-amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride); A_{2A} antagonist, SCH 58261 {2-(2-furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine}; A_{2B} antagonist, MRS 1754 {*N*-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide}; and A_3 agonist, 2Cl-IB-MECA {1-[2-chloro-6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-β-D-ribofuranuronamide}. The PKA inhibitors Rp-8-Br-cAMPS (8-bromoadenosine-3′,5′-cyclic monophosphorothioate) and PKI6-22 amide (H-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH_2) were purchased from Merck-Calbiochem (Whitehouse Station, NJ). The protein kinase C (PKC) inhibitors GF109203X {3-[1-(3-dimethylaminopropyl)indol-3-yl]-4-(1*H*-indol-3-yl) pyrrole-2,5-dione)} and Calphostin C were purchased from Merck-Calbiochem. The TBK1 inhibitor BX795 [3-(5-iodo-4-{3-[(thiophene-2-carbonyl)-amino]-propylamino}-pyrimidin-2-ylamino)-phenyl]-amide] was purchased from Santa Cruz Biotechnology. The NF-κB inhibitor PDTC (1-pyrrolidine carbodithioic acid), polyinosine-polycytosine [poly(I·C)], and *Escherichia coli* lipopolysaccharide (LPS) O111:B4 were purchased from Sigma-Aldrich (St. Louis, MO).

Cells and viruses. The human amnion cell line FL, the cervix adenocarcinoma cell line HeLa, and the cervix squamous cell carcinoma cell lines SiHa and CaSki were obtained from American Type Culture Collection (ATCC; Manassas, VA) and routinely cultured in RPMI 1640 (Wako Pure Chemical, Tokyo, Japan) containing 10% fetal bovine serum (FBS), 100 U of penicillin/ml, and 100 μg of streptomycin/ml. The normal keratinocyte cell line HaCaT was kindly obtained from Katsuhiko Enomoto of Akita University (Akita, Japan), and the normal fibroblast cell line Tig118 was purchased from the Health Science Research Resources Bank (Osaka, Japan). HaCaT and Tig118 cells are routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The HSV-1 strain VR3 and KOS were obtained from the ATCC and maintained in Vero cells.

Chinese hamster ovary cells stably expressing human adenosine A_1 receptor (CHO-A1) were provided from Karl-Norbert Klotz (Universität Wurzburg, Wurzburg, Germany), and details of this cell line were described previously (17). The Chinese hamster ovary cell line (CHO-K1) was used as a control. The CHO-K1 cells were grown in Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Tokyo, Japan) containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The CHO-A1 cells were grown in IMDM containing 10% FBS and 0.2 mg of G418 (a neomycin analog)/ml.

Infection experiments and cell viability assay. FL cells were seeded at 10^5 cells/ml in a 6-cm dish and cultured for 24 h. For analyses of the effects of imiquimod on the cell viability and virus replication, the cells were cultured in a medium containing imiquimod for 12 or 24 h before virus infection. The cells were then inoculated with HSV-1 at a multiplicity of infection (MOI) of 0.1 or 1, incubated for 1 h for absorption, and then cultured for a further 24 h. Virus titers in the supernatants were determined by plaque assay using Vero cells as described previously (8). Cell viability was determined with a modified 3-(4,5-dimethylthoazol-2-yl)-2,5-diphenyltetrazolium bromide assay using Cell Counting Kit 8 (Dojin Chemical, Kumamoto, Japan).

Immunofluorescence microscopic analysis. Cells were fixed with methanol at −20°C for 20 min. Fixed cells were incubated with a fluorescein-conjugated goat anti-HSV-1 antibody (Chemicon, Temecula, CA). The antibody was diluted in Dulbecco phosphate-buffered saline [PBS(−)] containing 0.1% bovine serum albumin at 1:1,000 and incubated at 37°C for 1 h. Fluorescence microscopy was carried out with an Olympus IX71 system (Olympus, Tokyo, Japan). The image capture conditions were set at a nonsaturating level, and the images were taken and processed at the same time and under the same conditions.

RT-PCR. Total cellular RNA was prepared from cells using an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Semiquantitative reverse transcription-PCR (RT-PCR) assays were performed using a One-Step RT-PCR kit (Qiagen). The quantitative nature of the PCR was validated by the linearity of the determination curve at various concentrations of RNA. In accordance with previous papers, the primer sets used were as follows: cystatin A (37), cystatin C (40); TLRs 1, 5, 6, 7, 8, 9, and 10 (13); TLR2, TLR4, and glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) (33); and TLR3 (28). Primer sets for adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 were as described by Schön et al. (31), except for the reverse primer of A_{2A} (5′-CTGCTTCAGCTGTCGTCGC G-3′). The primer sets of cystatin B were as follows: forward, 5′-GTC GCCGCAAGATGATGTGC-3′, and reverse, 5′-GAAATAGGTCAGCTC ATCATG-3′.

Microarray analysis. Total cellular RNA was prepared from FL cells that were cultured for 24 h with or without imiquimod at 10 μg/ml. Microarray analysis was performed using the 3D-Gene Human OligoChip 25K (Toray, Tokyo, Japan).

Western blot analysis. To prepare cell extracts, cell monolayers were washed once with ice-cold PBS(−) and lysed with 1% Nonidet P-40, 120 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, and 20 mM HEPES-NaOH (pH 7.5). The cell lysates were incubated on ice for 30 min and centrifuged for 10 min at 10,000 × *g* at 4°C. Supernatants were analyzed by SDS-PAGE and Western blotting as described previously (43). Mouse anti-cystatin A, goat anti-thymidine kinase (TK), mouse anti-VP16, mouse anti-glycoprotein C (gC), and rabbit anti-human adenosine receptor A_1 antibodies were purchased from Santa Cruz Biotechnology. Mouse anti-ICP4 and mouse anti-ICP0 antibodies were purchased from Virusys Co. (Taneytown, MD). Antibody bindings were detected using alkaline phosphatase-labeled goat anti-mouse, goat anti-rabbit, or swine anti-goat immunoglobulins antibodies (BioSource International, Camarillo, CA) as a secondary antibody and Western Blue enzyme substrate (Promega, Madison, WI).

ELISA. Human IFN-α multi-subtype and human IFN-β enzyme-linked immunosorbent assay (ELISA) kits were purchased from PBL Biomedical Laboratories (Piscataway, NJ). The amount of cystatin A protein in cells was determined by using an ELISA kit (USCN Life Science, Wuhan, China) after the cells were lysed using Western blotting as described above.

Enzyme-linked DNA-protein interaction assay (ELDIA). Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). The activated NF-κB in the nuclear extract was determined by using a BD Mercury Transfactor NF-κB p65 kit (BD Bioscience, San Jose, CA) according to the manufacturer's instructions.

siRNA-mediated suppression of gene expression. Small interfering RNA (siRNA) specific for human cystatin A [cystatin A siRNA(h); sc-44430], siRNA specific for human adenosine receptor A_1 [adenosine A1-R siRNA(h); sc-39848], and a control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. The siRNA (0.5 μg) was transfected into

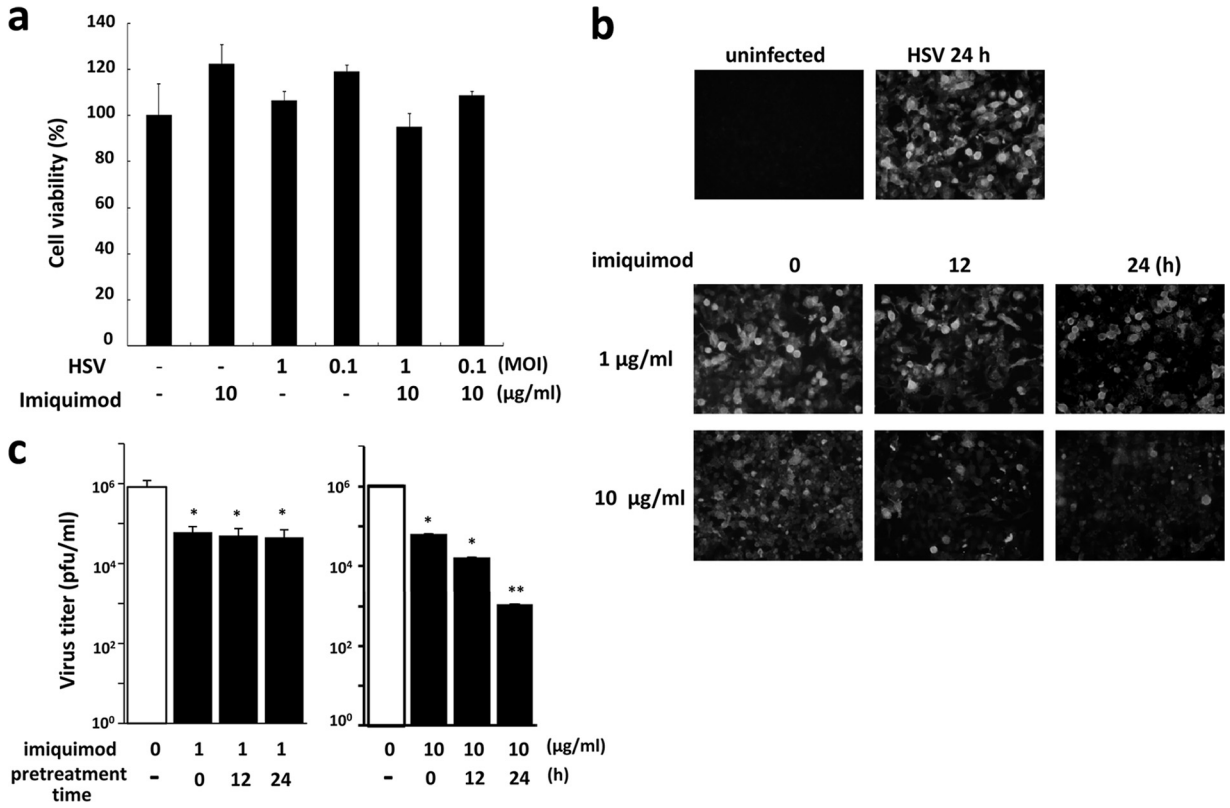

FIG 1 Imiquimod suppressed HSV-1 propagation in FL cells without affecting cell viability. (a) Cytotoxicity by imiquimod treatment and/or HSV-1 infection was measured by cell viability assay as described in the text. FL cells were cultured with or without imiquimod (10 μg/ml) for 24 h and infected with HSV-1 at an MOI of 0 (mock), 0.1, or 1.0 PFU/cell and cultured for a further 24 h. Cells without imiquimod or virus infection were used as a control (100%). Each experiment was carried out in triplicate. and the means ± the standard deviations (SD) are presented. (b) Fluorescence microscope imaging of HSV-1-infected FL cells. Cells were cultured in the presence or absence of imiquimod for 0, 12, or 24 h and then infected with HSV-1. At 24 h after infection, the cells were fixed with methanol and stained with a fluorescence-labeled anti-HSV-1 antibody. (c) Virus titers in the culture supernatants were measured by plaque assay. Cells were cultured in the presence or absence of imiquimod for 0, 12, or 24 h and infected with HSV-1 for 24 h as described in the text. Black and white bars indicate cells incubated with or without imiquimod, respectively. Each experiment was carried out in triplicate, and the means ± the SD are presented. *, $P < 0.05$; **, $P < 0.01$.

cells using FUGENE 6 reagent (Roche-Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. After 24 h of culture, some, but not all, of the cells were treated with 10 μg of imiquimod/ml or not for 24 h. The cells were then infected with HSV-1 VR3 at an MOI of 1. The virus titer in the culture supernatant was determined as described above.

Binding to adenosine receptor determined by competition assay. ^{3}H-labeled DPCPX, which is a selective antagonist of adenosine A_1 receptor, was purchased from Perkin-Elmer (Waltham, MA). CHO-A1 or CHO-K1 cells (17) were seeded into a 96-well plate at a density of 2×10^3 cells per well, followed by incubation overnight. The cells were incubated with [^{3}H]DPCPX (550 Bq/well) and competition chemicals (imiquimod and resiquimod [each at 100 μM] or DPCPX [1 μM]) in the IMDM containing 10% FBS and penicillin-streptomycin for 20 min in a CO_2 incubator. After incubation, the cells were washed four times with PBS (−) and lysed with lysis buffer (1% NP-40, 5% glycerol, 5 mM EDTA, 100 mM NaCl, 50 mM HEPES [pH 7.4]). The counts per minute (cpm) of [^{3}H]DPCPX in the lysate were measured using a LS6500 liquid scintillation counter (Beckman Coulter). Selective binding amounts of [^{3}H]DPCPX on adenosine receptor A_1 of CHO-A1 cells were calculated as the cpm value of CHO-A1 subtracted from the cpm value of CHO-K1 cells as a control. The cpm value without competitors was set to 100%.

Statistical analysis. Determination of statistical significance was carried out by using the Student *t* test.

RESULTS

Imiquimod treatment decreased the HSV-1 level and the release of infectious viruses in infected FL cells. FL cells were treated with imiquimod under various conditions (pretreatment times and concentrations), infected with HSV-1 at an MOI of 1 or 10, and then incubated for 24 h. Imiquimod treatment (1 or 10 μg/ml) did not show any cytotoxicity toward FL cells (Fig. 1a). The cell numbers with positive staining and intensity of staining with the fluorescein-labeled anti-HSV-1 antibody and the amounts of infectious virus particles in the culture medium were decreased by treatment with imiquimod in dose-dependent and pretreatment time-dependent manners (Fig. 1b and c). These results suggested that imiquimod reduced the levels of intracellular and extracellular viruses. Furthermore, imiquimod was effective in suppressing the release of HSV-1 even after inoculation of the virus (pretreatment time zero, Fig. 1b and c). On the other hand, resiquimod did not show anti-HSV-1 activity in FL cells at a concentration of 10 μg/ml (Fig. 2). The imiquimod-induced anti-HSV-1 activities were also observed another HSV-1 strain KOS (data not shown).

To evaluate the effect of imiquimod on the expression of HSV-1 proteins, Western blot analyses were performed (Fig. 3).

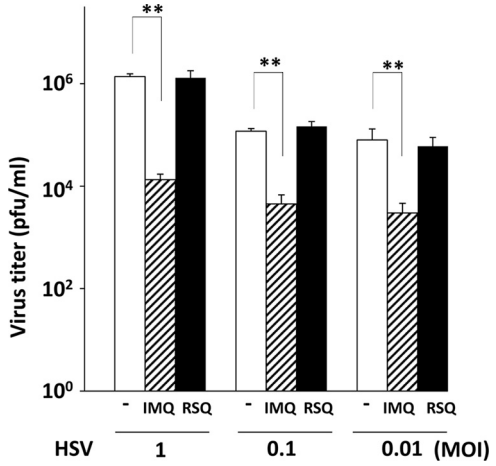

FIG 2 Resiquimod did not show anti-HSV-1 activity in FL cells. FL cells were treated with 10 μg of imiquimod (IMQ) or resiquimod (RSQ)/ml for 24 h and then infected with HSV-1 at an MOI of 0.01, 0.1 or 1 PFU/cell. At 24 h after infection, virus titers in the supernatants were determined by plaque assay. Each experiment was carried out in triplicate, and the means ± the SD are presented. **, $P < 0.01$.

Until 6 h postinfection, immediate-early (IE) proteins (ICP4 and ICP0) in imiquimod-treated infected cells were expressed at levels similar to those in untreated infected cells. By 12 h postinfection, expression of the IE proteins, early (E) protein (TK), late (L) protein (VP16), and true late (TL) protein (gC) in HSV-1-infected FL cells was significantly suppressed in imiquimod-treated cells compared to untreated cells. The results indicated that HSV-1 entered FL cells at similar levels regardless of the presence or absence of imiquimod, but the replication process after the initial expression of IE gene products was suppressed by imiquimod.

Cystatin A was induced in imiquimod-treated FL cells. To investigate the mechanism of the antiviral effect of imiquimod in

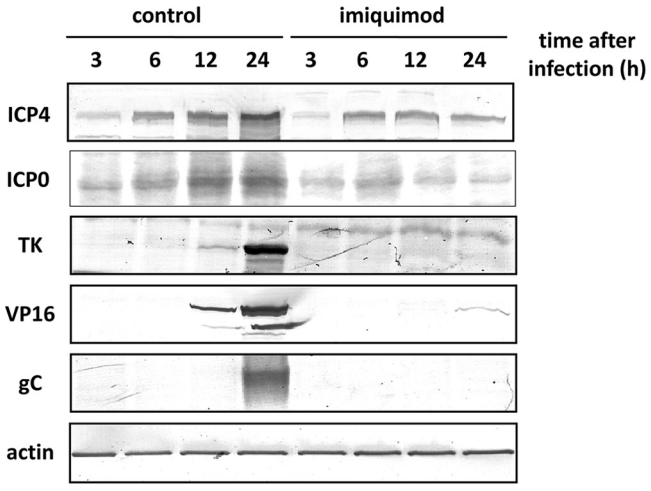

FIG 3 Effect of imiquimod on the expression of viral proteins in HSV-1-infected FL cells. The levels of HSV-1 proteins were determined by Western blotting. Cells were treated with 10 μg of imiquimod/ml for 24 h and then infected with HSV-1 at a MOI of 1. Cell lysates were collected at the indicated infectious periods. ICP4, infected-cell polypeptide 4; ICP0, infected-cell polypeptide 0; TK, thymidine kinase; VP16, virion polypeptide 16; gC, glycoprotein C. Analysis of actin was performed as a control.

TABLE 1 Modulation of IFN and IFN-stimulated gene expression by imiquimod treatment in FL cells

RefSeq_ID	Gene	Ratio[a]
NM_016562	Toll-like receptor 7 precursor	ND
NM_016610	Toll-like receptor 8 precursor	0.5
NM_005534	IFN-γ receptor β chain precursor	ND
NM_021268	IFN-α-17 precursor	1.0
NM_021057	IFN-α −14 precursor	0.8
NM_017999	Transcriptional regulator ISGF3 subunit γ	1.3
NM_002176	IFN-β precursor	1.1
NM_030980	IFN-stimulated 20-kDa exonuclease-like 2	1.3
NM_002200	IFN regulatory factor 5	ND
NM_004031	IFN regulatory factor 7	0.9
NM_000594	Tumor necrosis factor precursor	ND
NM_000575	IL-1 α precursor	0.9
NM_000882	IL-12 subunit α precursor	ND
NM_000600	IL-6 precursor	0.7
NM_000584	IL-8	1.2
NM_000586	IL-2 precursor	ND

[a] Imiquimod treatment/control ratio. ND, not detected.

FL cells, we examined the modulation of cellular gene expression by imiquimod using microarray analysis. The results indicated that the expression of IFNs, TLRs, or IFN-stimulated genes was not altered significantly more than 1.5-fold (Table 1). Indeed, IFN-α and IFN-β proteins were not detected in the supernatants of imiquimod-treated FL cells by ELISA (data not shown). These results suggested that the IFN system did not contribute to the anti-HSV-1 effect of imiquimod in FL cells.

Among cellular genes with increased expression of >10-fold in the imiquimod-treated cells, cystatin A showed the greatest increase (Table 2). Exogenously added cystatins were reported to show anti-HSV-1 activity (24), so we focused on the cystatin A induced by imiquimod. mRNAs of other members of cystatins, namely, cystatin B and C, were also upregulated by imiquimod (10 μg/ml) in FL cells by RT-PCR (Fig. 4a). However, cystatin A was induced more potently than cystatins B and C. Resiquimod did not induce cystatin A mRNA even at a concentration of 10 μg/ml (Fig. 4b).

Cystatin A suppressed HSV-1 propagation. We examined whether imiquimod-induced cystatin A contributes to the suppression of HSV-1 propagation. First, FL cells were treated with recombinant cystatin A protein (0 to 2 μg/ml) for 24 h and then infected with HSV-1. Exogenously added cystatin A significantly suppressed the release of HSV-1 infectious particles into the culture medium (Fig. 5a), whereas it did not show any cytotoxicity to FL cells in this condition (data not shown). The expression of viral proteins were also examined. Similar to the imiquimod treatment

TABLE 2 Highly upregulated genes by imiquimod treatment in FL cells determined by using microarray analysis

RefSeq_ID	Gene	Ratio[a]
NM_005213	Cystatin A	25.8
NM_032621	Brain-expressed X-linked protein	21.9
NM_152703	Sterile alpha motif domain containing 9-like	13.4
NM_014331	Cystine/glutamate transporter	10.3
NM_174941	Scavenger receptor cysteine-rich type 1 protein M160 precursor	10.0

[a] Imiquimod treatment/control ratio.

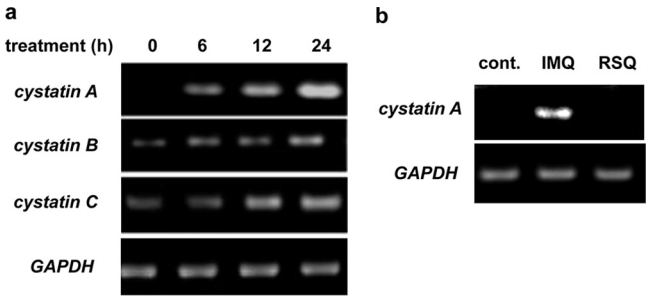

FIG 4 Effect of imiquimod and resiquimod on the expression of cystatins in FL cells. (a) Expression of cystatin A, B, and C in FL cells treated with imiquimod. FL cells were treated with 10 μg of imiquimod/ml. At the indicated times after treatment, total RNA was extracted and mRNA levels of cystatins were examined by RT-PCR. (b) Expression of cystatin A mRNA in FL cells treated with imiquimod (IMQ) or resiquimod (RSQ) (10 μg/ml each) determined by RT-PCR. The GAPDH level was determined as a control.

(Fig. 3), initial (prior to 6 h of infection) protein expression of IE gene products was similar in cells with or without cystatin A treatment (data not shown). This indicated that exogenously added cystatin A suppressed HSV-1 replication, but not the attachment

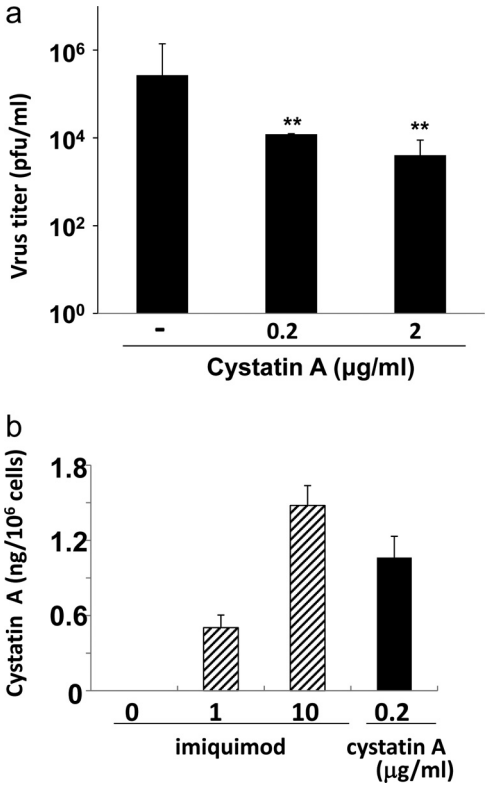

FIG 5 Internalization to cells and suppression of HSV-1 replication of exogenously added cystatin A protein in FL cells. (a) FL cells were treated with 0.2 or 2 μg of human recombinant cystatin A protein/ml for 24 h and then infected with HSV-1 at an MOI of 1. At 24 h after infection, culture supernatants were collected, and virus titers were determined by the plaque assay. Each experiment was carried out in triplicate, and the means ± the SD are presented. **, $P < 0.01$. (b) The amounts of intracellular cystatin A were measured by ELISA. After cells were cultured in the medium containing imiquimod or cystatin A for 24 h, they were collected, lysed, and processed for the measurement of cystatin A protein by ELISA. Each experiment was carried out in triplicate, and the means ± the SD are presented.

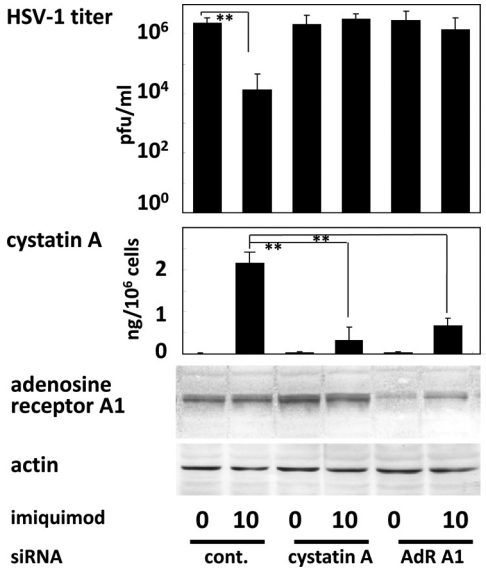

FIG 6 Effect of suppression of cystatin A and adenosine receptor A_1 expression by siRNA on imiquimod-induced cystatin A expression and HSV-1 proliferation. The siRNA specific for cystatin A or adenosine receptor A_1 was transfected into FL cells. After incubation for 24 h, the cells were treated in the presence or absence of imiquimod for 24 h. Cystatin A and adenosine receptor A_1 (AdR A1) levels in the cell lysates were determined by ELISA and Western blotting, respectively. The actin level was determined as a control. The cells transfected with siRNA and then treated (or not) with imiquimod were infected with HSV-1 at an MOI of 1. After 24 h of infection, virus titer in the culture supernatant was determined by a plaque-forming assay using Vero cells as an indicator. All experiments were carried out in triplicate. The data represent means ± the SD.

of HSV-1 to cells. In the FL cells treated with imiquimod, cystatin A proteins were detected in cells by ELISA (Fig. 5b), but not in the culture medium (data not shown). Exogenously added cystatin A (0.2 μg/ml) was detectable in the cells (approximately 1 ng/10^6 cells, Fig. 5b), in an amount comparable to that in the imiquimod-treated cells (approximately 1.5 to 2 ng/10^6 cells). In addition, exogenously added cystatin A did not alter the mRNA levels of cystatin A, so it did not influence the *de novo* synthesis of cystatin A. The lines of evidence indicated that exogenously added cystatin A was internalized into FL cells, and the amount of cystatin A incorporated into FL cells was comparable to that upregulated by imiquimod in cells.

To evaluate the contribution of cystatin A to anti-HSV-1 activity, the imiquimod-induced cystatin A expression was suppressed by transfection of the specific siRNA. Suppression of cystatin A expression was confirmed by ELISA. Anti-HSV-1 activity by imiquimod was diminished in cells transfected with siRNA (Fig. 6). These results suggested that cystatin A induced by imiquimod suppressed the proliferation of HSV-1 in FL cells.

Imiquimod-mediated cystatin A induction was dependent on the adenosine receptor A_1/PKA pathway. Imiquimod is known as a preferential agonist for TLR7; however, another TLR7 agonist, resiquimod, did not show anti-HSV-1 activity (Fig. 2). The mRNA species of all TLRs (TLRs 1 to 10) were detected in FL cells determined by RT-PCR (Fig. 7a). Whereas poly(I·C) (a TLR3 agonist) and *E. coli* LPS (a TLR4 agonist) activated NF-κB determined by DNA binding using ELDIA in FL cells, imiquimod and resiquimod did not (Fig. 7b). The results indicated that TLR7 and

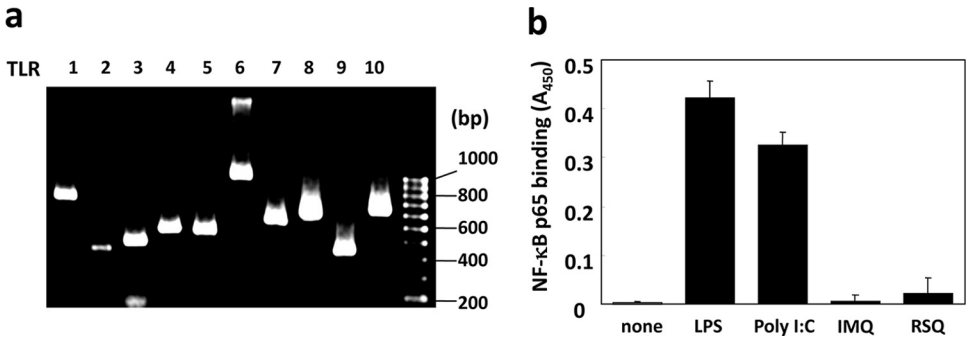

FIG 7 Expression and function of TLRs in FL cells. (a) mRNA expression of TLRs in FL cells determined by RT-PCR. Lanes 1 through 10 correspond to TLR 1 through 10, respectively. (b) Functional analysis of TLRs in FL cells. FL cells were treated with LPS (TLR4 agonist), poly(I·C) (TLR3 agonist), imiquimod (IMQ; TLR7 agonist), or resiquimod (RSQ; TLR7/8 agonist) at 37°C for 6 h. Nuclear extracts prepared from the treated cells were applied to ELDIA for analysis of activated NF-κB p65. The experiments were performed in triplicate. The data represent means ± the SD.

TLR8 were not functional in FL cells, whereas TLR3 and TLR4 were functional. This suggested that imiquimod transduces intracellular signals that elicit anti-HSV-1 activity independently of TLR7.

We investigated the molecular mechanism of cystatin A induction by imiquimod. Transcription of cystatin A is controlled by PKA, PKC, and Ca^{2+} (36, 38). PKA inhibitors (Rp-8-Br-cAMPs and PKI6-22 amide) suppressed imiquimod-induced cystatin A expression, whereas the PKC inhibitors (GF109203X and calphostin C) were not altered (Fig. 8a). We also examined pathways of NF-κB and TAK1-binding kinase 1 (TBK1), which are downstream molecules of MyD88- and TRIF-dependent pathways of TLR signaling, respectively. Neither NF-κB inhibitor (PDTC) nor TBK1 inhibitor (BX795) altered imiquimod-induced cystatin A expression (Fig. 8a). These results suggested that imiquimod upregulated cystatin A via PKA.

Schön et al. reported that imiquimod acted as not only an agonist for TLR7 but also an antagonist for adenosine receptors (31). We also observed that FL cells clearly expressed adenosine receptor A_1 as detected by RT-PCR, whereas the other types of

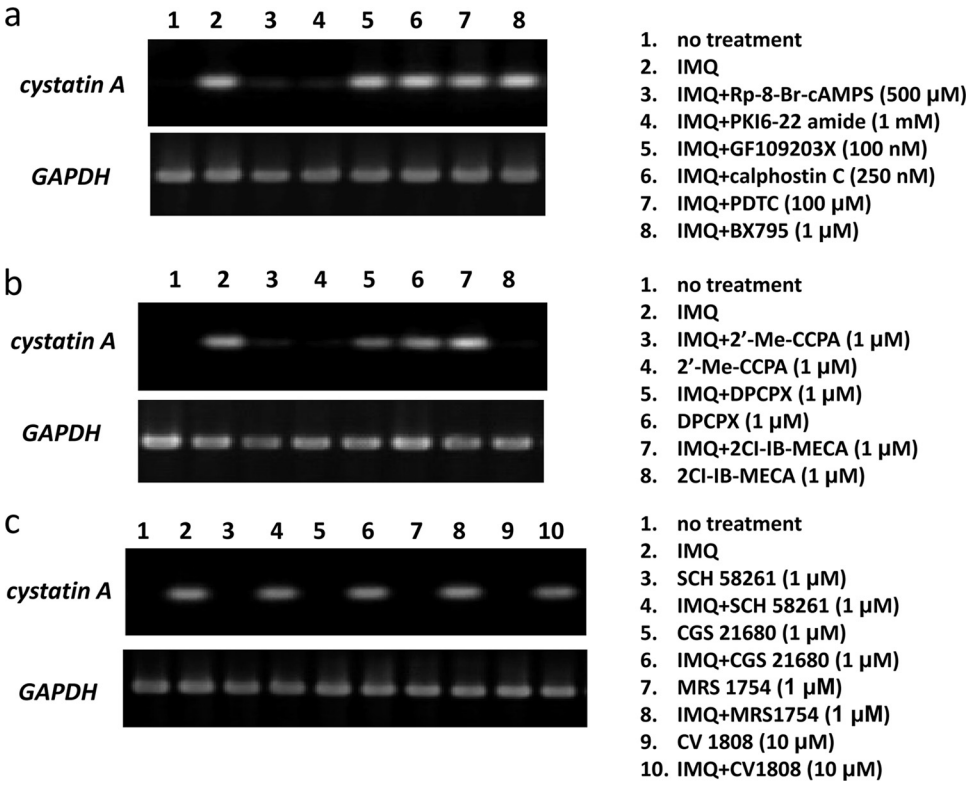

FIG 8 Effect of antagonists and agonists for adenosine receptors and inhibitors for PKA, PKC, NF-κB, and TBK-1 on the imiquimod (IMQ)-induced cystatin A expression. The FL cells were treated with reagents indicated for 24 h. After treatment, total RNA was isolated, and the cystatin A mRNA level was determined by RT-PCR. The GAPDH level was determined as a control. The reagents used were PKA inhibitors (Rp-8-Br-cAMPS and PKI6-22 amide), PKC inhibitors (GF109203X and calphostin C), NF-κB inhibitor (PDTC), TBK1 inhibitor (BX795), adenosine receptor A_1 agonist (2′-Me-CCPA), A_1 antagonist (DPCPX), A_3 agonist (2Cl-IBMECA), A_{2A} antagonist (SCH 58261), A_{2A} agonist (CGS 21680 hydrochloride), A_{2B} antagonist (MRS 1754), and $A_{2A/B}$ agonist (CV 1808).

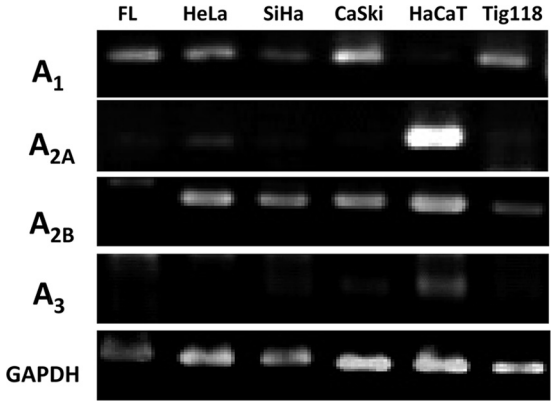

FIG 9 Expression of adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 in various cell lines. The mRNA expressions of adenosine receptors in FL, HeLa, SiHa, CaSki, HaCaT, and Tig118 cells were analyzed by RT-PCR. GAPDH was amplified as a control.

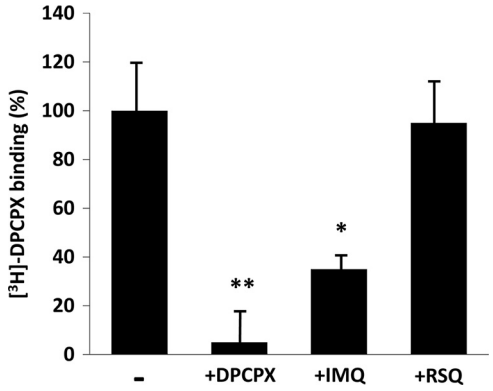

FIG 10 Competition with imiquimod and resiquimod of the binding of adenosine receptor antagonist [^{3}H]DPCPX to CHO-K1 cells stably expressing human adenosine receptor A_1 (CHO-A1 cells). [^{3}H]DPCPX (500 Bq/well) was added to the cell culture of CHO-K1 cells and CHO-A1 cells in the presence or absence of competitor (DPCPX, 1 μM; imiquimod [IMQ], 100 μM; or resiquimod [RSQ], 100 μM). After the cells were washed, the radioactivity (cpm) of the cell lysate was determined. The values obtained from CHO-A1 cells were subtracted from the value obtained from CHO-K1 cells. The value without competitor is set as 100%. **, $P < 0.01$; *, $P < 0.05$.

adenosine receptors did not (Fig. 9). The adenosine receptors are G protein-coupled receptors, and they modulate PKA activities and the intracellular concentration of cAMP via interaction with specific ligands such as adenosine and ATP. Adenosine receptor A_1 transduces a negative signal, namely, downregulates PKA activity, whereas adenosine receptors A_{2A} and A_{2B} transduce positive signals (7, 11). We therefore examined the effect of agonists and antagonists specific for adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) on the induction of cystatin A by imiquimod. An A_1 antagonist DPCPX alone upregulated cystatin A mRNA, and the induction was not altered by imiquimod, whereas an A_1 agonist 2′-Me-CCPA suppressed imiquimod-induced cystatin A mRNA induction, and 2′-Me-CCPA alone did not alter cystatin A expression (Fig. 8b). Antagonists and agonists specific for other families of adenosine receptors did not alter the expression of cystatin A or the induction of cystatin A by imiquimod (Fig. 8b and c).

Since adenosine receptor A_1 transduces negative signals, imiquimod was suggested to act as an antagonist for adenosine receptor A_1, similar to DPCPX, and induced cystatin A via PKA. Furthermore, TLRs, including TLR7, were not involved in cystatin A expression induced by imiquimod. In addition to the agonist/antagonist experiments described above, transfection of adenosine receptor A_1-specific siRNA significantly suppressed not only imiquimod-induced cystatin A expression but also anti-HSV-1 activity in FL cells (Fig. 6). These results suggested that the adenosine receptor A_1-mediated signaling is responsible for the anti-HSV1 activity induced by imiquimod.

The interaction of imiquimod to adenosine receptor A_1 was examined by competition assay using a tritium-labeled adenosine receptor A_1 antagonist DPCPX. Selective binding amounts of [^{3}H]DPCPX on CHO-K1 cells stably expressing human adenosine receptor A_1 (CHO-A1 cells) were determined in the presence of unlabeled DPCPX, imiquimod, or resiquimod. Under the condition in which DPCPX almost completely blocked specific [^{3}H]DPCPX binding by 1 μM, imiquimod significantly blocked the binding by 100 μM, whereas resiquimod did not (Fig. 10). This result indicated that imiquimod interacted with adenosine receptor A_1, whereas resiquimod did not. However, imiquimod shared less affinity with adenosine receptor A_1 than did DPCPX.

Imiquimod induced cystatin A expression and anti-HSV-1 activity in other cells. The effect of imiquimod on the expression of cystatin A mRNAs in HeLa, SiHa, CaSki, HaCaT, and Tig118 cells was investigated (Fig. 11). Cystatin A was significantly induced by imiquimod treatment in HeLa, SiHa, and CaSki cells. HaCaT cells expressed relatively high levels of cystatin A in nontreated cells, and the cystatin A expression levels were not altered by imiquimod. Tig118 fibroblast cells did not show cystatin A mRNA expression under nonstimulation and imiquimod-treated conditions. Adenosine receptor A_1 mRNA was detected in these cells except in HaCaT cells (Fig. 9). Adenosine receptor A_{2B} mRNA was detected in these cells, except for FL cells. HaCaT cells strongly expressed adenosine receptor A_{2A} mRNA.

We also examined the effect of imiquimod treatment on HSV-1 propagation in these cells. Consistent with the induction of cystatin A, anti-HSV-1 activity was induced by imiquimod in HeLa, SiHa, and CaSki cells (Fig. 11). These results suggested that cystatin A induction and anti-HSV-1 activity induced by imiquimod were observed in various, but not all, cells other than FL cells.

DISCUSSION

Imiquimod induces IFN preferentially in pDCs, resulting in an innate antiviral immune response. The IFN induces many IFN-stimulated antiviral genes, such as 2′-5′-oligoadenyl-synthetase, dsRNA-activated protein kinase, and myxovirus resistance protein A (27, 32). We found that imiquimod suppressed HSV-1 propagation in FL cells; however, we found no evidence that the expression of IFNs, TLRs, IFN regulatory factors, or other proinflammatory cytokines were altered by imiquimod by microarray analysis. This suggested that the anti-HSV-1 activity of imiquimod does not depend on the IFN system.

Microarray analysis indicated marked upregulation of cystatin A, which is a host cysteine protease inhibitor and whose typical target is cathepsins. Exogenously added cystatin A suppressed propagation of HSV-1 as reported previously (24), which suggested that it internalized into FL cells (Fig. 5). Suppression of

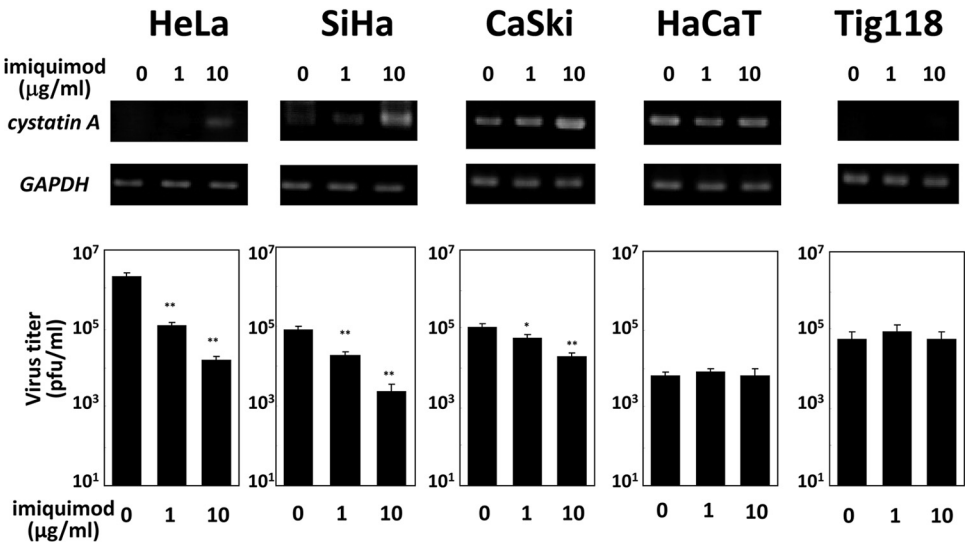

FIG 11 Effect of imiquimod on the expression of cystatin A and HSV-1 replication in various cell lines. The cells HeLa, SiHa, CaSki, HaCaT, and Tig118 were treated with 1 or 10 μg of imiquimod/ml for 24 h. Total RNA was prepared, and the cystatin A mRNA levels were determined by RT-PCR. GAPDH was amplified as a control. The imiquimod-treated cells were infected with HSV-1 at an MOI of 1. After 24 h (for HeLa cells) or 48 h (for other cells) of infection, the culture supernatants were collected, and the virus titers were determined by a plaque-forming assay using Vero cells as an indicator. The experiments were carried out in triplicate. The data represent means ± the SD.

cystatin A expression by siRNA decreased the imiquimod-induced anti-HSV-1 activity (Fig. 7). Lines of evidence indicated that cystatin A conferred a major role in the anti-HSV-1 activity of imiquimod in FL cells.

Imiquimod is also reported to be an antagonist for adenosine receptors, and it induces the inflammatory response via both the TLR7 and adenosine receptor pathways (31). We found that the cystatin A induction by imiquimod was suppressed by the adenosine receptor A_1 agonist, 2′-Me-CCPA, and PKA inhibitors (Fig. 8). TLR7 was not functional in FL cells, whereas TLR7 mRNA was detected by RT-PCR. Moreover, the cystatin A induction was mediated by neither the MyD88- nor the TRIF-dependent pathway. These findings suggested that the anti-HSV-1 effect of imiquimod in FL cells is mediated via the adenosine receptor A_1 pathway. Imiquimod appeared to suppress HSV-1 replication but not virus entry. This is because viral proteins derived from IE genes (ICP4 and ICP0) were at similar levels during the early stage of infection (until 6 h after infection) in the presence or absence of imiquimod.

Resiquimod is an analog of imiquimod and an agonist for both TLR7 and TLR8 (9, 30). Resiquimod showed neither suppression of HSV-1 propagation nor induction of cystatin A expression. We demonstrated that imiquimod interacted with adenosine receptor A_1, whereas resiquimod did not. The anti-HSV-1 activity of imiquimod independent to IFN system was observed in various, but not all, nonimmune cells, and was closely related to cystatin A induction. That cystatin A was not induced by imiquimod in HaCaT cells may have some connection to the fact that HaCaT highly expressed adenosine receptor A_{2A} but not A_1.

Cystatins are one of the host cysteine protease inhibitors, and the typical target is cathepsin. Three types (A, B, and C) of cystatins have been identified according to their structure and distribution in the body (41). Cystatins are involved in a variety of physiological processes, including antigen processing and presentation and extracellular matrix degradation (41). Cystatin A is expressed abundantly in the epithelium of skin tissues and contributes to the local innate immune defense (23). Therefore, cystatin A is believed to have important roles in the protection against infections in the epithelium. It is well known that patients with atopic dermatitis are prone to Kaposi's varicelliform eruption that is caused by the infection and cutaneous expansion of HSV-1. Since skin barrier function is defective in atopic dermatitis, including a downregulation of cystatin A in the corneal layer, it is conceivable that a decreased level of cystatin A is related to cutaneous infection of HSV-1 in atopic dermatitis.

The antiviral mechanisms of cystatin A have not been revealed yet. Proteolytic cleavage steps are essential for the infectious processes of various viruses, including hepatitis C (3), human immunodeficiency (39), and influenza (16) viruses. There are reports regarding the ability of the cystatin family to prevent viral replication, in which the antiviral activities of several cystatins were evaluated by inhibiting the cysteine protease activities of viruses (1, 2, 4, 24). The HSV-1 tegument protein VP1-2 must be cleaved by a serine or cysteine protease in order for the viral DNA to be released into the nucleus (12). A serine protease inhibitor prevents the release of viral DNA from the capsid and suppresses HSV-1 replication. Peri et al. reported that cystatins partially inhibit HSV-1 propagation (24). They speculated that the ubiquitin-specific cysteine protease $UL36^{USP}$, which is an N-terminal fragment generated by proteolytic cleavage of pUL36 of HSV-1, is a target for cystatins. On the other hand, cystatins are specific inhibitors of cathepsins, thus the contribution of cathepsins to the process of HSV-1 proliferation needs to be examined.

In conclusion, the inhibitory effect of imiquimod on HSV-1 propagation was not mediated by TLR7 or type I IFN but was associated with the induction of cystatin A by which antagonized the adenosine receptor A_1 pathway in nonimmune FL cells. This direct antiviral effect, which depended on cystatin A, is a novel antiviral mechanism of imiquimod but not resiquimod. The difference in these TLR7 agonists can be seen not only in the agoniza-

tion of TLR8 but also in the antagonization of adenosine receptors.

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