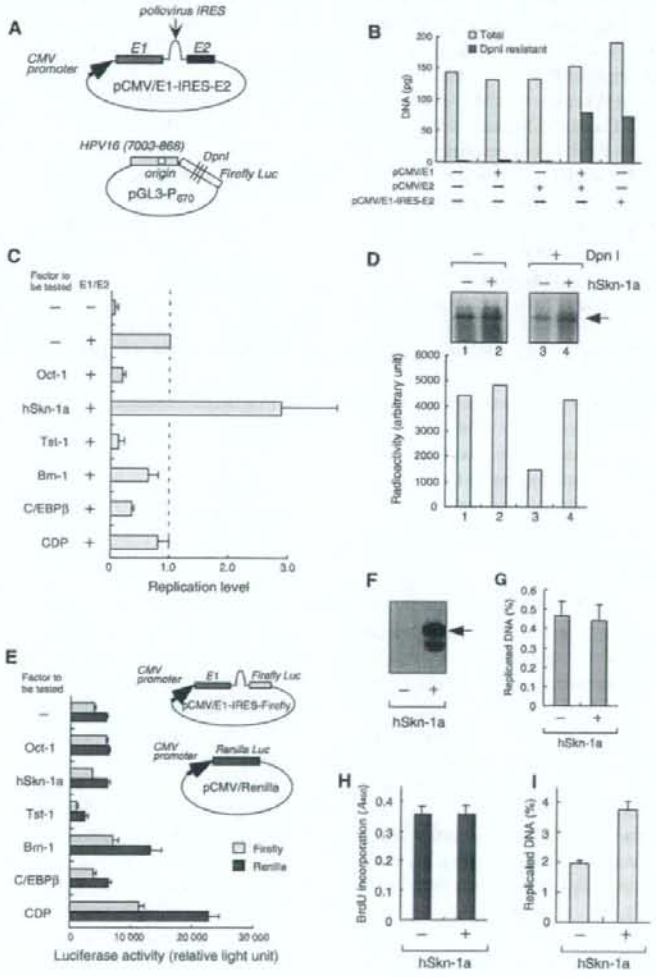
between E1 and E2 ORFs, can express both E1 and E2 proteins from a single cytomegalovirus (CMV) immediate early promoter (Fig. 1A). HEK293 cells were transfected with pGL3-P_{670} together with pCMV/E1-IRES-E2 or with a mixture of expression plasmids for E1 (pCMV/E1) and E2 (pCMV/E2). Seventy-two hours later, the episomal DNA was extracted from the cells by Hirt procedure. The DNA sample (total episomal DNA) was digested with *Dpn*I, which can cleave input methylated DNA but cannot cleave the replicated unmethylated DNA. The levels of total episomal and replicated pGL3-P_{670} were measured by quantitative PCR analysis (Fig. 1B). The HPV16 origin-mediated DNA replication occurred when both E1 and E2 were co-expressed. Similar results were obtained by using Southern blot analysis (data not shown), verifying that the quantitative PCR analysis can yield consistent results for HPV replication, as reported previously [21]. Because pCMV/E1-IRES-E2 supported the replication as efficiently as the mixture of pCMV/E1 and pCMV/E2, pCMV/E1-IRES-E2 was used in the following experiments.

We examined the co-expression of the selected transcription factors for the effect on the transient replication of pGL3-P_{670} and found that hSkn-1a enhanced the replication. One of the expression plasmids for hSkn-1a, Oct-1, Tst-1, Brn-1, C/EBPβ and CDP was co-transfected into HEK293 cells together with



pGL3-P_{670} and pCMV/E1-IRES-E2 in the transient replication assay (Fig. 1C). The relative amount of the replicated pGL3-P_{670} was determined by dividing the amount of the *Dpn*I-resistant pGL3-P_{670} by that of the total episomal pGL3-P_{670}. The effect of the factor was presented as the replication level, which was the ratio of the relative amount obtained with the factor to that obtained without the factor. hSkn-1a enhanced the replication by two- to three-fold. The enhancement was confirmed by detection of the replicated *Dpn*I-resistant DNA by Southern blotting (Fig. 1D). The other POU-domain factors, Oct-1, Tst-1 and Brn-1, repressed the replication. C/EBPβ also repressed the replication. The ubiquitous transcription repressor CDP showed little effect on the replication. The replication enhancement by hSkn-1a was similarly observed in HeLa cells (data not shown), indicating that the enhancement is not restricted to HEK293 cells.

The enhancement by hSkn-1a was not due to the enhanced expression of E1 and E2 because the expression of hSkn-1a did not affect the transcription from the CMV promoter and the IRES-dependent translation. HEK293 cells were co-transfected with pCMV/Renilla, which expresses *Renilla* luciferase from the CMV promoter, and pCMV/E1-IRES-Firefly, which was produced by the replacement of E2 in pCMV/E1-IRES-E2 with the firefly luciferase gene, together with one of the expression plasmids for the transcription factors. The two luciferase activities in the cell lysates were measured at 72 h after the transfection to monitor the effects of the transcription factor on the transcription from the CMV promoter (*Renilla*) and on the IRES-dependent translation (firefly). As shown in Fig. 1E, hSkn-1a showed only a marginal effect on the both luciferase activities, indicating that the transcription from the CMV promoter and the IRES-dependent translation were not affected by the over-expression of hSkn-1a.

Although Brn-1 and CDP enhanced firefly and *Renilla* luciferase activities (Fig. 1E), these factors were incapable of enhancing the transient replication of pGL3-P_{670} (Fig. 1C), suggesting that the levels of E1 and E2 produced from pCMV/E1-IRES-E2 were sufficient to induce the transient replication of pGL3-P_{670} efficiently and that the level of the replication was independent of the levels of E1 and E2 under the conditions used in the present study.

Western blot analysis revealed that exogenous hSkn-1a was efficiently expressed from the expression plasmid, whereas endogenous hSkn-1a was not detected in HEK293 cells (Fig. 1F).

The expression of hSkn-1a affected neither the replication from the Epstein–Barr virus latent origin of plasmid replication (oriP), nor the replication of cellular DNA. HEK293 cells were transfected with the oriP-containing plasmid (pREP4) with or without the hSkn-1a expression plasmid, and the replicated pREP4 was quantitated at 72 h after the transfection. The levels of the replicated pREP4 with and without hSkn-1a were similar, indicating that hSkn-1a did not enhance the oriP-dependent DNA replication (Fig. 1G). HEK293 cells transfected with the hSkn-1a expression plasmid were incubated for 24 h and then labeled for 24 h with bromodeoxyuridine (BrdU) that was added to the culture medium. The level of the nuclear BrdU, which was incorporated into the newly synthesized cellular DNA, was not affected by the expression of hSkn-1a (Fig. 1H). These data strongly suggest that the hSkn-1a-mediated enhancement of replication is specific to the HPV origin-containing plasmid in HEK293 cells.

Furthermore, hSkn-1a enhanced the replication of an authentic HPV16 full-genome DNA. The full-genome DNA of HPV16 was excised from a bacterial plasmid and self-ligated to generate a complete circular viral genome. HEK293 cells were transfected with the HPV16 genome and pCMV/E1-IRES-E2 with or without the hSkn-1a expression plasmid, and the level of replicated HPV16 genome was determined at 72 h after the transfection. The genome replication level with hSkn-1a was approximately two-fold higher than that without hSkn-1a (Fig. 1I), indicating that the enhancing effect of hSkn-1a is not restricted to the HPV16 origin-containing plasmid, but also extended to the complete viral genome.

Domain of hSkn-1a required for the replication enhancement

Both DNA-binding and transactivation domains of hSkn-1a were required for the replication enhancement. hSkn-1a consists of the centrally located DNA-binding domain (POU-domain) flanked by the N- and C-terminal transactivation domains (Fig. 2A). An hSkn-1a mutant N331A, which has shown to be deficient in DNA-binding to its target sequence [22], and the POU-domain alone did not enhance the replication of pGL3-P_{670} (Fig. 2B).

It should be noted that the transcription from HPV16 P_{670} promoter is activated by the hSkn-1a POU-domain alone (Fig. 2C), and a previous study demonstrated that the P_{670} activation is mediated by replacement of YY1 transcriptional repressor, which has been bound to P_{670}, with hSkn-1a [23]. Considering the different domain requirement, hSkn-1a enhances the replication by some mechanism distinct from the P_{670} activation.

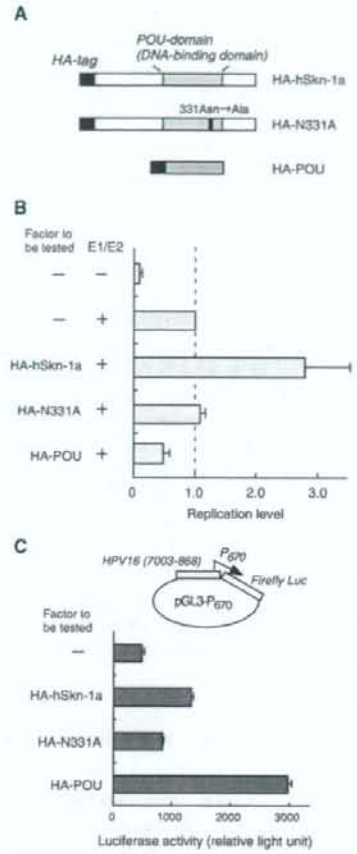


Fig. 2. Domain of hSkn-1a required for the replication enhancement. (A) Schematic representation of the structures of wild-type, DNA-binding deficient and POU-domain forms of hSkn-1a. (B) Effects of DNA-binding deficient and POU-domain forms of hSkn-1a on HPV16 transient replication. Replicated pGL3-P_{670} was measured by real-time PCR with the extract from HEK293 cells that had been co-transfected with three plasmids: pGL3-P_{670}, pCMV/E1-IRES-E2 and a plasmid to be tested (containing hSkn-1a DNA fragments). The relative replication of pGL3-P_{670} has been normalized and is expressed as the ratio of replicated pGL3-P_{670} in the presence of E1/E2 alone. The scale bar represents the mean ± SD of three independent transfection experiments. (C) Effects of DNA-binding deficient and POU-domain forms of hSkn-1a on transcription from HPV16 P_{670} promoter. HEK293 cells were transfected with 200 ng of pGL3-P_{670} and 100 ng of expression plasmids for HA-hSkn-1a, HA-N331A, HA-POU or pHM6 (backbone plasmid), with 5 ng of pCMV/*Renilla*. The luciferase activities of the cell lysate were measured at 48 h after the transfection. Transfection efficiency was normalized using *Renilla* luciferase activity. The scale bar represents the mean ± SD of three independent transfection experiments.

HPV16 genome region responsible for the hSkn-1a-mediated replication enhancement

The cis-element(s) required for the hSkn-1a-mediated replication enhancement was located within the region from nucleotides 7838–100 (designated as the core region), which contains the replication origin. Three fragments of the HPV16 DNA, from nucleotides 7003–100 containing previously identified one hSkn-1a binding site (from nucleotides 7733–7738) [23,24], from nucleotides 7838–100, and from nucleotides 281–864 containing previously identified two hSkn-1a binding sites (from nucleotides 560–569 and from nucleotides 581–590) [25], were cloned into pGL3-Basic (the backbone of pGL3-P_{670}) to produce three plasmids, pGL3-(7003/100), pGL3-(7838/100) and pGL3-(281/864), respectively (Fig. 3A). As shown in Fig. 3B, the

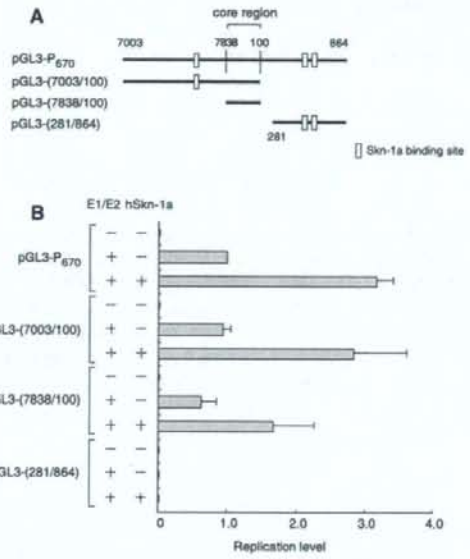


Fig. 3. HPV16 DNA segments responsible for the hSkn-1a-mediated replication enhancement. (A) Schematic representation of the HPV16 DNA segments used in the transient replication assay. Previously identified hSkn-1a binding sites are indicated as open boxes. (B) Effects of hSkn-1a on replication of plasmids containing segments near the HPV16 origin. Replicated pGL3-P_{670}, pGL3-(7003/100), pGL3-(7838/100) or pGL3-(281/864) was measured by real-time PCR with the extract from HEK293 cells that had been co-transfected with (plus) or without (minus) plasmids expressing E1/E2 and hSkn-1a, as indicated. The relative replication of each plasmid has been normalized and is shown as the ratio of replicated pGL3-P_{670} with E1/E2 but without hSkn-1a. The scale bar represents the mean ± SD of three independent transfection experiments.

replication of pGL3-(7003/100) and pGL3-(7838/100) was enhanced by hSkn-1a by approximately two-fold. The replication of pGL3-(281/864), which lacks the origin, was not detected either in the absence or the presence of hSkn-1a. The data clearly indicate that the core region is essential for the replication of pGL3-P_{670} and that the hSkn-1a-mediated replication enhancement does not take place without the core region.

Binding of hSkn-1a to the core region *in vitro*

An electrophoretic mobility shift assay (EMSA) identified two binding sites for hSkn-1a in the core region. Because the core region does not contain the previously identified three hSkn-1a-binding sites (Fig. 3A), additional binding sites for hSkn-1a were explored by EMSA. A bacterially expressed hSkn-1a fused with glutathione *S*-transferase (GST-hSkn-1a) was incubated with radiolabeled DNA probes having nucleotide sequences of the HPV16 genome from nucleotides 7835–7884 (a), from nucleotides 7875–20 (b), from nucleotides 11–62 (c) and from nucleotides 49–100 (d) (Fig. 4A). The complex of GST-hSkn-1a with the probes was detected by mobility shift. As shown in Fig. 4B, GST-hSkn-1a bound to probes b and d, but not to probes a and c.

The nuclear extract from HeLa cells expressing hSkn-1a bound to probes b and d (Fig. 4C). The HeLa/hSkn-1a cells, which express hSkn-1a in response to doxycycline in culture medium [26], were used to obtain nuclear extracts with or without hSkn-1a. The incubation of probes b or d with the nuclear extract containing hSkn-1a gave rise to newly shifted bands, which were not formed by the incubation with the nuclear extract not containing hSkn-1a (Fig. 4C, compare lanes 4 and 8 with lanes 3 and 7, respectively). These bands completely disappeared upon addition of the antibody against hSkn-1a (Fig. 4C, lanes 10 and 12), indicating that these shifted bands contained hSkn-1a. Competition with unlabeled probes b or d depleted the shifted bands (Fig. 4D), demonstrating the sequence-specific binding of hSkn-1a to probes b and d.

The hSkn-1a binding sites #1 and #2 were identified in probes b and d, respectively. The nucleotide sequences of probes b and d contain ATGAATTA (from nucleotides 15–8) and ATGCACCA (from nucleotides 83–90), respectively, which differ in one base from the hSkn-1a-binding consensus sequence (WTGCAWNN) (Fig. 4A,E). Nucleotide substitutions of GTC for ATG were introduced into sites #1 and #2 in probes b and d, respectively, to produce mutated probes mb and md (Fig. 4E). The level of GST-hSkn-1a/mb complex was approximately half of the level of the GST-hSkn-1a/b complex (Fig. 4E, lanes 2 and 4). The level of GST-hSkn-1a/md complex was severely reduced (Fig. 4E, lanes 6 and 8). The results indicate that hSkn-1a binds to sites #1 and #2 in a sequence-specific manner. Site #1 partially overlaps with the E1-binding site, and site #2 resides between the TATA sequence for the early promoter and the E6 coding region (Fig. 4A).

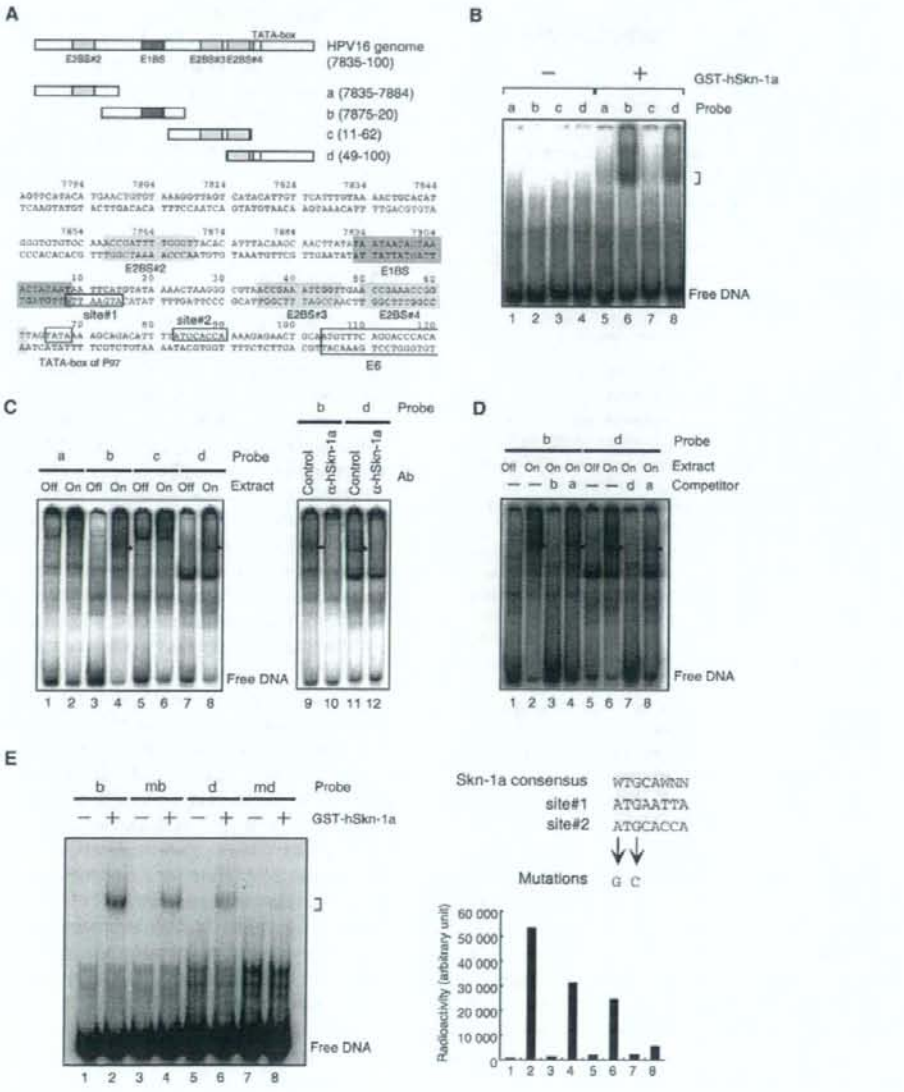
Binding of hSkn-1a to the core region in cells

A chromatin immunoprecipitation (ChIP) showed that hSkn-1a bound to the core region of pGL3-(7838/100) in HEK293 cells. ChIP assays using anti-hSkn-1a or control sera were conducted with HEK293 cells transfected with pGL3-(7838/100) and

Fig. 4. Binding of hSkn-1a to the HPV16 origin region *in vitro*. (A) The DNA probes used in the EMSA. Numbers in parentheses indicate nucleotide numbers of the HPV16 DNA (Los Alamos National Laboratory database, NM, USA). Nucleotide sequences from nucleotides 7785–7904 and nucleotides 1–120 of HPV16 DNA are presented. The binding sites for E1 and E2 are boxed in dark or light gray, respectively, and labeled E1BS for E1, E2BS#2, E2BS#3 and E2BS#4 for E2. Two binding sequences for hSkn-1a identified by EMSAs in the present study are boxed and indicated as sites #1 and #2. The TATA-box sequence for the P_{97} promoter and the coding region of E6 are also boxed. (B) EMSA detecting the complex of the ^{32}P-labeled probes and GST-hSkn-1a. The probe/GST-hSkn-1a complex was electrophoresed on a 5% polyacrylamide gel and visualized by autoradiography. The ^{32}P-labeled probe/GST-hSkn-1a complex is indicated by a square bracket. (C) EMSA detecting the complex of the ^{32}P-labeled probes and hSkn-1a in the HeLa nuclear extract. The HPV16 probes were incubated with nuclear extracts containing hSkn-1a (lanes 2, 4, 6 and 8) or not containing hSkn-1a (lanes 1, 3, 5 and 7). In the right panel, the antibody against hSkn-1a (lanes 10 and 12) or control rabbit IgG (lanes 9 and 11) was added to the reaction mixture. The ^{32}P-labeled probe/hSkn-1a complex is indicated by asterisks. (D) EMSA showing competition between unlabeled probes and the ^{32}P-labeled probe/hSkn-1a complex in the HeLa nuclear extract. The ^{32}P-labeled probes b or d were incubated with nuclear extracts containing hSkn-1a (lanes 2–4 and 6–8) or not containing hSkn-1a (lanes 1 and 5), in the presence of a 50-fold exess of unlabeled probe b (lane 3), unlabeled probe d (lane 7), unlabeled probe a (lanes 4 and 8), or in the absence of competitors (lanes 1, 2, 5 and 6). The ^{32}P-labeled probe/hSkn-1a complex is indicated by asterisks. (E) EMSA detecting the complex of the ^{32}P-labeled mutated probes and GST-hSkn-1a. Potential binding sequences for hSkn-1a and base substitutions introduced in probes b and d are shown in the right upper panel. W indicates A or T. The levels of the shifted band (square bracket) were measured and are shown in the right-hand panel.

the plasmid expressing hSkn-1a. Immunoprecipitates with the anti-hSkn-1a serum contained more core region DNA than the control IgG precipitates (Fig. 5B, lanes 5 and 6). The level of core region DNA in the samples precipitated with the anti-hSkn-1a serum was three-fold higher than that precipitated with the control antibody (three-fold enrichment) (Fig. 5C). Without the plasmid expressing hSkn-1a, the anti-hSkn-1a serum did not enrich the core region DNA in the precipitate (data not shown), indicating that exogenous hSkn-1a binds to the core region DNA in cells.

The nucleotide substitutions in sites #1 and #2 of pGL3-(7838/100) reduced the levels of the enrichment by the anti-hSkn-1a serum. Nucleotide substitutions of GTC for ATG (mutations corresponding to those



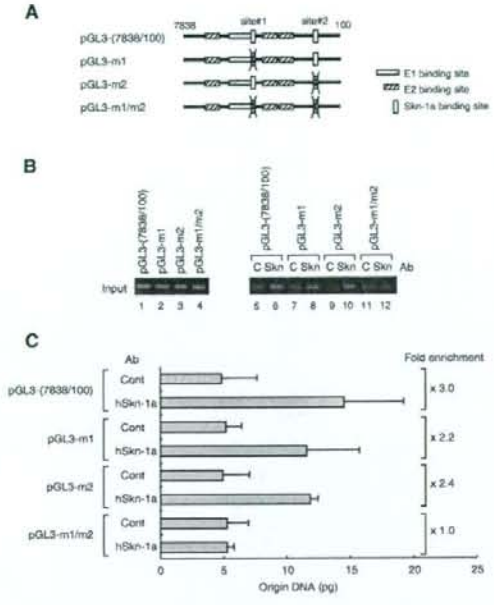


Fig. 5. Binding of hSkn-1a to the HPV16 origin region *in vivo*. (A) Schematic representation of pGL3-(7838/100) with mutations in the hSkn-1a binding sites. The mutations shown in Fig. 4E were introduced into the corresponding positions of pGL3-(7838/100) to produce pGL3-m1, pGL3-m2 and pGL3-m1/m2. The binding sites for E1, E2 and hSkn-1a are indicated. (B) Chromatin immunoprecipitation analysis detecting complex of hSkn-1a and the origin region DNA. HEK293 cells transfected with pGL3-(7838/100), pGL3-m1, pGL3-m2 or pGL3-m1/m2 together with pHM/hSkn-1a were cultured for 72 h and then treated with formaldehyde for cross-linking. The chromatin/hSkn-1a complex was immunoprecipitated with anti-hSkn-1a serum (Skn) or control rabbit IgG (C). DNA was extracted from the precipitates and used as a template for PCR amplification of the origin DNA fragments (from nucleotides 7851 to 90). The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Part (1%) of the total input chromatin was used for PCR analyses (input). (C) The level of immunoprecipitated origin DNA was determined by real-time PCR. Fold enrichment of the precipitated DNA fragments is indicated on the right. The scale bar represents the mean ± SE of two independent experiments.

introduced into mb and md) were introduced into sites #1 and site #2 and both of pGL3-(7838/100) to produce pGL3-m1, pGL3-m2 and pGL3-m1/m2, respectively (Fig. 5A). As shown in Fig. 5B (lanes 7 to 12) and Fig. 5C, the enrichment of the core region DNA in the precipitates obtained with pGL3-m1 and pGL3-m2 was reduced and not detected with pGL3-m1/m2, suggesting that hSkn-1a binds to sites #1 and #2 in a sequence-specific manner in HEK293 cells.

Effect of mutations in hSkn-1a binding sites on replication enhancement

The double mutations in both sites #1 and #2 abolished the hSkn-1a-mediated enhancement of the HPV16 origin-dependent replication. Transient replication assays were conducted with pGL3-(7838/100), pGL3-m1, pGL3-m2 and pGL3-m1/m2 (Fig. 6). The overall levels of replication of pGL3-m1, pGL3-m2 and pGL3-m1/m2 were lower than that of pGL3-(7838/100), probably due to the mutations in the core region sequences [27]. The hSkn-1a-mediated enhancement of the replication of pGL3-m1 and pGL3-m2 was reduced and that of pGL3-m1/m2 was totally abolished (Fig. 6A). Similar results were obtained by Southern blot analysis (Fig. 6B). The results strongly suggest that the binding of hSkn-1a to sites #1 and #2 is necessary for the replication enhancement by hSkn-1a.

Discussion

In the present study, we have shown that hSkn-1a enhances the transient replication of pGL3-P_{670}, a plasmid containing the HPV16 replication origin, in HEK293 cells. The enhancement is mediated by the sequence-specific binding of hSkn-1a to the two sites, from nucleotides 8–15 and from nucleotides 83–90, near the origin. The replication of a plasmid containing the HPV11 origin was similarly enhanced by hSkn-1a (data not shown), suggesting that the enhancing effect of hSkn-1a may be common to the other HPV types.

Although hSkn-1a affects cellular DNA synthesis of undifferentiated cultured cells, the enhancement of HPV DNA replication in HEK293 cells is not caused by the hSkn-1a-mediated activation of cellular DNA synthesis machinery. Primary human keratinocytes inoculated with the recombinant retrovirus expressing hSkn-1a proliferate transiently, express the marker genes for the differentiation, and form stratified layers [15]. The cervical cancer cell lines HeLa, SiHa, CaSki and C33A transfected with an expression plasmid for hSkn-1a trigger the resumption of partial differentiation and cease growing, and eventually result in apoptosis [26]. However, a previous study has shown that the growth capacity of HEK293 cells is not affected by hSkn-1a [26], indicating that hSkn-1a does not up- or down-regulate the expression of genes directly associated with DNA synthesis of HEK293 cells. Thus, HEK293 cells appear to be an appropriate system to detect the hSkn-1a functions apart from those inducing transient cellular DNA synthesis or apoptosis.

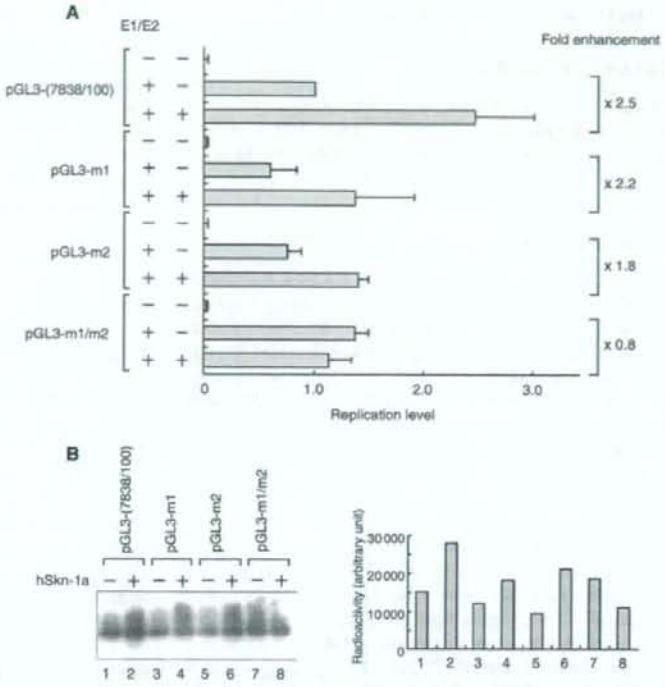


Fig. 6. Effect of hSkn-1a on the replication of mutated origin-containing plasmids. (A) Replicated pGL3-(7838/100), pGL3-m1, pGL3-m2 or pGL3-m1/m2 was measured with the extract from HEK293 cells that had been co-transfected with (plus) or without (minus) plasmids expressing E1/E2 and hSkn-1a, as indicated. The relative replication of each plasmid has been normalized and is shown as the ratio of replicated pGL3-(7838/100) with E1/E2 but without hSkn-1a. The scale bar represents the mean ± SD of three independent transfection experiments. (B) Southern blot analysis of *Dpn*I-resistant replicated pGL3-P_{670}, pGL3-m1, pGL3-m2, or pGL3-m1/m2 in the absence or presence of hSkn-1a co-expression. The signal in the autoradiogram was quantitated and is shown in the right-hand panel.

Mouse Skn-1a was shown to interact with cellular histone acetyltransferases, CREB-binding protein (CBP) and p300 [22]. As is the case with the host genome, the HPV DNA associates with cellular histone octamers in cells to form nucleosomes. In general, the nucleosome structure restricts the access of DNA-binding proteins to their recognition sequences. CBP/p300 acetylates the lysine of the N-terminal tails in histones H3 and H4 and loosens the binding of DNA to a histone octamer by neutralization of the positive charge of histones [28,29]. Thus, the acetylation of histones facilitates recruitment of cellular factors required for transcription and replication to their recognition sequences. Cellular Hbo1 acetyltransferase, which was isolated as an interacting protein with human origin recognition complex, was shown to be involved in the initiation of cellular DNA replication [30]. It is possible that hSkn-1a recruits CBP/p300 to the HPV origin and enhances the HPV replication through acetylation of histones of a viral nucleosome near the origin.

The enhanced expression of E1 from the viral late promoter, such as P_{670} for HPV16 and P_{742} for HPV31, which is located within the *E7* gene, is the initial event for HPV DNA to replicate. CDP [20], YY1 [31], hSkn-1a [23], C/EBPβ [25] and probably unidentified cellular factor(s) are involved in the regulation of the late promoter activity. CDP and YY1 bind to the late promoter region of HPV16 and suppress the transcription in the undifferentiated cells [23,32]. Although the precise mechanism is not yet elucidated, the suppression is released in the differentiating keratinocytes. At least hSkn-1a and C/EBPβ both emerge in the differentiating keratinocytes, bind to the late promoter region of HPV16 and enhance the transcription of the *E1* gene [23,25]. Thus, hSkn-1a is likely to be involved in the three important steps required for the efficient HPV DNA replication in the differentiating keratinocytes: activation of *E1* gene transcription from the late promoter, the transient activation of cellular DNA synthesis machinery and the enhancement of the HPV DNA replication through the binding to the core origin region. Further studies are necessary to fully understand the HPV DNA replication that proceeds only in differentiating keratinocytes, in which multiple regulatory factors (including yet unidentified factors) coordinate along with the process of differentiation.

Experimental procedures

Culture of HEK293 cells

HEK293 cells, a human embryonic kidney cell line transformed with the adenovirus *E1* gene, were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C.

Construction of plasmids

A plasmid expressing both HPV16 E1 and E2, pCMV/E1-IRES-E2, was constructed. The DNA fragment encoding the poliovirus IRES was amplified by PCR with pSRα-IRES [33] (a gift from T. Mizuno, Riken, Saitama, Japan) as a template, and cloned into the *Sma*I site located downstream of the *E1* gene of pCMV4/E1 (a gift from P. Howley, Harvard Medical School, Boston, MA, USA). Next, the E2 DNA fragment, which had been amplified by PCR with pCMV4/E2 (a gift from P. Howley) as a template, was cloned into downstream of the IRES sequence to generate pCMV/E1-IRES-E2.

The expression plasmids for hSkn-1a and for C/EBPβ were described previously [23,25]. The expression plasmid for DNA-binding deficient hSkn-1a, N331A, in which asparagine at the amino acid position 331 was replaced with alanine, was constructed from pHM/hSkn-1a with a oligonucleotide, 5′-AGG GTC TGG TTC TGC GCC CGA CGC CAA AAG GAG-3′ (mismatched bases underlined), using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

The expression plasmids for Tst-1 and for Brn-1 were provided by M. Wegner (Hamburg University, Germany). The expression plasmids for Oct-1 and for CDP were provided by W. Herr (Lausanne University, Switzerland) and E. Neufeld (Boston Children's Hospital, MA, USA), respectively.

The HPV16 origin-containing plasmid pGL3-P_{670} was described previously [25]. HPV16 DNA fragments from nucleotides (the HPV Sequence Database of Los Alamos National Laboratory) 7003–100, from nucleotides 7838–100 and from nucleotides 281–864, were amplified by PCR and inserted between *Sma*I and *Nco*I sites of pGL3-Basic (Promega, Madison, WI, USA) to generate pGL3-(7003/100), pGL3-(7838/100) and pGL3-(281/864), respectively. All nucleotide substitutions in pGL3-(7838/100) were introduced by using standard PCR techniques with KOD-plus polymerase (Toyobo, Osaka, Japan) and verified by DNA sequencing.

HPV16 transient replication assay

HEK293 cells (4×10^5) were grown on a 60-mm dish for 18 h and then transfected with 5 ng of pGL3-P_{670}, 600 ng of pCMV/E1-IRES-E2 and 400 ng of the expression plasmid for the transcription factor by using a FuGENE6 reagent (Roche Applied Science, Indianapolis, IN, USA). At 72 h after the transfection, the episomal DNA was recovered by a modified Hirt procedure [34]. Briefly, the cells were lysed with a lysis buffer consisting of 25 mM Tris–HCl (pH 7.5), 5 mM EDTA, 0.6% SDS and 25 $\mu g \cdot mL^{-1}$ of RNase A, followed by the addition of a buffer consisting of 3 M CsCl, 1 M potassium acetate and 0.67 M acetic acid. The mixture was centrifuged at 14 000 *g* to precipitate proteins and genomic DNA of the cells. The clear supernatant was applied to a QIAprep spin column (Qiagen, Valencia, CA, USA). After washing the column with a buffer consisting of 10 mM Tris-HCl (pH 7.5), 80 mM potassium acetate, 40 mM EDTA and 60% ethanol, the DNA samples trapped in the column were eluted with TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). The purified DNA samples were digested with *Dpn*I to cut the methylated DNA, which had been used for the transfection, at 37 °C for 5 h. The origin-containing plasmid in both the *Dpn*I-digested and undigested samples was quantitated by a real-time PCR analysis using an ABI PRISM7700 Sequence Detector with Power SYBR Green PCR Kit (Applied Biosystems, Foster City, CA, USA). The 150 bp fragment of the firefly luciferase gene, from nucleotides 641–790 of pGL3-Basic, which encompasses three *Dpn*I sites, was amplified with primers, 5′-CCT TCG ATA GGG ACA AGA CAA TTG-3′ and 5′-TAT CCG GAA TGA TTT GAT TGC CAA A-3′. The relative amount of the replicated reference plasmid, pGL3-P_{670} or pGL3-(7838/100), was determined by dividing the amount of the *Dpn*I-resistant pGL3-P_{670} by that of the total episomal pGL3-P_{670}. The effect of the factor to be tested was presented as the relative replication, which was the ratio of the relative amount obtained with the factor to that obtained without the factor.

The Southern blot analysis to detect the *Dpn*I-resistant replicated pGL3-P_{670} was conducted as described previously [23].

The replication level of a plasmid containing oriP of Epstein–Barr virus in HEK293 cells was measured similarly by using pREP4 (Invitrogen, Carlsbad, CA, USA), which contains oriP and the coding region for the viral oriP-binding protein, EBNA1. A real-time PCR analysis was performed to quantitate the total episomal and *Dpn*I-resistant pREP4 using specific primers for hygromycin gene in pREP4, 5′-GGT CGC GGA GGC CAT GGA TGC GA-3′ and 5′-GTT TGC CAG TGA TAC ACA TGG GGA-3′. The incorporation of BrdU into nuclei of HEK293 cells with or without hSkn-1a expression was measured by using BrdU Cell Proliferation Assay Kit (Exalpha Biological, Watertown, MA, USA). The replication level of the HPV16 full-genome was measured by using a re-circularized HPV16 genome [23]. A real-time PCR analysis was performed to quantitate the total episomal and *Dpn*I-resistant HPV16 genome using primers 5′-CCG GTC GAT GTA TGT CTT GTT GCA GAT CAT-3′ and 5′-GCT CAT

AAC AGT AGA GAT CAG TTG TCT CTG-3′ to amplify the HPV16 genome fragment from nucleotides 501–640.

Luciferase reporter assay

The *E2* gene in pCMV/E1-IRES-E2 was replaced with the firefly luciferase gene to produce, pCMV/E1-IRES-Firefly. HEK293 cells were seeded onto 24-well plates (4×10^4 cells·well^{-1}) and cultured for 18 h. The cells were transfected with a mixture of 240 ng of pCMV/E1-IRES-Firefly, 5 ng of the plasmid expressing *Renilla* luciferase, pCMV/Renilla (pRL-CMV; Promega) and 160 ng of the expression plasmid for transcription factor by using a FuGENE6 reagent. At 72 h after the transfection, firefly and *Renilla* luciferase activities were measured with one-third of the lysate by using Dual-Glo luciferase assay Kit (Promega) and an ARVO MX luminescence counter (Perkin-Elmer, Boston, MA, USA). The luciferase activities were normalized to protein concentration of the lysate determined by DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

Electrophoretic mobility shift assay

GST-hSkn-1a was purified as described previously [23]. The nuclear extracts containing or not containing hSkn-1a were prepared by Dignam's procedure from HeLa/hSkn-1a cells [26], in which expression of hSkn-1a is induced by the addition of doxycycline to the medium. A mixture of 200 ng of GST-hSkn-1a or 100 μg of the nuclear extract, double-stranded ^{32}P-labeled oligonucleotides (0.4 pmol) and 1 μg of poly (dI/dC) in a final volume of 10 μL of a binding buffer consisting of 20 mM Tris–HCl (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol and 40 μg·mL^{-1} BSA, was incubated on ice for 30 min. Then the samples were loaded on 5% polyacrylamide gels and electrophoresed in 0.5× Tris-borate/EDTA buffer at 4 °C. The gels were dried. ^{32}P-labeled oligonucleotides were visualized by autoradiography on a BAS2500 image analyzer (Fuji Film, Tokyo, Japan). The sequences of double-stranded oligonucleotides are: a (from nucleotides 7835–7884), 5′-AAC TGC ACA TGG GTG TGT GCA AAC CGA TTT TGG GTT ACA CAT TTA CAA GC-3′; b (from nucleotides 7825–20), 5′-ATT TAC AAG CAA CTT ATA TAA TAA TAC TAA ACT ACA ATA ATT CAT GTA TA-3′; c (from nucleotides 11–62), 5′-TTC ATG TAT AAA ACT AAG GGC GTA ACC GAA ATC GGT TGA ACC GAA ACC GGT T-3′; d (from nucleotides 49–100), 5′-AAC CGA AAC CGG TTA GTA TAA AAG CAG ACA TTT TAT GCA CCA AAA GAG AAC T-3′; mb, 5′-ATT TAC AAG CAA CTT ATA TAA TAA TAC TAA ACT ACA ATA ATT GAC GTA TA-3′; md, 5′-AAC CGA AAC CGG TTA GTA TAA AAG CAG ACA TTT TGT CCA CCA AAA GAG AAC T-3′. Nucleotides used for substitution mutations are underlined. The anti-hSkn-1a serum used in the supershift assay was purchased from Santa Cruz Biotechnology (C-20X, Santa Cruz, CA, USA).

Chromatin immunoprecipitation assay

HEK293 cells (1×10^6) were grown on a 100-mm dish for 20 h and then transfected with 2 μg of the HPV16 origin-containing plasmid and 4 μg of pHM/hSkn-1a using FuGENE6. At 72 h after the transfection, the cells were incubated for cross-linking with 1% formaldehyde for 5 min at 37 °C and then treated for 5 min with 125 mM glycine for quenching. The cells were lysed in 200 μL of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0) supplemented with protease inhibitor mixture (Roche Applied Science), incubated on ice for 10 min, and sonicated using a Bioruptor (Cosmobio, Tokyo, Japan). One hundred microlitre of the sample was mixed with 900 μL of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris–HCl, pH 8.0) and precleared with salmon sperm DNA/protein G-agarose beads. The supernatant was incubated overnight at 4 °C with anti-hSkn-1a serum (Santa Cruz) or normal rabbit IgG. The chromatin–antibody complex was purified by the incubation with the agarose beads for 2 h at 4 °C. The beads were washed sequentially for 5 min at 4 °C in wash buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, Tris–HCl, pH 8.0), wash buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, Tris–HCl, pH 8.0), wash buffer III (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0) and TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) twice. The chromatin was extracted with 200 μL of elution buffer (1% SDS, 0.1 M $NaHCO_3$, 10 mM dithiothreitol) and heated at 65 °C for 4 h to reverse the cross-links, followed by proteinase K digestion overnight at 37 °C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The purified DNA was used as a template to amplify the origin DNA fragment (from nucleotides 7851–90) by PCR (PCR primers: forward, 5′-GTG CAA ACC GAT TTT GGG TTA CAC ATT TAC-3′; reverse, 5′-TGG TGC ATA AAA TGT CTG CTT TTA TAC TAA-3′). PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. The amounts of the origin DNA fragment in one-tenth of the purified samples were quantitated by a real-time PCR analysis using an ABI PRISM7700 with Power SYBR Green PCR Kit.

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References

1 zur Hausen H (1996) Papillomavirus infections: a major cause of human cancers. *Biochim Biophys Acta* **1288**, F55–F78.

2 Fehrmann F & Laimins LA (2003) Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation. *Oncogene* **22**, 5201–5207.

3 Longworth MS & Laimins LA (2004) Pathogenesis of human papillomaviruses in differentiating epithelia. *Microbiol Mol Biol Rev* **68**, 362–372.

4 Stenlund A (2003) Initiation of DNA replication: lessons from viral initiator proteins. *Nat Rev Mol Cell Biol* **4**, 777–785.

5 Lee KY, Broker TR & Chow LT (1998) Transcription factor YY1 represses cell-free replication from human papillomavirus origins. *J Virol* **72**, 4911–4917.

6 O'Connor MJ, Stunkel W, Koh CH, Zimmermann H & Bernard HU (2000) The differentiation-specific factor CDP/Cut represses transcription and replication of human papillomaviruses through a conserved silencing element. *J Virol* **74**, 401–410.

7 Narahari J, Fisk JC, Melendy T & Roman A (2006) Interactions of the cellular CCAAT displacement protein and human papillomavirus E2 protein with the viral origin of replication can regulate DNA replication. *Virology* **350**, 302–311.

8 Boner W, Taylor ER, Tsirimonaki E, Yamane K, Campo MS & Morgan IM (2002) A functional interaction between the human papillomavirus 16 transcription/replication factor E2 and the DNA damage response protein TopBP1. *J Biol Chem* **277**, 22297–22303.

9 Lee D, Sohn H, Kalpana GV & Choe J (1999) Interaction of E1 and hSNF5 proteins stimulates replication of human papillomavirus DNA. *Nature* **399**, 487–491.

10 Eckert RL, Crish JF & Robinson NA (1997) The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. *Physiol Rev* **77**, 397–424.

11 Ryan AK & Rosenfeld MG (1997) POU domain family values: flexibility, partnerships, and developmental codes. *Genes Dev* **11**, 1207–1225.

12 Seitz CS, Lin Q, Deng H & Khavari PA (1998) Alterations in NF-kappaB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-kappaB. *Proc Natl Acad Sci USA* **95**, 2307–2312.

13 Maytin EV, Lin JC, Krishnamurthy R, Batchvarova N, Ron D, Mitchell PJ & Habener JF (1999) Keratin 10 gene expression during differentiation of mouse epidermis requires transcription factors C/EBP and AP-2. *Dev Biol* **216**, 164–181.

14 Hildesheim J, Foster RA, Chamberlin ME & Vogel JC (1999) Characterization of the regulatory domains of the human skn-1a/Epoc-1/Oct-11 POU transcription factor. *J Biol Chem* **274**, 26399–26406.

15 Hildesheim J, Kuhn U, Yee CL, Foster RA, Yancey KB & Vogel JC (2001) The hSkn-1a POU transcription factor enhances epidermal stratification by promoting keratinocyte proliferation. *J Cell Sci* **114**, 1913–1923.

16 Andersen B, Weinberg WC, Rennekampff O, McEvilly RJ, Bermingham JR, Hooshmand F, Vasilyev V, Hansbrough JF, Pittelkow MR, Yuspa SH *et al.* (1997) Functions of the POU domain genes Skn-1a/i and Tst-1/Oct-6/SCIP in epidermal differentiation. *Genes Dev* **11**, 1873–1884.

17 Maytin EV & Habener JF (1998) Transcription factors C/EBP alpha, C/EBP beta, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vitro and in vivo. *J Invest Dermatol* **110**, 238–246.

18 Zhu S, Oh HS, Shim M, Sterneck E, Johnson PF & Smart RC (1999) C/EBPbeta modulates the early events of keratinocyte differentiation involving growth arrest and keratin 1 and keratin 10 expression. *Mol Cell Biol* **19**, 7181–7190.

19 Nepveu A (2001) Role of the multifunctional CDP/Cut/Cux homeodomain transcription factor in regulating differentiation, cell growth and development. *Gene* **270**, 1–15.

20 Ai W, Toussaint E & Roman A (1999) CCAAT displacement protein binds to and negatively regulates human papillomavirus type 6 E6, E7, and E1 promoters. *J Virol* **73**, 4220–4229.

21 Taylor ER & Morgan IM (2003) A novel technique with enhanced detection and quantitation of HPV-16 E1- and E2-mediated DNA replication. *Virology* **315**, 103–109.

22 Sugihara TM, Kudryavtseva EI, Kumar V, Horridge JJ & Andersen B (2001) The POU domain factor Skin-1a represses the keratin 14 promoter independent of DNA binding. A possible role for interactions between Skn-1a and CREB-binding protein/p300. *J Biol Chem* **276**, 33036–33044.

23 Kukimoto I & Kanda T (2001) Displacement of YY1 by differentiation-specific transcription factor hSkn-1a activates the P(670) promoter of human papillomavirus type 16. *J Virol* **75**, 9302–9311.

24 Yukawa K, Butz K, Yasui T, Kikutani H & Hoppe-Seyler F (1996) Regulation of human papillomavirus transcription by the differentiation-dependent epithelial factor Epoc-1/skn-1a. *J Virol* **70**, 10–16.

25 Kukimoto I, Takeuchi T & Kanda T (2006) CCAAT/enhancer binding protein beta binds to and activates

the P670 promoter of human papillomavirus type 16. *Virology* **346**, 98–107.

26 Enomoto Y, Enomoto K, Kitamura T & Kanda T (2004) Keratinocyte-specific POU transcription factor hSkn-1a represses the growth of cervical cancer cell lines. *Oncogene* **23**, 5014–5022.

27 Russell J & Botchan MR (1995) cis-Acting components of human papillomavirus (HPV) DNA replication: linker substitution analysis of the HPV type 11 origin. *J Virol* **69**, 651–660.

28 Bannister AJ & Kouzarides T (1996) The CBP co-activator is a histone acetyltransferase. *Nature* **384**, 641–643.

29 Ogryzko VV, Schiltz RL, Russanova V, Howard BH & Nakatani Y (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**, 953–959.

30 Iizuka M, Matsui T, Takisawa H & Smith MM (2006) Regulation of replication licensing by acetyltransferase Hbo1. *Mol Cell Biol* **26**, 1098–1108.

31 Ai W, Narahari J & Roman A (2000) Yin yang 1 negatively regulates the differentiation-specific E1 promoter of human papillomavirus type 6. *J Virol* **74**, 5198–5205.

32 Sato K, Takeuchi T, Kukimoto I, Mori S, Yasugi T, Taketani Y & Kanda T (2007) Human papillomavirus type 16 P670 promoter is negatively regulated by CCAAT displacement protein. *Virus Genes* **35**, 473–481.

33 Mizuno T, Yamagishi K, Miyazawa H & Hanaoka F (1999) Molecular architecture of the mouse DNA polymerase alpha-primase complex. *Mol Cell Biol* **19**, 7886–7896.

34 Arad U (1998) Modified Hirt procedure for rapid purification of extrachromosomal DNA from mammalian cells. *Biotechniques* **24**, 760–762.

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Expression of CD1d and Ligand-Induced Cytokine Production Are Tissue Specific in Mucosal Epithelia of the Human Lower Reproductive Tract▽

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Mucosal epithelia of the human lower reproductive tract (vagina, cervix, and penile urethra) are exposed to sexually transmitted microbes, including *Chlamydia trachomatis*. The in vivo susceptibility of each tissue type to infection with *C. trachomatis* is quite distinct. CD1d is expressed on the surface of antigen-presenting cells, including mucosal epithelial cells, and interacts specifically with invariant NKT cells. Invariant NKT cells play a role in both innate and adaptive immune responses to microbes. Here we assessed CD1d expression in normal reproductive tissues by using immunohistochemistry. Immortalized epithelial cell lines from the human lower reproductive tract (vagina, endocervix, and penile urethra) were examined for CD1d expression and for ligand-induced cytokine production induced by CD1d cross-linking. CD1d expression in normal tissue was strong in the vagina but weak in the endocervix and penile urethra. Gamma interferon exposure induced CD1d transcription in all of the cell types studied, with the strongest induction in vaginal cells. Flow cytometry revealed cell surface expression of CD1d in vaginal and penile urethral epithelial cells but not in endocervical cells. Ligation of surface-expressed CD1d by monoclonal antibody cross-linking promoted interleukin-12 (IL-12) and IL-15, but not IL-10, production in vaginal and penile urethral cells. No induction was demonstrated in endocervical cells. CD1d-mediated cytokine production in penile urethral cells was abrogated by *C. trachomatis* infection. Basal deficiency in CD1d-mediated immune responsiveness may result in susceptibility to sexually transmitted agents. Decreased CD1d-mediated signaling may help *C. trachomatis* evade detection by innate immune cells.

Mucosal epithelia of the human lower reproductive tract are exposed to sexually transmitted microbes. However, the in vivo susceptibility of each tissue to infection by *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, herpes simplex virus (HSV), and cytomegalovirus is quite distinct. In women, the cervix is typically more vulnerable than the vagina (7, 22, 26). Many previous studies have demonstrated that human female reproductive tract epithelial cells participate in immunological function and are regulated by the actions of inflammatory cytokines, chemokines, and female hormones (13, 20, 27, 37).

CD1d is a major histocompatibility complex-like glycoprotein that presents self or microbial lipid antigen to natural killer T (NKT) cells (33). In humans, a specific subset of NKT cells expresses an invariant Vα24-JaQ/Vβ11 T-cell receptor and can recognize CD1d on the surface of antigen-presenting cells (APCs) through this receptor. CD1d is expressed not only in typical APCs (macrophages, dendritic cells [DCs], and B cells) but also in intestinal epithelial cells (4, 8), foreskin keratinocytes (5), and penile urethral epithelial cells (21). CD1d plays a role in both innate and adaptive immunity to various bacteria, viruses, fungi, and parasites (reviewed in reference 28). Activation of CD1d-restricted invariant NKT (iNKT) cells enhances host resistance to some microbes in a manner dependent on the level of CD1d expression on APCs (29, 30). In contrast, the activation of iNKT cells promotes susceptibility to some microbes (3, 28). For instance, in the murine lung, the immune responses of iNKT cells to two very closely related microbes, *Chlamydia pneumoniae* and *Chlamydia muridarum*, are protective and harmful, respectively (19).

The activation of CD1d-restricted iNKT cells in response to microbial invasion is antigen dependent, but these antigens can be derived from the invading microbe or possibly from host lipid antigens (6, 16, 23). Intracellular signaling mediated by

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surface CD1d utilizes NF-κB, a well-known immune-related transcription factor (32, 38). CD1d-restricted NKT cells can act directly on infected cells, killing CD1d-expressing cells (28). They can also modulate adaptive immune cells by altering Th1-Th2 polarization. Recognition of CD1d by iNKT cells can cause rapid release of both interleukin-4 (IL-4) and gamma interferon (IFN-γ) from NKT cells (2). The resultant T helper cell polarization induced by activated iNKT cells differs, depending on the nature of the invading microbe (19). Therefore, CD1d and CD1d-restricted NKT cells serve as a natural bridge between innate and adaptive immune responses to microbes.

The function of CD1d can be addressed experimentally by monoclonal antibody (MAb) cross-linking of cell surface-expressed CD1d (11, 38). CD1d ligation by cross-linking with an anti-CD1d MAb (51.1) induces tyrosine phosphorylation in the cytoplasmic tail of CD1d, subsequent intracellular signaling through NF-κB, and autocrine cytokine release from CD1d-bearing cells. IL-10 is induced by CD1d cross-linking in intestinal epithelial cells (11), whereas IL-12 is induced in monocytes and immature DCs (38).

The expression and function of CD1d in human lower reproductive tract mucosae have been poorly studied, although previous work has demonstrated that CD1d expression is negligible in the upper reproductive tract (endometrial glands) (8). Here we assessed the CD1d expression profiles in a variety of mucosal epithelial sites in the female and male reproductive tracts (endometrium, cervix, vagina, and penile urethra) and addressed the hypothesis that varied expression of CD1d at these sites may invoke tissue-specific differences in epithelial immune responsiveness. Additionally, we have recently demonstrated that *C. trachomatis* infection results in the downregulation of surface-expressed CD1d in human penile urethral epithelial cells, the most commonly infected cell type in the male, and that this is caused by proteasomal degradation (21). Here, *C. trachomatis*-infected and noninfected penile epithelial cells were examined for antibody cross-linking of surface CD1d to confirm that *C. trachomatis* infection alters CD1d-mediated autocrine cytokine production.

MATERIALS AND METHODS

Immunohistochemistry. Immunostaining for CD1d was performed on formalin-fixed, paraffin-embedded sections of normal human endometrium, cervix (endocervix and ectocervix), vagina, and penis tissues (obtained under Institutional Review Board approval through Brigham and Women's Hospital, Harvard Medical School, and the University of Tokyo). Two to 10 tissue samples from each site were examined. Optimal immunostaining required antigen retrieval via microwave exposure in 0.01 M citrate buffer. A mouse anti-CD1d MAb (NOR3.2; 1:500; Abcam Inc., Cambridge, MA) or an irrelevant, isotype-matched mouse MAb (negative control anti-chlamydial protein antibody, a kind gift from Li Shen, Boston University) was used as the primary reagent. Immunostaining was amplified and detected by standard avidin-biotin-horseradish peroxidase methodology (Vector Laboratories, Burlingame, CA) and diaminobenzidine color development (DakoCytomation, Carpinteria, CA). Nuclei were counterstained by standard hematoxylin protocols (Vector Laboratories). Analyses were performed at a magnification of ×200.

Cell lines and IFN-γ treatment. The endocervical (End1/E6/E7), vaginal (VK2/E6E7) (generous gifts from D. J. Anderson, Boston University, Boston, MA), and penile urethral (PURL) epithelial cell lines used in this study were established from primary epithelial cells that were immortalized by transduction with the retroviral vector LXSN-16E6E7 (12, 21). Cells were cultured in keratinocyte serum-free medium supplemented with bovine pituitary extract, recombinant epidermal growth factor, and calcium chloride (KSFM; Invitrogen Corporation, Carlsbad, CA). For experiments involving IFN-γ, cells were exposed to 100 ng/ml IFN-γ (Sigma-Aldrich Inc., St. Louis, MO) for 1 to 9 h.

Cross-linking with an anti-CD1d MAb. Epithelial cells were cultured in KSFM in 12-well plates and used at near (80%) confluence. Ten micrograms per milliliter anti-CD1d MAb 51.1 (a kind gift from R. S. Blumberg, Harvard Medical School, Boston, MA) or an isotype control MAb (DakoCytomation, Glostrup, Denmark) was added to cell monolayers, which were then incubated for 1 h at 37°C. Cells were then washed with phosphate-buffered saline (PBS), and 10 μg/ml goat anti-mouse immunoglobulin (Ig) antibody (Chemicon International, Temecula, CA) was added as a cross-linker for 30 min of incubation at 37°C. The cells were washed in PBS one additional time and incubated in the serum-free growth medium without antibiotics for 0 to 24 h.

Flow cytometric analysis. Single-color flow cytometry was performed to determine cell surface CD1d expression patterns. Reproductive tract epithelial cells were detached from culture plates with 0.05% EDTA in PBS, washed in cold PBS, and incubated with NOR3.2 MAb (1 μg/ml) in PBS for 30 min on ice. For indirect staining experiments, cells were incubated with R-phycoerythrin-conjugated anti-mouse IgG (DakoCytomation, Glostrup, Denmark) for 30 min on ice. Controls were exposed to an isotype-matched irrelevant MAb (1 μg/ml; DakoCytomation, Glostrup, Denmark). After washing, the cells were analyzed for PC5 via standard flow cytometry.

Reverse transcription (RT)-PCR and semiquantitative PCR. One microgram of total RNA was used for the reverse transcriptase reaction with oligo(dT) and an RNA PCR kit (Applied Biosystems, Foster City, CA). Total cDNA reaction samples were used as templates for the amplification of each gene fragment with the PCR Core kit (Applied Biosystems). A primer pair set for each gene was synthesized by Invitrogen Corporation as follows: CD1d (453 bp), 5′-GCTGCA ACCAGGACAAGTGGACGAG-3′ (forward) and 5′-AGGAACAGCAAGCA CGCCAGGACT-3′ (reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 889 bp), 5′-GGAAGGTGAAGGTCGGAGTC-3′ (forward) and 5′-AAGGTG GAGGAGTGGGTGTC-3′ (reverse). Expected single-band PCR products were quantitated with an image analyzer (Scion Image, Frederick, MD) and normalized to GAPDH.

For quantitative PCR, cDNAs were produced via RT of 1 μg of total RNA extracted from the cells as described above with an Omniscript RT kit (Qiagen, Inc., Valencia, CA). Two microliters of fivefold-diluted cDNA was amplified in a thermal cycler (7300 Real-Time PCR System; Applied Biosystems) with a QuantiTect SYBR green PCR kit (Qiagen, Inc.) and the following primer pair sets: IL-12 p35, commercially available pair (R&D Systems, Minneapolis, MN); IL-10, 5′-AGCTCAGCACTGCTCTGTTG-3′ (forward) and 5′-GCATTCTTC ACCTGCTGCTCCA-3′ (reverse) (11); IL-15, 5′-TTCACTTGAGTCCGGAG ATGC-3′ (forward) and 5′-GCATCCAGATTCTGTTACATTCCC-3′ (reverse); β-actin, 5′-GAAATCGTGCGTGACATTAAGG-3′ (forward) and 5′-TCAGG CAGCTCGTAGCTTCT-3′ (reverse). The IL-12 and IL-15 mRNA levels were normalized to that of β-actin, the internal control.

Cytokine ELISAs. Levels of secreted IL-12 and IL-15 were quantified by solid-phase sandwich enzyme-linked immunosorbent assay (ELISA; BioSource International, Inc., Camarillo, CA). Briefly, anti-IL-12 or -IL-15 specific antibody was used to coat microtiter plates and 50-μl volumes of culture supernatant samples or cytokine standards were added. Detection required the addition of a biotinylated secondary antibody and standard streptavidin-peroxidase methodology. A standard curve was produced by absorbance measurements at 450 nm for standard samples, and each unknown sample was measured and plotted on the standard curve.

***C. trachomatis* infection.** Nearly confluent PURL cells were overlaid with *C. trachomatis* serovar F (strain F/IC-Cal-13) elementary bodies suspended in a sucrose-phosphate-glutamate solution at a predetermined dilution that resulted in 70% of the cells becoming infected (multiplicity of infection 1). Plates were centrifuged for 1 h, supernatants were aspirated after centrifugation, and cells were cultured for 24 h at 37°C in KSFM.

Statistical analysis. Quantitative PCR and ELISA data are presented as means ± standard deviations. Experiments were performed independently at least three times. mRNA and protein levels (CD1d, IL-12, or IL-15) were compared to those without treatment or with isotype controls by paired, two-tailed Student *t* tests. A *P* value of < 0.05 was considered significant.

RESULTS

Expression of CD1d in human normal lower reproductive tract epithelia. Since CD1d expression in human normal intestinal epithelial cells (4) and epidermal keratinocytes (5) has been demonstrated by immunohistochemistry with the anti-CD1d NOR3.2 MAb, we examined immunostaining of human

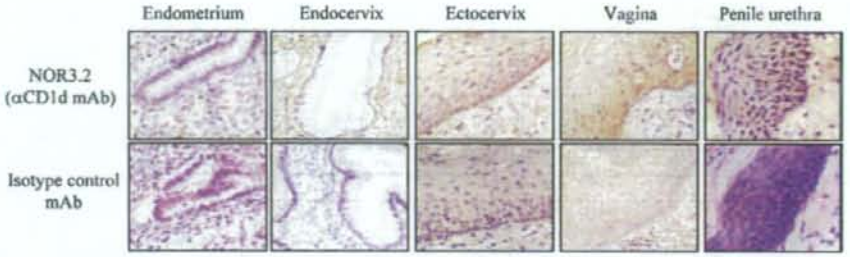

FIG. 1. Immunostaining of human normal reproductive tract epithelia for CD1d. Immunostaining for CD1d was performed on formalin-fixed, paraffin-embedded tissue sections of normal human endocervix, ectocervix, vagina, penile urethra, and proliferative-phase endometrium after antigen retrieval. CD1d was detected with NOR3.2, a CD1d-specific MAb (1:500) (upper panels). An isotype-matched control MAb was used as a negative control (lower panels) (magnification, ×200). Results are representative of 2 to 10 normal tissue samples from each site.

normal endocervix, ectocervix, vagina, penile urethra, and endometrium with NOR3.2 or isotype control MAbs (Fig. 1). Vaginal epithelium was immunoreactive with the NOR3.2 MAb. CD1d was expressed strongly in the epithelial cells of basal and suprabasal cell layers with negligible immunoreactivity in the intermediate and superficial cell layers. Like the vaginal epithelium, ectocervical and penile urethral epithelia were immunoreactive with NOR3.2 in their basal and suprabasal cell layers, although their immunoreactivity was weaker than that of the vagina. In contrast, immunoreactivity with NOR3.2 was barely detectable in endocervical gland epithelial cells and completely absent in endometrial epithelial glandular cells. In all of the tissues tested, CD1d immunoreactivity was absent in submucosal stromal cells, although several small NOR3.2-reactive cells were detected among the stromal cells that may represent lymphoid cells or DCs. The latter finding is consistent with the previous data obtained from normal skin (5).

Expression of CD1d in genital tract epithelial cells and its induction by IFN-γ. To quantitatively evaluate the CD1d expression levels in epithelial cells representing genital tract mucosal sites, we used immortalized cell lines primarily derived from endocervical, vaginal, and penile urethral epithelia in in vitro assays. These immortalized epithelial cells have been demonstrated to be characteristic of basal and parabasal cell-like cells in each tissue by comparing immunological markers and patterns of differentiation with those of normal tissues (12). Endometrial cells were also not used for this portion of our studies since endometrial epithelia lacked immunoreactivity to NOR3.2 in vivo. Semiquantitative RT-PCR demonstrated basal CD1d expression in endocervical, vaginal, and penile urethral epithelial cells at the transcriptional level (Fig. 2). Basal mRNA levels were clearly the highest in vaginal cells, followed by penile urethral and endocervical cells. The strong expression of CD1d in immortalized vaginal cells was consistent with CD1d immunoreactivity patterns in normal human tissues.

Previous studies have shown IFN-γ-mediated induction of CD1d in some APCs, including macrophages (9), keratinocytes (5), and intestinal epithelial cells (10). The CD1d promoter is upregulated by IFN-γ, possibly through IFN-γ-responsive elements present within the transcription initiation site (9). We therefore addressed the IFN-γ-mediated induction of CD1d expression in reproductive tract epithelial cells (Fig. 2). CD1d transcription was rapidly upregulated by IFN-γ exposure in all of the cell types studied; however, the level and duration of CD1d induction varied among the cell types. Endocervical cells demonstrated minimal CD1d induction, while vaginal and penile urethral cells exhibited more robust CD1d induction. Maximal induction was noted in vaginal cells. In endocervical and penile urethral cells, CD1d transcription increased at 1 h after exposure and returned to basal levels by 6 h after exposure. In vaginal cells, CD1d remained elevated 6 h after of IFN-γ exposure.

To investigate more thoroughly whether CD1d molecules were expressed on the plasma membrane of each cell line, cells were immunostained with the NOR3.2 antibody and analyzed by flow cytometry (Fig. 3). Vaginal and penile urethral cells both expressed CD1d molecules at the cell surface, although expression was stronger in vaginal cells. In contrast, endocervical cells did not express CD1d at the surface despite our prior

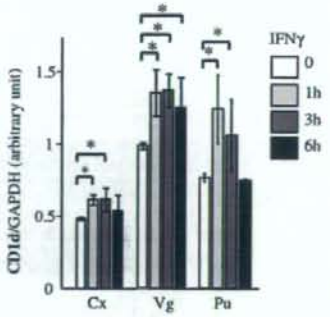

FIG. 2. CD1d expression in human reproductive tract epithelial cell lines. Immortalized epithelial cell lines derived from the cervix (Cx), vagina (Vg), and penile urethra (Pu) were cultured with or without IFN-γ (100 ng/ml) for 1 to 6 h. cDNA was produced via RT of 1 μg of total RNA extracted from these cells and then amplified by PCR with primer pairs set in the CD1d and GAPDH genes. Expected single-band PCR products were quantitated with an image analyzer (Scion Image, Frederick, MD) and normalized to GAPDH. Mean values with standard deviations are presented. Asterisks indicate comparisons (exposure versus nonexposure) with statistical significance ($P < 0.05$; $n = 6$).

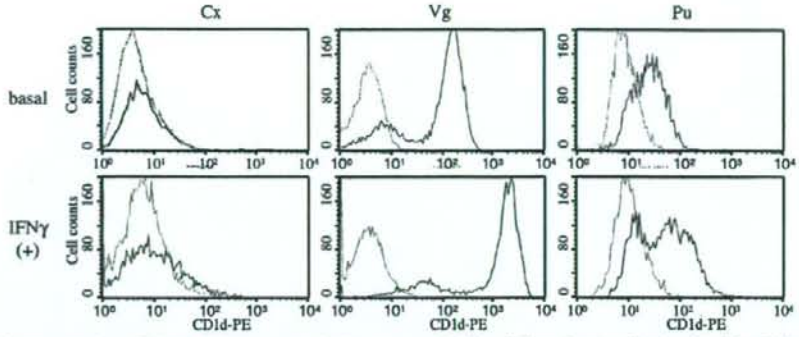

FIG. 3. Cell surface expression of CD1d in human reproductive tract epithelial cell lines. Immortalized epithelial cell lines derived from the cervix (Cx), vagina (Vg), and penile urethra (Pu) were cultured with or without IFN-γ (100 ng/ml) for 24 h. The cells were stained with the anti-CD1d MAb NOR3.2 and a phycoerythrin (PE)-conjugated goat anti-mouse Ig secondary antibody (bold line). Background level staining of the cells with an isotype-matched control antibody is also shown (thin line). The upper and lower panels show cell surface CD1d levels in nontreated (basal) and IFN-γ-treated cells [IFN-γ (+)]. The histograms shown are representative of at least three separate experiments.

demonstration of CD1d mRNA in these cells by RT-PCR. Flow cytometric analysis also revealed that the cell surface expression of CD1d clearly increased in vaginal cells after exposure to IFN-γ. Similar exposures in penile urethral cells resulted in much less dramatic changes in cell surface CD1d expression. CD1d expression at the endocervical epithelial cell surface was negligible as revealed by flow cytometry, even in the presence of IFN-γ.

Autocrine cytokine production induced by CD1d ligation. Surface CD1d interacts specifically with iNKT cells bearing an invariant Vα24-JaQ/Vβ11 T-cell receptor. The interaction not only activates NKT cells but also induces phosphorylation of CD1d, intracellular signaling, and the release of cytokines from the CD1d-bearing cell. Anti-CD1d MAb 51.1 can be used for CD1d cross-linking and represents an in vitro model for CD1d ligation (11, 38). IL-10, but not tumor necrosis factor alpha or IL-8, is induced by CD1d cross-linking in intestinal epithelial cells (11); IL-12 is induced in peripheral blood monocytes and immature DCs (38). IL-15 is also produced by a variety of mucosal epithelial cells that play important roles in mucosal immunity to microbes, including intraepithelial lymphocytes and NK and NKT cells (14, 15). To address the function of CD1d in reproductive tract mucosal epithelial cells, here we investigated CD1d ligation-induced autocrine cytokine production from reproductive tract-derived epithelial cell lines. First, penile urethral cells were exposed to an anti-CD1d MAb (51.1) or to an isotype control MAb. Both exposures were followed by exposure to a secondary anti-mouse IgG antibody cross-linker, and the cells were examined for IL-10, IL-12, and IL-15 production (Fig. 4). RT-PCR revealed that transcription of both IL-12 (p35) and IL-15 increased upon cross-linking; IL-10 transcription did not (Fig. 4A). Increases in IL-12 p35 occurred relatively rapidly, while those of IL-15 were comparatively delayed. To examine the autocrine production of IL-12 and IL-15, protein secretion into the culture medium was assessed by ELISA (Fig. 4B). IL-12 p70 secretion peaked at 12 h after cross-linking, while IL-15 secretion increased continuously throughout the first 24 h after exposure. Alterations in

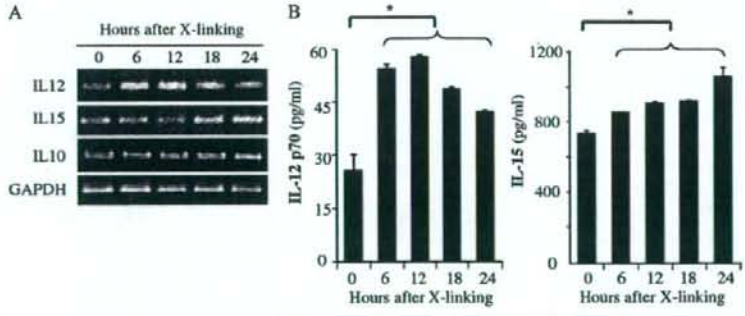

FIG. 4. Autocrine cytokines production by CD1d cross-linking in penile urethral epithelial cells. Ten micrograms per milliliter anti-CD1d MAb (51.1) was added to a monolayer of epithelial cells and incubated for 1 h. After washing with PBS, 10 μg/ml goat anti-mouse Ig antibody was added as a cross-linker for 30 min. The cells were incubated in serum-free growth medium without any antibiotics for 0 to 24 h. (A) cDNA was produced via RT of 1 μg of total RNA extracted and amplified by PCR with primer pairs for IL-10, IL-12 p35, IL-15, and GAPDH. PCR products were separated over an ethidium bromide-containing agarose gel. (B) Autocrine cytokine secretion from the epithelial cell at each time point was assessed by ELISA for IL-12 p70 and IL-15. Mean values with standard deviations are presented. Asterisks indicate comparisons (before versus after cross-linking [X-linking]) with statistical significance ($P < 0.05$; $n = 4$).

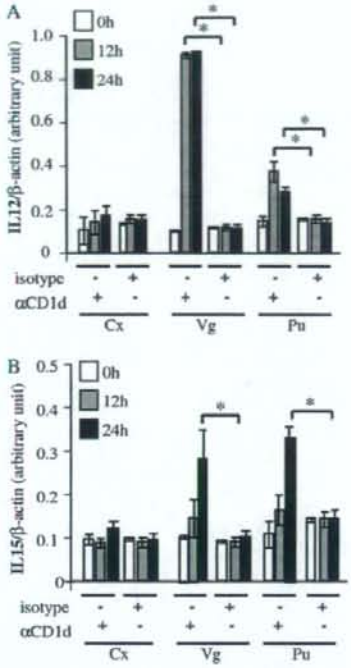

FIG. 5. Quantitative analysis of cytokine production after CD1d cross-linking. CD1d cross-linking was performed as described in the legend to Fig. 4. Anti-CD1d 51.1 (αCD1d) and isotype-matched control (isotype) MAbs were used as primary antibodies for cross-linking. Cells were harvested at 0, 12, and 24 h after cross-linking. IL-12 p35 (A) and IL-15 (B) mRNA levels were analyzed by quantitative RT-PCR by SYBR green methodology. IL-12 p35 and IL-15 mRNA levels were normalized to the β-actin mRNA level. Mean mRNA levels and standard deviations are plotted against time. Asterisks indicate comparisons (isotype versus αCD1d at each time point) with statistical significance ($P < 0.05$; $n = 6$). Cx, cervix; Vg, vagina; Pu, penile urethra.

cytokine secretion patterns paralleled those seen at the mRNA level. CD1d cross-linking appears to induce the production of IL-12 and IL-15 in penile urethral epithelial cells.

Next, we quantitatively estimated the transcription of IL-12 and IL-15 after CD1d cross-linking in the other available organotypic reproductive tract epithelial cell types (Fig. 5). Quantitative RT-PCR demonstrated that IL-12 p35 mRNA levels peaked rapidly 12 h after cross-linking in vaginal and penile urethral cells (Fig. 5A). This induction was particularly strong in vaginal cells and remained elevated for at least 24 h. IL-15 transcription rose, albeit less dramatically, from 12 to 24 h after cross-linking in vaginal and penile urethral cells. These alterations in IL-12 and IL-15 transcription were consistent with the RT-PCR and ELISA data shown in Fig. 4. Endocervical cells did not respond to MAb cross-linking (Fig. 5). Taken together, our results demonstrate autocrine production of IL-12 and IL-15 by vaginal and penile urethral cells, an effect that appears to be mediated through pathways involving CD1d.

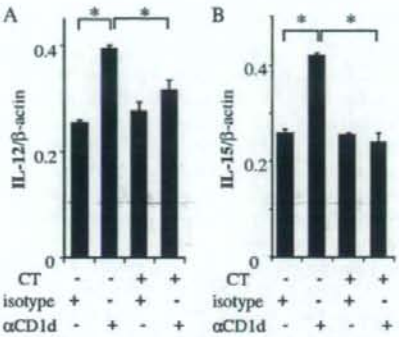

FIG. 6. Abrogation of autocrine cytokine production via CD1d intracellular signaling. Penile urethral epithelial cells were infected (CT+) with *C. trachomatis* serovar F (multiplicity of infection = 1, 70% infection) and harvested at 24 h postinfection. Control cells (CT−) remained uninfected. Thereafter, antibody cross-linking with the isotype-matched control (isotype) or 51.1 (αCD1d) MAb was performed as described in the legend to Fig. 5. The cells were harvested 12 h after cross-linking. The levels of IL-12 p35 (A) and IL-15 (B) mRNAs were analyzed by quantitative RT-PCR and normalized to β-actin. Mean values with standard deviations are presented. Asterisks indicate comparisons (isotype versus αCD1d) with statistical significance ($P < 0.05$; $n = 4$).

***C. trachomatis* infection abrogates CD1d-mediated cytokine production.** We have previously demonstrated that CD1d is downregulated by *C. trachomatis* infection through cellular and chlamydial degradation pathways (21). We therefore examined whether CD1d-mediated cytokine production was abrogated in *C. trachomatis*-infected cells (Fig. 6). In this investigation, we again used immortalized human penile urethral epithelial cells, which represent the most commonly infected cell type in the male reproductive tract. To our knowledge, there have been no published reports on *C. trachomatis* infection of human vaginal cells. We have therefore limited our further experimentation to those human cells known to be infected with this organism that also express basal levels of CD1d that allow for downregulation. Penile urethral epithelial cells were grown to a monolayer, infected with *C. trachomatis*, and cross-linked as described previously. Uninfected cells were used as a control. Quantitative RT-PCR revealed the typical increase in IL-12 p35 transcription 12 h after CD1d cross-linking in uninfected cells but no increase in *C. trachomatis*-infected cells (Fig. 6A). A similar abrogation of the increase in IL-15 transcription was also seen in cells infected with *C. trachomatis* (Fig. 6B). These differences in CD1d-mediated cytokine production between *C. trachomatis*-infected and noninfected cells were clearly significant ($P = 0.0005$ for IL-12, $P = 0.0084$ for IL-15). Note that IL-12 has been reported to be increased in endocervical secretions after *C. trachomatis* infection (35). Indeed, a minimal increase in the basal level of IL-12 mRNA was observed in *C. trachomatis*-infected cells in our experiments, although this increase was not statistically significant. In short, our results indicate that *C. trachomatis* interferes with CD1d-mediated cytokine production in infected epithelial cells from human reproductive tract mucosa.

DISCUSSION

We have demonstrated in this study that human lower reproductive tract epithelial cells possess surface-expressed CD1d molecules and that they exhibit CD1d-mediated cytokine production. CD1d is known to be expressed in DCs, macrophages, B cells, and epithelial cells. It appears to serve as an essential bridge between innate and adaptive immune responses within the local mucosa, the first responders to invasion by microbes. As shown in our immunohistochemical investigations, epithelial cells represent the primary CD1d-bearing cells within the lower reproductive tract mucosa in humans. Expression at these sites varies, however, with strong expression in the vagina, ectocervix, and penile urethra and very low expression in the endocervix and endometrium. The interaction of CD1d with CD1d-restricted iNKT cells is lipid antigen dependent. This lipid antigen can be derived from invading microbes or possibly from host lipid antigens. Since most sexually transmitted microbes invade the epithelial cells within the reproductive tract mucosa, these cells could be important in the presentation of pathogen-derived lipid antigens via cell surface CD1d molecules. This would be predicted to activate CD1d-restricted iNKT cells and rapidly induce an adaptive immune response to invading microbes.

Our immunochemical data demonstrated that squamous epithelial cells in basal and parabasal cell layers react strongly with anti-CD1d MAbs, in patterns replicating those seen in normal human skin (5). The distribution of CD1d-bearing epithelial cells within the basal and parabasal cell layers may be required for effective interactions between CD1d and the iNKT cells that reside within submucosal tissues. These interactions may occur primarily through CD1d expressed on the basal membrane. CD1d expression patterns in our model cell lines were similar to those of basal or parabasal cells in corresponding normal tissues. Indeed, the immortalized cell lines used in this study have been previously characterized by others as being similar to epithelial cells present in basal or parabasal cell layers in vivo (12). Fichorova et al. have established and used an ectocervical epithelial cell line in several of their investigations. We were not able to examine this ectocervical cell line in our in vitro assays because of its slow growth characteristics. Still, our immunohistochemical study demonstrated that CD1d immunoreactivity and distribution patterns in the ectocervix were similar to those in the penile urethra and the vagina. We hypothesize that ectocervical cells may therefore possess CD1d functions similar to those of the vaginal and penile urethral cells studied here.

Like intestinal epithelia, the endocervical epithelium contains monolayers of glandular epithelial cells. However, we show here that CD1d distribution in the endocervix is quite distinct from that reported in the intestine. Previous studies have demonstrated that CD1d localizes to the apical and lateral membranes of the small intestine and colon (31). By contrast, CD1d immunoreactivity in the endocervix was negligible at apical and lateral membranes. Our flow cytometric and RT-PCR analyses confirmed that endocervical epithelial cells do not present CD1d molecules on the cell surface despite CD1d transcription. Canchis et al. have previously reported that endometrial glandular cells also do not express CD1d (8), a result consistent with our immunohistochemical data. The absence of cell surface CD1d expression in the endocervical and endometrial epithelia may make these sites vulnerable to pathogen attack.

Although IFN-γ induced CD1d production at the mRNA level in all of the reproductive tract epithelial cells studied, the induction patterns of CD1d were quite distinct, depending on cell types. Flow cytometry more clearly revealed the differences in CD1d induction by IFN-γ exposure. Cell surface expression of CD1d was induced markedly in vaginal cells and minimally in penile urethral cells and remained unchanged in endocervical cells. The minimal increase in surface CD1d expression in penile urethral cells may reflect the transient induction in CD1d transcription noted after IFN-γ. In contrast, the induction of both CD1d transcription and translation upon IFN-γ-exposure in vaginal cells appeared significantly more prolonged.

Our investigations have also demonstrated that the human reproductive tract epithelial cells expressing CD1d on their surfaces have the capacity to produce cytokines after CD1d ligation. Vaginal and penile urethral cells increase their production of the Th1 cytokines IL-12 and IL-15 (but not IL-10) in a CD1d-mediated fashion. Since the ectocervix expressed CD1d in a distribution similar to that in the vagina and penile urethra, CD1d-bearing ectocervical epithelial cells may also possess similar CD1d-mediated cytokine production characteristics. Colgan et al. have demonstrated that anti-CD1d 51.1 MAb cross-linking of CD1d expressed at the apical surface of the plasma membrane induces IL-10 in an intestinal epithelial cancer cell line (T84 cells) (11). However, T84 cells are polarized and are known to possess distinct biological plasma membrane characteristics between apical and basolateral sites (24). As discussed in the Colgan report, it is unclear whether signal transduction through basolateral CD1d is the same as that through apical CD1d. Although our CD1d cross-linking experiments used the same anti-CD1d MAb, CD1d ligation induced not IL-10 but IL-12 and IL-15 production in reproductive tract epithelial cells. These cells, however, do not exhibit documented cell surface polarization. Yue et al. have also demonstrated that CD1d cross-linking by several MAbs, including 51.1, induces IL-12 in monocytes and immature DCs (38). Thus, the nature of the cytokine production and signaling induced by CD1d cross-linking may differ, depending on cell type and/or cell surface polarization.

IL-12 is a central mediator in both innate and adaptive immunity and is crucial in the prevention of infectious diseases and tumors (34). IL-12 induces IFN-γ-producing NK, NKT, T helper, and cytotoxic T cells and thereby bridges innate and adaptive immune responses. Yue et al. have demonstrated that CD1d cross-linking rapidly induces phosphorylation of IκB. This, in turn, promotes NF-κB activation and IL-12 production in monocytes and immature DCs (38). Colgan et al. have shown that CD1d cross-linking induces tyrosine phosphorylation of the cytoplasmic tail of CD1d in intestinal epithelial cells (11), invoking a mechanism by which CD1d-bearing cells signal downstream pathways that result in cytokine modulation. Here we show the rapid release of IL-12 upon CD1d cross-linking in reproductive tract epithelial cells.

The activation of NF-κB is also associated with IL-15 production. NF-κB binding to the IL-15 promoter region is essential for IL-15 transcription (36). The activation of NF-κB up-

regulates the expression of tumor necrosis factor alpha, IL-6, IL-12, IL-15, and IL-18 (1). Our investigations demonstrate that CD1d ligation can induce IL-15, as well as IL-12, production. Like IL-12, IL-15 is released both from classical APCs such as macrophages and DCs and from epithelial cells (25, 30). IL-15 is known to play a key role in mucosal immune responses within the reproductive tract. Using an IL-15 knockout (KO) mouse model, Gill et al. demonstrated that IL-15 is required for the generation of both innate and adaptive immune responses against transvaginal infection with HSV type 2 (HSV-2) (14). They reported that administration of recombinant IL-15 within the murine reproductive tract enables IL-15 KO mice to survive an otherwise lethal infection with HSV-2 and to generate specific adaptive immune responses to HSV-2 (15). Hirose et al. reported that IL-15 is synthesized by epithelial cells after bacterial infection of the intestinal mucosa. Further, this response is accompanied by early stimulation and IFN-γ production by intraepithelial lymphocytes (18). Taken together, our data suggest that interaction of CD1d with cross-linking receptors on iNKT cells can promote IL-12 and IL-15 release from the CD1d-bearing epithelial cells in the human reproductive tract mucosa. This may promote IFN-γ production by T helper, NK, and NKT cells and generate rapid innate and adaptive immune responses. In addition, IFN-γ derived from resident mucosal lymphocytes will upregulate epithelial cell CD1d expression in a paracrine fashion.

We have recently demonstrated that *C. trachomatis* downregulates the cell surface expression of CD1d by both cellular and chlamydial degradation pathways (21). Bilenki et al. have demonstrated that activation of iNKT cell by α-galactosylceramide exacerbates host susceptibility to *C. muridarum* infection in the lung while NKT KO and CD1 KO mice acquire increased resistance to the infection (3). In contrast, their group has also revealed that NKT KO and CD1 KO mice display increased susceptibility to pulmonary infection with another chlamydial species, *C. pneumoniae*; α-galactosylceramide treatment promotes resistance to this infection (19). These divergent immune responses to two distinct chlamydial species are thought to be due to differences in the cytokine profiles induced by activated iNKT cells (19). The production of IFN-γ or IL-4 by iNKT cells induces a Th1 or Th2 adaptive immune response, respectively. Since we here addressed CD1d-mediated cytokine production derived from reproductive tract epithelial cells, the resulting polarization of iNKT cells in response to *C. trachomatis* remains unstudied. As shown here, IL-12 and IL-15 induction after CD1d cross-linking is abrogated in the *C. trachomatis*-infected epithelial cell while the baseline level of these cytokines remains stable. The inhibition of CD1d-mediated cytokine production may be a mechanism by which *C. trachomatis*-infected cells evade (at least temporarily) the bridging of innate and adaptive immune responses that would otherwise occur upon the interaction of CD1d and iNKT cells.

The human endocervix is one of the most common sites of pathogen invasion in the human female reproductive tract, acting as the primary transmission site for many microbes, including HSV, cytomegalovirus, *C. trachomatis*, and *N. gonorrhoeae* (7, 22, 26). In contrast, the vaginal wall appears relatively resistant to microbial passage. Previous studies have demonstrated that Toll-like receptors (TLRs), which serve as sentinels in pathogen recognition and help to generate host innate immunity, are expressed differentially in distinct mucosal tissues of the female reproductive tract. The immortalized cervical and vaginal epithelial cell lines used in this study express all TLRs, with the exception of TLR4 and -8 (17). Fichorova et al. reported that the absence of TLR4 is associated with susceptibility to *N. gonorrhoeae* in the endocervix (13). Our data add a relative deficiency of surface-expressed CD1d to the immune characteristics of the human endocervical epithelium, a quality that may add to its susceptibility to pathogen invasion. In the male reproductive tract, the minimal induction of surface CD1d by IFN-γ in penile urethral cells may influence their susceptibility to many microbes, including *C. trachomatis*. CD1d expression and CD1d activation of neighboring iNKT cells may play an important role in the generation of innate and adaptive immune responses to microbial infection of the penile urethra, vagina, and ectocervix. This may help to explain the distinct differences in sexual pathogen susceptibility among various sites within the human male and female reproductive tracts.

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REFERENCES

1. **Andreakos, E., R. O. Williams, J. Wales, B. M. Foxwell, and M. Feldmann.** 2006. Activation of NF-κB by the intracellular expression of NF-κB-inducing kinase acts as a powerful vaccine adjuvant. Proc. Natl. Acad. Sci. USA **103:**314459–14464.
2. **Behar, S. M., and S. A. Porcelli.** 2007. CD1-restricted T cells in host defense to infectious diseases. Curr. Top. Microbiol. Immunol. **314:**215–250.
3. **Bilenki, L., S. Wang, J. Yang, Y. Fan, A. G. Joyee, and X. Yang.** 2005. NK T cell activation promotes *Chlamydia trachomatis* infection in vivo. J. Immunol. **175:**3197–3206.
4. **Blumberg, R. S., C. Terhorst, P. Bleicher, F. V. McDermott, C. H. Allan, S. B. Landau, J. S. Trier, and S. P. Balk.** 1991. Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. J. Immunol. **147:**2518–2524.
5. **Bonish, B., D. Jullien, Y. Dutronc, B. B. Huang, R. Modlin, F. M. Spada, S. A. Porcelli, and B. J. Nickoloff.** 2000. Overexpression of CD1d by keratinocytes in psoriasis and CD1d-dependent IFN-γ production by NK-T cells. J. Immunol. **165:**4076–4085.
6. **Brigl, M., L. Bry, S. C. Kent, J. E. Gumperz, and M. B. Brenner.** 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. Nat. Immunol. **4:**1230–1237.
7. **Byard, R. W., N. Z. Mikhael, G. Orlando, R. M. Cyr, and M. Prefontaine.** 1991. The clinicopathological significance of cytomegalovirus inclusions demonstrated by endocervical biopsy. Pathology **23:**318–321.
8. **Canchis, P. W., A. K. Bhan, S. B. Landau, L. Yang, S. P. Balk, and R. S. Blumberg.** 1993. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. Immunology **80:**561–566.
9. **Chen, Q. Y., and N. Jackson.** 2004. Human CD1d gene has TATA Boxless dual promoters: an SP1-binding element determines the function of the proximal promoter. J. Immunol. **172:**5512–5521.
10. **Colgan, S. P., V. M. Morales, J. L. Madara, J. E. Polischuk, S. P. Balk, and R. S. Blumberg.** 1996. IFN-γ modulates CD1d surface expression on intestinal epithelia. Am. J. Physiol. **271:**C276–283.
11. **Colgan, S. P., R. M. Hershberg, G. T. Furuta, and R. S. Blumberg.** 1999. Ligation of intestinal epithelial CD1d induces bioactive IL-10: critical role of the cytoplasmic tail in autocrine signaling. Proc. Natl. Acad. Sci. USA **96:**13938–13943.
12. **Fichorova, R. N., and D. J. Anderson.** 1999. Differential expression of immunobiological mediators by immortalized human cervical and vaginal epithelial cells. Biol. Reprod. **60:**508–514.
13. **Fichorova, R. N., A. O. Cronin, E. Lien, D. J. Anderson, and R. R. Ingalls.** 2002. Response to *Neisseria gonorrhoeae* by cervicovaginal epithelial cells occurs in the absence of Toll-like receptor 4-mediated signaling. J. Immunol. **168:**2424–2432.

14. **Gill, N., K. L. Rosenthal, and A. A. Ashkar.** 2005. NK and NKT cell-independent contribution of interleukin-15 to innate protection against mucosal viral infection. J. Virol. **79:**4470–4478.
15. **Gill, N., and A. A. Ashkar.** 2007. Adaptive immune responses fail to provide protection against genital HSV-2 infection in the absence of IL-15. Eur. J. Immunol. **37:**2529–2538.
16. **Gumperz, J. E., C. Roy, A. Makowska, D. Lum, M. Sugita, T. Podrebarac, Y. Koezuka, S. A. Porcelli, S. Cardell, M. B. Brenner, and S. M. Behar.** 2000. Murine CD1d-restricted T cell recognition of cellular lipids. Immunity **12:** 211–221.
17. **Herbst-Kralovetz, M. M., A. J. Quayle, M. Ficarra, S. Greene, W. A. Rose II, R. Chesson, R. A. Spagnuolo, and R. B. Pyles.** 2008. Quantification and comparison of Toll-like receptor expression and responsiveness in primary and immortalized human female lower genital tract epithelia. Am. J. Reprod. Immunol. **59:**212–224.
18. **Hirose, K., H. Suzuki, H. Nishimura, A. Mitani, J. Washizu, T. Matsuguchi, and Y. Yoshikai.** 1998. Interleukin-15 may be responsible for early activation of intestinal intraepithelial lymphocytes after oral infection with *Listeria monocytogenes* in rats. Infect. Immun. **66:**5677–5683.
19. **Joyce, A. G., H. Qiu, S. Wang, Y. Fan, L. Bilenki, and X. Yang.** 2007. Distinct NK T cell subsets are induced by different Chlamydia species leading to differential adaptive immunity and host resistance to the infections. J. Immunol. **178:**1048–1058.
20. **Kawana, K., Y. Kawana, and D. J. Schust.** 2005. Female steroid hormones utilize Stat protein-mediated pathways to modulate the expression of T-bet in epithelial cells: a mechanism for local immune regulation in the human reproductive tract. Mol. Endocrinol. **19:**2047–2059.
21. **Kawana, K., A. J. Quayle, M. Ficarra, J. A. Ibana, L. Shen, Y. Kawana, H. Yang, L. Marrero, S. Yavagal, S. J. Greene, Y. X. Zhang, R. B. Pyles, R. S. Blumberg, and D. J. Schust.** 2007. CD1d degradation in Chlamydia trachomatis-infected epithelial cells is the result of both cellular and chlamydial proteasomal activity. J. Biol. Chem. **282:**7368–7375.
22. **Levine, W. C., V. Pope, A. Bhoomkar, P. Tambe, J. S. Lewis, A. A. Zaidi, C. E. Farshy, S. Mitchell, and D. F. Talkington.** 1998. Increase in endocervical CD4 lymphocytes among women with nonulcerative sexually transmitted diseases. J. Infect. Dis. **177:**167–174.
23. **Mattner, J., K. L. Debord, N. Ismail, R. D. Goff, C. Cantu III, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, K. Hoebe, O. Schneewind, D. Walker, B. Beutler, L. Teyton, P. B. Savage, and A. Bendelac.** 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature **434:**525–529.
24. **McCormick, B. A., C. A. Parkos, S. P. Colgan, D. K. Carnes, and J. L. Madara.** 1998. Apical secretion of a pathogen-elicited epithelial chemoattractant activity in response to surface colonization of intestinal epithelia by Salmonella typhimurium. J. Immunol. **160:**455–466.
25. **Mention, J. J., M. B. Ahmed, B. Bégue, U. Barbe, V. Verkarre, V. Asnafi, J. F. Colombel, P. H. Cugnenc, F. M. Ruemmele, E. McIntyre, N. Brousse, C. Cellier, and N. Cerf-Bensussan.** 2003. Interleukin 15: a key to disrupted intraepithelial lymphocyte homeostasis and lymphomagenesis in celiac disease. Gastroenterology **125:**730–745.
26. **Nyirjesy, P.** 2001. Nongonococcal and nonchlamydial cervicitis. Curr. Infect. Dis. Rep. **3:**540–545.
27. **Rasmussen, S. J., L. Eckmann, A. J. Quayle, L. Shen, Y. X. Zhang, D. J. Anderson, J. Fierer, R. S. Stephens, and M. F. Kagnoff.** 1997. Secretion of proinflammatory cytokines by epithelial cells in response to chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis. J. Clin. Investig. **99:**77–87.
28. **Shiraki, Y., Y. Ishibashi, M. Hirumu, A. Nishikawa, and S. Ikeda.** 2006. Cytokine secretion profiles of human keratinocytes during *Trichophyton tonsurans* and *Arthroderma benhamiae* infections. J. Med. Microbiol. **55:**1175–1185.
29. **Sköld, M., and S. M. Behar.** 2003. Role of CD1d-restricted NKT cells in microbial immunity. Infect. Immun. **71:**5447–5455.
30. **Sköld, M., X. Xiong, P. A. Illarionov, G. S. Besra, and S. M. Behar.** 2005. Interplay of cytokines and microbial signals in regulation of CD1d expression and NKT cell activation. J. Immunol. **175:**3584–3593.
31. **Somnay-Wadgaonkar, K., A. Nusrat, H. S. Kim, W. P. Canchis, S. P. Balk, S. P. Colgan, and R. S. Blumberg.** 1999. Immunolocalization of CD1d in human intestinal epithelial cells and identification of a β2-microglobulin-associated form. Int. Immunol. **11:**383–392.
32. **Stanic, A. K., J. S. Bezbradica, J. J. Park, L. Van Kaer, M. R. Boothby, and S. Joyce.** 2004. The ontogeny and function of Va14Ja18 natural T lymphocytes require signal processing by protein kinase Cθ and NF-κB. J. Immunol. **172:**4667–4671.
33. **Taniguchi, M., and T. Nakayama.** 2000. Recognition and function of Vα14 NKT cells. Semin. Immunol. **12:**543–550.
34. **Trinchieri, G.** 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat. Rev. Immunol. **3:**133–146.
35. **Wang, C., J. Tang, P. A. Crowley-Nowick, C. M. Wilson, R. A. Kaslow, and W. M. Geisler.** 2005. Interleukin (IL)-2 and IL-12 responses to Chlamydia trachomatis infection in adolescents. Clin. Exp. Immunol. **142:**548–554.
36. **Washizu, J., H. Nishimura, N. Nakamura, Y. Nimura, and Y. Yoshikai.** 1998. The NF-κB binding site is essential for transcriptional activation of the IL-15 gene. Immunogenetics **48:**1–7.
37. **Wira, C. R., J. V. Fahey, C. L. Sentman, P. A. Pioli, and L. Shen.** 2005. Innate and adaptive immunity in female genital tract: cellular responses and interactions. Immunol. Rev. **206:**306–335.
38. **Yue, S. C., A. Shaulov, R. Wang, S. P. Balk, and M. A. Exley.** 2005. CD1d ligation on human monocytes directly signals rapid NF-κB activation and production of bioactive IL-12. Proc. Natl. Acad. Sci. USA **102:**11811–11816.

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Expression of surface CD1d in the extravillous trophoblast cells of early gestational placenta is downregulated in a manner dependent on trophoblast differentiation

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ABSTRACT

CD1d is a specific ligand for the invariant Vα24Vβ11-natural killer T (iNKT) cells that play an important role in placental development during early human pregnancy. The localization and regulation of placental CD1d expression remain unclear. Immunohistochemistry of human early gestational placentas revealed CD1d was present in villous and extravillous trophoblast (EVT) but not in syncytiotrophoblast or decidual cells. CD1d immunoreactivity in EVT cells decreased with EVT differentiation. Flow cytometry of primary cultured human trophoblast cells confirmed cell-surface expression of CD1d decreased with time in culture. These changes in CD1d expression occur at the level of transcription. TGF-β1 secreted from the cultured EVT cells accumulated with time in culture and directly suppressed CD1d expression, as evidenced by monoclonal antibody neutralization of TGF-β1 effects. Thus, trophoblast differentiation is characterized by TGF-β1-mediated decreases in trophoblast cell CD1d expression. This effect may support appropriate activation of decidual iNKT cells at the maternal-fetal interface.

Micro-immune responses at the human maternal-fetal interface are required for maintenance of early pregnancy, including embryo implantation and placental formation [1–3]. Extravillous trophoblast (EVT) cells of the placenta have been shown to express the MHC class I-like molecule CD1d [4]. Unlike MHC I, CD1d presents self- or microbe-derived glycolipid rather than peptide and its immune effectors are typically natural killer T (NKT) cells [5]. In humans, a specific subset of NKT cells expresses an invariant Vα24Jα18/Vβ11 TCR (iTCR) and can recognize CD1d on the surface of APCs through this receptor. The activation of invariant NKT (iNKT) cells is antigen dependent, but the antigen itself can be derived from an invading microbe or possibly the host itself [6]. Recognition of CD1d by iNKT cells causes rapid release of IL-4 and IFN-γ from the iNKT cell and thereby modulates the Th1/Th2 polarization of adaptive immune cells [5]. iNKT cells also appear to play an essential role in allograft tolerance [7,8].

At the maternal-fetal interface, decidual (maternal) CD1d-restricted iNKT cells modulate the immune microenvironment during normal placental formation [4]. Activation of iNKT cells with α-galactosylceramide, a ligand for iTCR on CD1d-bearing cells, induces pregnancy loss in mice [9] although the gestational stage of loss and the mechanisms involved vary by mouse strain [9,10]. iNKT cell-mediated early losses occur in all mice strains studied but mid-gestation pregnancy loss exhibits strain-dependent variations [10].

Although the expression of CD1d has been reported in villous trophoblast from normal human placenta [4], the lack of a culture system for villous trophoblast cells has hampered more detailed descriptions of its expression pattern and regulation. Our primary culture system for trophoblast cells isolated from normal placenta during early pregnancy [11] allows such experimentation.

Viewing the importance of iNKT cells in early pregnancy maintenance, we hypothesize that CD1d plays a role in the regulation of iNKT cell function at the human maternal-fetal interface. Here, we demonstrate the normal expression of human placental CD1d *in vivo* to be isolated to trophoblast cells and to decrease with the level of trophoblast invasion into the maternal decidua. This change in CD1d expression is mediated at the transcriptional level by TGF-β1.

Materials and methods

Samples. All sample collections and experiments in this study were approved by the Ethical Committee of the Medical Faculty, University of Tokyo.

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