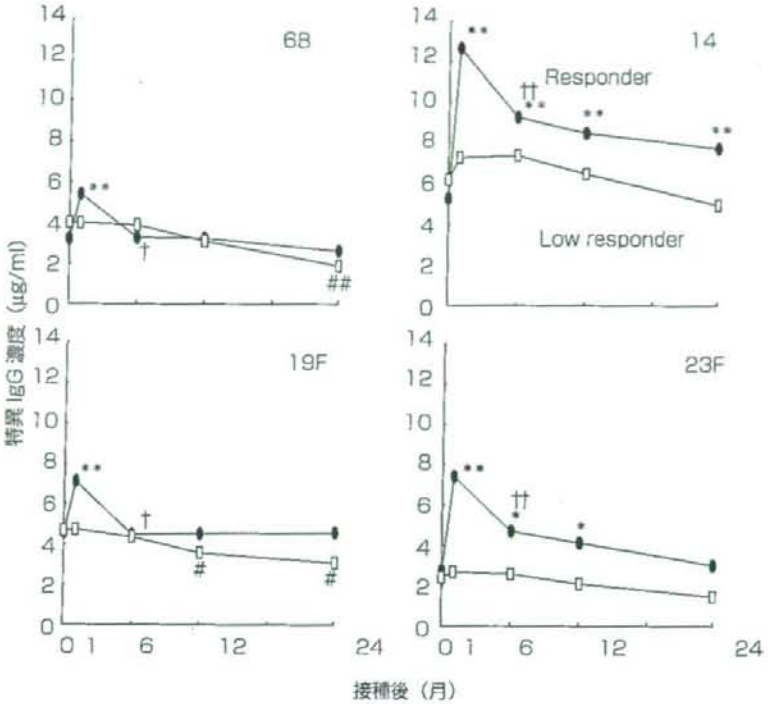

図1. 慢性肺疾患患者84例における肺炎球菌ワクチン接種後2年間の特異IgG濃度の推移．応答者58例の相乗平均値（●），低応答者26例の相乗平均値（□）
（文献6より改変）

た原因と推察される．一方，血清中特異IgG濃度が高いにもかかわらず，慢性肺疾患患者は肺炎球菌に易感染性である事も注目すべき点である．全体症例，応答者では接種1カ月後に有意な特異IgG濃度の増加を認めるが，低応答者では血清型14を除いた他の血清型でIgG抗体の増加が見られていない．また，接種後2年間の血清中特異IgG濃度の推移は，4つの血清型で全く異なることが判る（図1）．応答者の血清中特異IgG濃度は，どの血清型においても，ワクチン接種1カ月後から6カ月後までに速やかに減少している．特異IgG濃度が接種前のレベルに低下するまでの期間は，血清型6Bで0.87年，血清型19Fで1.1年，血清型23Fで2.5年，血清型14では8.3年であった．これらの成績は高齢者でも慢性肺疾患患者のような特定のリスクグループに対しては初回接種3年以内の再接種が必要であることを示唆している．また，低応答者に対しては肺炎球菌ワクチン再接種の効果も期待できないことから，新たな救済策が必要である．

4. 肺炎球菌ワクチン再接種後の血清型特異免疫応答

Mufsonらは14価ワクチンを接種した56～79歳の15名に6年後に23価ワクチンを接種し，その後の血清中特異抗体濃度の動態を検討した[8]．初回接種後に，12価の特異的抗体レベルは接種前の3.1倍の上昇が認められたのに対し，再接種後には平均1.5倍の上昇が認められた．Davidsonらは，慢性疾患患者において23価肺炎球菌ワクチン接種の初回接種と6年以上後の再接種によ

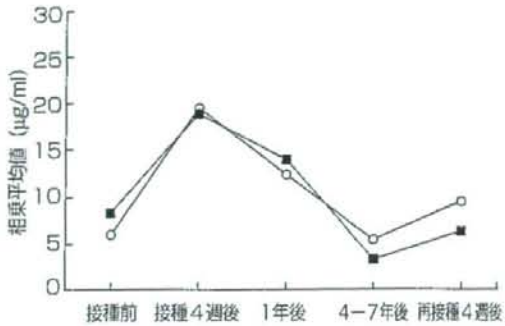

図2．肺炎球菌ワクチン初回接種と4～7年後の再接種時の6つの血清型に対する血清中特異IgG濃度の相乗平均値の推移．50～68歳（○），69歳以上（■）（文献9より改変）

表3．接種前後の各血清型および全血清型の特異IgG濃度の増加比

血清型	初回接種	再接種	P
1	2.04	2.21	0.34
4	1.8	1.51	0.4
7F	3.05	1.79	0.001
14	3.52	1.78	0.002
18C	3.27	1.97	0.004
19F	3.22	1.84	0.003
全血清型	2.73	1.84	0.001

（文献9から改変）

る免疫誘導能についてRIA法で比較した[9]．彼らは12価の血清中特異抗体濃度を測定し，初回接種後の抗体濃度が全値の1.4倍以上となった割合（平均54%）と再接種後の抗体濃度が全値の1.4倍以上となった割合（平均54.7%）を比較し，両者の特異抗体応答は同等であったと報告している．

一方，比較的最近，Torlingらは過去に肺炎の治療歴のある高齢者に対する肺炎球菌ワクチンの再接種を試み，血清免疫学的応答を第二世代EIA法で測定している[9]．彼らは6種の血清型について特異IgG濃度を測定し，初回接種4～7年後の再接種4週後の抗体濃度（7.47μg/ml）は再接種前より有意に増加するが，初回接種後のピーク（19.06μg/ml）より有意に低かったと報告している．また，これらの結果を50～68歳と69歳以上の2群に分けた場合，その初回接種および再接種後の特異IgG濃度は同等であった（図2）．また，接種前後の増加比は，初回接種が2.73に対し，再接種では1.84であった．表3には各血清型の初回接種，再接種時の増加比を示している．また，彼らは6つの血清型の増加比のうち2血清型以上で2以上であった人を応答者と定義した場合に，低応答者の頻度が初回接種では16%であり，再接種では41%に増加したとしている．

5．肺炎球菌ワクチンの再接種と副反応

Jacksonらは50歳から74歳までの年齢層で，過去に肺炎球菌ワクチン接種歴のない901名，少なくとも5年前に肺炎球菌ワクチン接種歴のある513名を対象として，接種前の血清中特異IgG濃度とワクチン接種による副反応について検討した[10]．その結果，接種後6日以内の発熱は初回接種，再接種いずれにおいてもほとんど認められなかったが，局所反応は再接種群が，初回接種群に比較して有意に多かった．また，接種後2日間における症状としては腕の痛みが頻繁に認められ，初回接種が57%に対し，再接種では74%であった．さらに，再接種と局所副反応の関連性について検討したところ，大きな局所反応（少なくとも10.2cm以上）の頻度が再接種群（11%）で初回接種群（3%）に比較して有意に多かった．しかしながら，これらの局所反応は接種後平均3日以内に自然に消失した．さらに，免疫能の正常な成人において，接種前の血清型特異IgG濃度と大きな局所反応との関連性を検討したところ，4，14，23Fのすべての血清型において高い血清中特異IgG濃度を有する人においてより大きな局所反応が認められた．Torlingらも再接種による副反応について報告し，局所反応は63%と高率であるが，ほとんどの副反応は再接種24時間以内に出現し，48時間以内に消失したとしている[9]．これらの副作用情報から，

肺炎球菌ワクチン再接種は安全であり，その副反応により再接種が禁忌となることは無いと結論できる．

6．5年後の再接種の是非

これまでに，米国ACIPは65歳以上の免疫能の正常な高齢者で，肺炎球菌ワクチン接種後5年以上経過し，かつ前回接種が65歳未満であった場合には再接種をAランクで推奨している（表1）．また，機能的または解剖学的無脾症の11歳以上で前回接種5年以上経過した人，あるいは10歳以下で前回接種後3年経過した人にも同様のAランク推奨をしている．しかしながら，前述の如く本邦においては1988年の肺炎球菌ワクチンの認可時に再接種は禁忌とされたままであり，その後の添付文書の改訂は実施されていない．高齢者や慢性肺疾患患者における肺炎球菌ワクチン接種後の血清中特異IgG濃度の推移，再接種後の安全性と免疫応答の成績から，これらの対象において初回接種5年後以降の再接種は妥当と考えられる．本邦ではほとんどの初回接種が65歳以上の高齢者を対象とされること，年齢による再接種後の免疫応答に差がないことから[9]，再接種の対象は肺炎球菌ワクチン接種後5年以上経過し，前回接種が65歳以上の高齢者が適切と考えている．我が国の平均寿命が男性78歳，女性85歳であることを勘案すれば，肺炎球菌ワクチンの効果を最大限に活用するべきであろう．

おわりに

肺炎球菌感染症の頻度の高い米国アラスカ州の原住民では，55歳以上では6年毎の肺炎球菌ワクチン再接種が実施されている[11]．しかしながら，再接種後の特異IgG応答は初回接種時より低下し，また低応答者の頻度も増加する事実から，65歳以上の高齢者における2回以上の再接種の是非については今後の検討が必要と考えられる．本邦では通常65歳以上の高齢者に初回接種されることから，当面は初回接種の5年後の再接種の認可に向けて国民の意識を高めていく必要がある．

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Transcription factor human Skn-1a enhances replication of human papillomavirus DNA through the direct binding to two sites near the viral replication origin

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Keywords
DNA replication; hSkn-1a; human papillomavirus; keratinocyte differentiation; transcription factor

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Human papillomavirus type 16 (HPV16) DNA replication, which requires two viral proteins E1 and E2, occurs only in the differentiating epithelium. Besides the general factors necessary for cellular DNA synthesis, other unidentified cellular factors are assumed to be involved in the regulation of HPV DNA replication. In the present study, we found that the POU-domain transcription factor human Skn-1a, which induces the terminal differentiation of keratinocytes and activates the HPV16 late promoter, enhanced the transient replication of a plasmid containing the HPV16 replication origin in HEK293 cells when co-transfected with a plasmid expressing E1 and E2. An electrophoretic mobility shift assay with a bacterially expressed human Skn-1a or an extract of HeLa cells over-expressing human Skn-1a revealed the presence of two human Skn-1a binding sites that are distinct from the known three sites, near the replication origin. A chromatin immunoprecipitation analysis showed that human Skn-1a bound to these sites in cells. Nucleotide substitutions in the sites abolished the binding of human Skn-1a and the human Skn-1a-mediated replication enhancement. The data strongly suggest that, through the binding to the two sites, human Skn-1a enhances HPV DNA replication.

Human papillomavirus (HPV), a small icosahedral virus, has a double-stranded circular DNA genome of approximately 8000 bp [1]. All HPV types, classified based on the homology of genomic DNA, share a common genetic organization: the early region encoding nonstructural viral proteins (E1, E2, E4, E5, E6 and E7), the late region encoding two capsid proteins (L1 and L2) and the noncoding regulatory region between the *L1* and *E6* genes, which includes the viral DNA replication origin [2].

The life cycle of HPV is tightly associated with epithelial differentiation [3]. HPV infects the basal cells of the epithelium through microlesions and their genomes are maintained as multicopy episomes in the nucleus. When the host cells initiate terminal differentiation, the HPV DNA starts to replicate and to be transcribed efficiently. HPV virions are generated in the upper layers of the epidermis or mucosa and released from them.

HPV DNA replication is initiated by the binding of viral E2 protein to the E2-binding sites near the replication origin [4]. Then, E2 protein recruits viral E1 protein, which is a replicative DNA helicase, to the origin. E1 protein assembles into an active helicase hexamer, thereby inducing the unwinding of the origin DNA to start replication [4]. The host cell supplies the general replication proteins, such as DNA polymerases, proliferating cell nuclear antigen and anabolic systems that synthesize the deoxynucleotide substrates.

Abbreviations
BrdU, bromodeoxyuridine; C/EBP, CCAAT/enhancer binding protein; CBP, CREB-binding protein; CDP, CCAAT-displacement protein; ChIP, chromatin immunoprecipitation; CMV, cytomegarovirus; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; HPV, human papillomavirus; IRES, internal ribosomal entry site; oriP, origin of plasmid replication.

Except for the general replication proteins, several cellular proteins have been shown to affect the HPV replication. Ubiquitous transcription repressors, YY1 and CCAAT-displacement protein (CDP), inhibit the replication of HPV origin-containing plasmids in cell culture systems and in cell-free replication systems through their binding to the origin sequences [5–7]. TopBP1, a protein involved in cellular DNA replication and repair, binds to HPV16 E2 and up-regulates the HPV16 replication [8]. Ini1, a subunit of the human SWI/SNF chromatin-remodeling complex, binds to E1 [9]. However, a cellular protein that enhances the HPV DNA replication in the differentiating epithelium has not been identified to date.

The proliferation and differentiation of keratinocytes are both tightly regulated by cellular transcription factors, including AP-1 family proteins [10], POU-domain proteins [11], nuclear factor-kappa B family proteins [12] and CCAAT/enhancer binding proteins (C/EBPs) [13]. The keratinocyte-specific POU-domain factor, human Skn-1a (hSkn-1a), plays a critical role in keratinocyte differentiation [14,15]. hSkn-1a, which is expressed in suprabasal cells, induces the transient cell proliferation required for keratinocyte stratification [15]. Tst-1, another POU-domain factor, which is expressed in the differentiating epithelium, has a function similar to that of Skn-1a [16]. The C/EBP family protein, C/EBPβ, whose expression is up-regulated during keratinocyte differentiation [17], causes cell-growth arrest and induces the expression of marker proteins for the differentiated keratinocytes [18]. The transcription repressor CDP [19], which is expressed abundantly in the basal cells, disappears during differentiation [20], suggesting that the release of the CDP-mediated transcription repression of some genes promotes the differentiation.

In the present study, we examined hSkn-1a, Oct-1, Tst-1, Brn-1, C/EBPβ and CDP for their ability to enhance the transient replication of a plasmid containing the HPV16 replication origin in HEK293 cells co-transfected with a plasmid expressing E1 and E2, and found that hSkn-1a enhanced the replication. An electrophoretic mobility shift assay showed that hSkn-1a bound to two sites near the origin. Nucleotide substitutions in these sites abolished the replication enhancement, suggesting that the replication enhancement was mediated by the sequence-specific binding of hSkn-1a.

Results

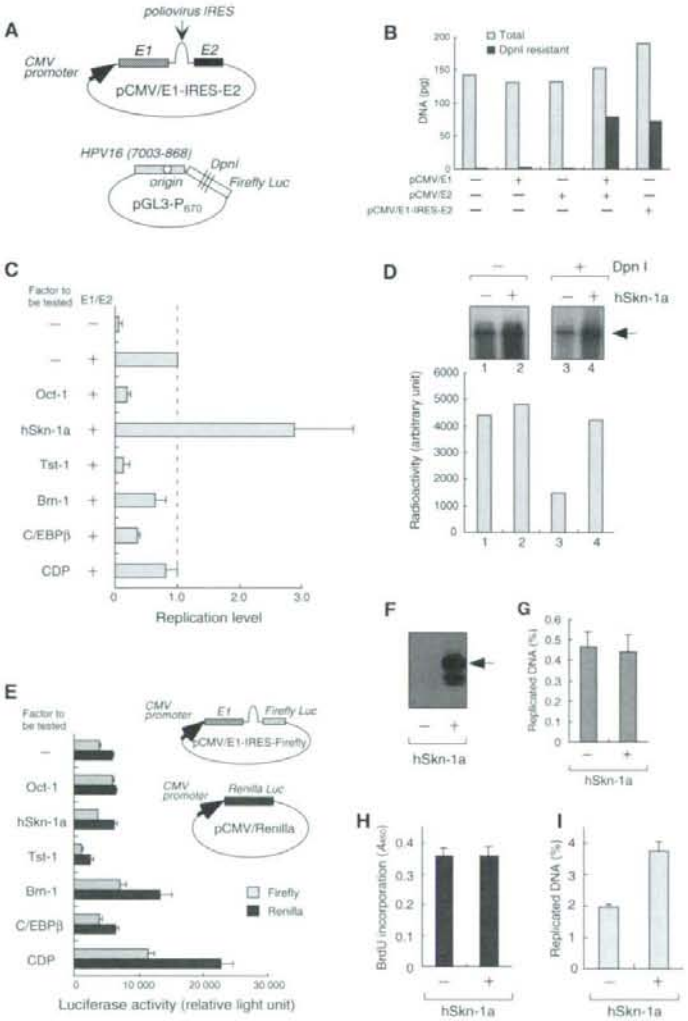
Enhancement of transient replication of the HPV16 origin-containing plasmid by hSkn-1a

An HPV16 origin-containing plasmid, pGL3-P_{670}, which has HPV16 DNA sequences from nucleotides 7003–7904 and 1–868 (Fig. 1A), replicated in HEK293 cells transfected with pCMV/E1-IRES-E2, an expression plasmid for HPV16 replication proteins E1 and E2. pCMV/E1-IRES-E2, which was newly constructed by inserting the internal ribosomal entry site (IRES)

Fig. 1. Enhancement of the transient replication of pGL3-P_{670} by hSkn-1a. (A) Schematic representation of the HPV16 E1/E2 co-expression plasmid and the HPV16 origin-containing plasmid. The *E1* and *E2* genes are transcribed simultaneously from pCMV/E1-IRES-E2, in which the *E1* and *E2* genes are connected with poliovirus IRES. pGL3-P_{670} contains the HPV16 DNA replication origin and the firefly luciferase gene. (B) The amounts of the replicated pGL3-P_{670}. Total episomal and the replicated (*Dpn*I-resistant) pGL3-P_{670} were measured by real-time PCR with Hirt extract from HEK293 cells that had been co-transfected with the plasmids indicated at the bottom. (C) Effects of hSkn-1a, Oct-1, Tst-1, Brn-1, C/EBPβ and CDP on the replication of pGL3-P_{670}. Replicated pGL3-P_{670} was measured by real-time PCR with Hirt 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 (for factor hSkn-1a, Oct-1, Tst-1, Brn-1, C/EBPβ or CDP). The relative replication of pGL3-P_{670} (in the presence of a factor tested) has been normalized and is expressed as the ratio of replicated pGL3-P_{670} in the absence of the factors indicated above. The scale bar represents the mean ± SD of three independent transfection experiments. (D) Southern blot analysis of replicated pGL3-P_{670} in the absence or presence of hSkn-1a co-expression. The signal indicated by the arrow in the autoradiogram was quantitated and presented at the bottom. (E) Effects of hSkn-1a, Oct-1, Tst-1, Brn-1, C/EBPβ and CDP on transcription from the CMV promoter and the IRES-dependent translation. The firefly and *Renilla* luciferase activities of the lysate derived from HEK293 cells that had been transfected with pCMV/E1-IRES-Firefly and pCMV/*Renilla* together with the expression plasmid for transcription factor were measured and normalized to the protein concentration of the lysate. The scale bar represents the mean ± SD of three independent transfection experiments. (F) Western blot analysis of total extracts of HEK293 cells that had been transfected with or without the hSkn-1a expression plasmid. The antibody against hSkn-1a (Santa Cruz) was used to detect hSkn-1a. Western blot analysis was performed as described [23]. (G) Effects of hSkn-1a on Epstein–Barr virus oriP-dependent plasmid replication. pREP4 was measured by real-time PCR with Hirt extract from HEK293 cells that had been transfected with pREP4 with or without the hSkn-1a expression plasmid. The ratio of replicated plasmid to total episomal plasmid is indicated. The scale bar represents the mean ± SD of three independent experiments. (H) Effects of hSkn-1a on cellular DNA replication. The level of the incorporation of BrdU was measured by BrdU Cell Proliferation Assay Kit and indicated as A_{450}. The scale bar represents the mean ± SD of three independent experiments. (I) Effects of hSkn-1a on HPV16 full-genome replication. The level of the HPV16 genome replication was measured by real-time PCR with Hirt extract from HEK293 cells that had been transfected with HPV16 complete genome with or without the hSkn-1a expression plasmid. The scale bar represents the mean ± SE of two independent experiments.

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.

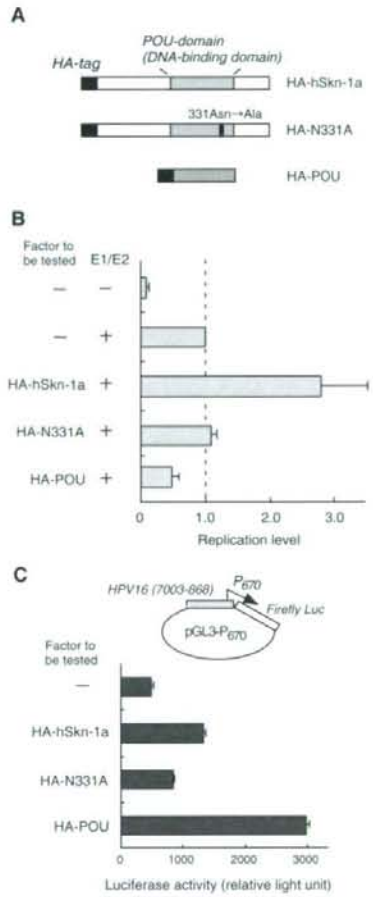


Fig. 2. Domain of hSkn-1a required for the replication enhancement. (A) Schematic representation of the structures of wild-type, DNA-binding deficient and POU-domain forms of hSkn-1a. (B) Effects of DNA-binding deficient and POU-domain forms of hSkn-1a on HPV16 transient replication. Replicated pGL3-P_{670} 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

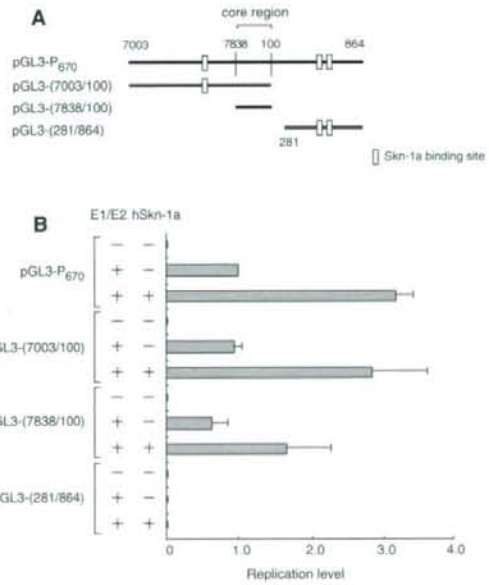


Fig. 3. HPV16 DNA segments responsible for the hSkn-1a-mediated replication enhancement. (A) Schematic representation of the HPV16 DNA segments used in the transient replication assay. Previously identified hSkn-1a binding sites are indicated as open boxes. (B) Effects of hSkn-1a on replication of plasmids 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).

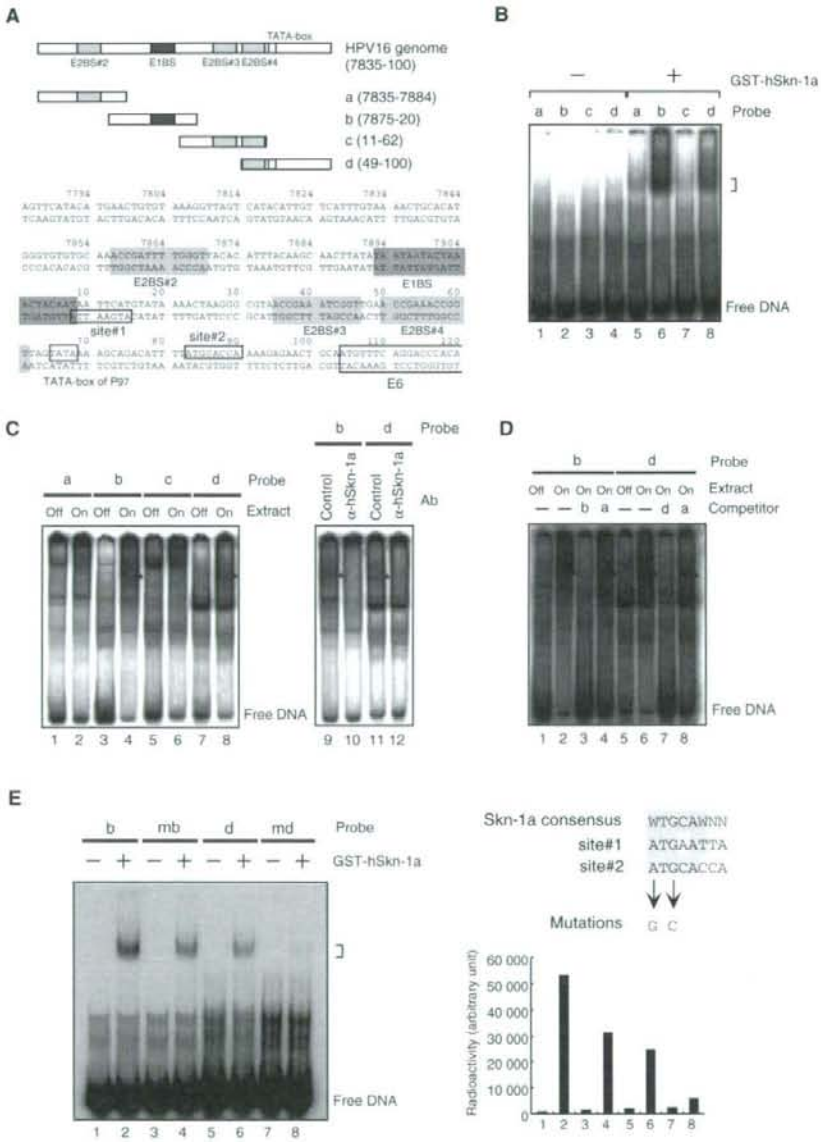
Binding of hSkn-1a to the core region in cells

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



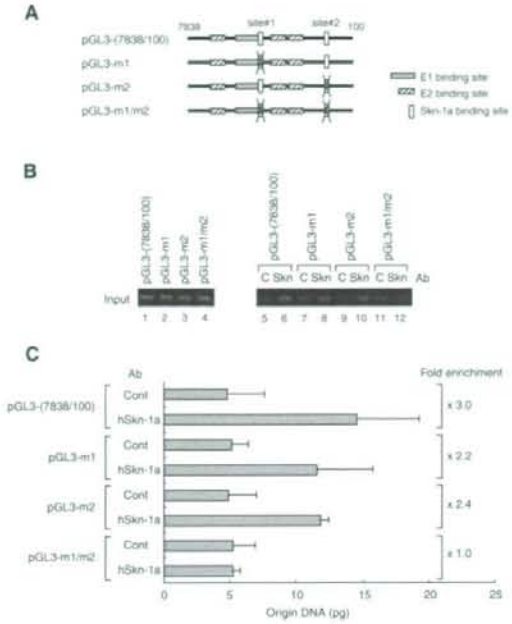


Fig. 5. Binding of hSkn-1a to the HPV16 origin region *in vivo*. (A) Schematic representation of pGL3-(7838/100) with mutations in the hSkn-1a binding sites. The mutations shown in Fig. 4E were introduced into the corresponding positions of pGL3-(7838/100) to produce pGL3-m1, pGL3-m2 and pGL3-m1/m2. 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.

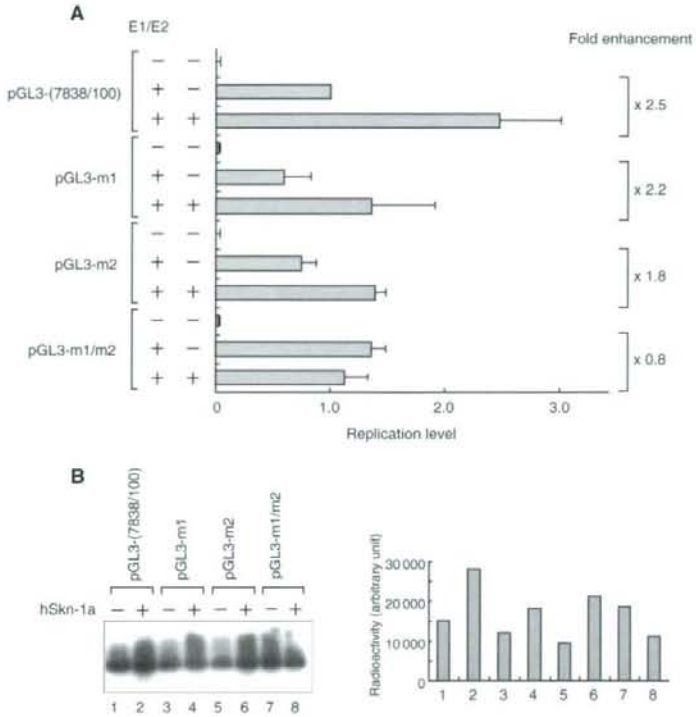


Fig. 6. Effect of hSkn-1a on the replication of mutated origin-containing plasmids. (A) Replicated pGL3-(7838/100), pGL3-m1, pGL3-m2 or pGL3-m1/m2 was measured with the extract from HEK293 cells that had been co-transfected with (plus) or without (minus) plasmids expressing E1/E2 and hSkn-1a, as indicated. 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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Commentary

Development of an HPV vaccine for a broad spectrum of high-risk types

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Key words: high-risk HPV, VLP vaccine, minor capsid protein L2, type-common neutralization epitope

Cervical cancer, the second most frequent gynecological malignancy in the world, is caused by infection with high-risk human papillomaviruses (HPV16, 18, and other 13 types). Vaccines protecting women from infection with high-risk HPVs can reduce prevalence of cervical cancer without costly screening for cervical precancerous lesions. Two vaccines containing HPV16 and 18 virus-like particles (VLPs), which were produced by self-assembly of the major capsid protein L1 of each HPV type, successfully induced type-specific neutralizing antibodies in the recipients of the large scale clinical trials and have been approved by several countries. Although HPV16 and HPV18 account for approximately 50% and 20% of cervical cancer, respectively, the remaining major issue to be addressed is how to prevent infection with other high-risk HPVs. Our and other studies have indicated that the HPV minor capsid protein L2 has several type-common neutralization epitopes and that immunization of animals with peptides having the L2-epitopes protects them against experimental challenge. Recently, we have shown that a type of chimeric VLP, HPV16 VLPs to which the type-common L2-epitope was added, induced in rabbits both the anti-HPV16 L1 neutralizing antibody at a high level and the anti-L2 cross-neutralizing antibody at lower but sufficient levels to be protective shown in the other animal experiments. Thus, this type of chimeric VLP seems likely to be one of the next-generation vaccine candidates for a broad spectrum of high-risk HPV types. Future issues were discussed about the HPV vaccine development.

Cervical Cancer and High-Risk HPVs

Cervical cancer is the second most frequent gynecological malignancy in the world.[1] World Health Report (2004) estimated that approximately 500,000 new cases appear and about 239,000 die annually. Industrialized countries have succeeded in greatly reducing deaths from cervical cancer through a screening program that allows early detection of precancerous lesions. Presently 80% of the patients are in developing countries, where the program is too expensive to implement. Cervical cancer is a major health inequity between developed and developing countries.

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Cervical cancer and its precursor lesions are caused by genital infection with human papillomaviruses (HPVs). HPVs are classified into more than 100 genotypes based on the homology of genomic DNA, and the types found in cervical cancer are grouped as high-risk types (types of 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 73).[2] The distribution of high-risk HPVs in humans slightly varies from region to region; HPV16 and HPV18 account for about 50% and 20%, respectively, of the cases worldwide.[2,3]

HPVs are a nonenveloped small icosahedral virus with a circular double-stranded DNA genome of 8 k base pairs (bp). The life cycle of HPVs is closely associated with epithelial differentiation. HPVs reach and infect the basal cells of the stratified epithelia through small epithelial lesions. In the basal cells the viral DNA is maintained as episomes in the nuclei. When the host cells initiate terminal differentiation, the HPV DNA starts to replicate and to be transcribed. HPV virions are generated in the upper layers of the epidermis or mucosa and released from them.[4,5]

HPV oncoproteins, E6 and E7, make the virus propagation possible in the terminally differentiating cells by reactivating cellular DNA synthesis machinery and preventing apoptotic death of the cells active for unnatural DNA synthesis. The E7 protein, binding to pRb, mediates release of the cellular E2F transcription factor from a pRb-E2F complex. The E6 protein, binding to p53, directs its degradation through a ubiquitin-mediated pathway. The accidental integration of the viral genome into the host chromosome produces cell continuously expressing E6 and E7. These cells, which lack the functions of pRb and p53, are immortalized and become malignant after accumulation of genetical alterations.[4,5] Thus, a vaccine that prevents HPV infection could reduce the incidence of the cancer.

Structure of HPV Capsid

The HPV capsid consists of the major capsid protein L1 (mol. wt.; around 55,000) and the minor capsid protein L2 (mol. wt.; around 76,000). Seventy-two capsomeres (each is an L1-pentamer) are arranged on a skew T = 7d icosahedral surface lattice of the capsid. Twelve capsomeres contact five neighbors and the others, six. Three-dimensional image reconstruction suggests that L2 is located in the center of pentavalent capsomeres at the virion vertices.[6]

Although cell cultures supporting efficient HPV replication are not available, the expression of L1 either alone or together with L2 in surrogate systems results in the spontaneous formation of particles without viral DNA, the virus-like particle (VLP) or the L1/L2-capsid, respectively.[7,8] These particles are morphologically undistinguishable from HPV virions. Expression of L1 and L2 in

cells harboring episomal copies of an expression plasmid results in packaging the episomal DNA into the L1/L2-capsids to produce infectious pseudovirions.[9] The pseudovirions are used as a surrogate virus to detect neutralizing activity of anti-HPV antibodies.[10]

VLP Vaccine for Types 16 and 18

The VLPs are highly immunogenic in animals[11] and humans[12] and induce a type-specific neutralizing antibody that recognizes a conformation-dependent epitope.[13] Rabbits immunized with cottontail rabbit papillomavirus (CRPV) VLP are resistant to CRPV challenge. Transfer of serum or immunoglobulin G from the immunized rabbits protects naive rabbits against the challenge, strongly suggesting that the protection is mediated by neutralizing antibodies. Similarly, immunization of calves with VLP of Bovine Papillomavirus 4 (BPV4) is extremely effective to protect animals against BPV4 challenge.[14] The immunization with VLP also elicits cell-mediated responses including induction of CTL and memory B-cells. Although the exact role of various immune mechanisms in the protective efficacy of the VLP vaccine remains to be determined, these data clearly indicate that VLP is suitable for prophylactic vaccine antigen.

Merck & Co., Inc., developed the prophylactic vaccine (Gardasil®) using VLPs of HPV16 and 18 (and low-risk HPV6 and 11 associated with benign genital warts) as antigens. The vaccine successfully induced type-specific neutralizing antibodies in the recipients and protected them against infection with these HPVs in the large-scale clinical trials.[15] GlaxoSmithKline Co., developed the similar prophylactic vaccine (Cervarix®) using VLPs of HPV16 and 18 as antigens and showed preventive effect against HPV16 and 18 with limited cross-protection against HPV31 and 45, the most closely related types to HPV16 and 18, respectively.[16] The US Food and Drug Administration and European Medicine Agency (EMEA) have approved Gardasil® in 2006 and EMEA has approved Cervarix® in 2007. Although HPV16 and 18 together account for approximately 70% of cervical cancer, one of the remaining problems to be addressed is how to prevent infection with other high-risk HPVs. Multivalent vaccine combining VLPs of all of the high-risk HPVs may not be practical.

Type-Common Neutralization Epitope in L2

We have identified type-common neutralization epitopes in HPV16 L2. Rabbit antisera induced by synthetic peptides with sequences of HPV16 L2 amino acids (aa) 18 to 38, aa 56 to 75, aa 61 to 75, aa 64 to 81 or aa 96 to 115 have been shown to neutralize the HPV16 pseudovirions and cross-neutralize the pseudovirions of one or more of HPV18, 31 and 58.[17] Also, mouse monoclonal antibody recognizing an epitope in aa 17 to 36 neutralizes pseudovirions of HPV5, 6, 16, 18, 31, 45, 52, 58 and bovine papillomavirus type 1 (BPV1), and authentic virions of HPV11.[18] These data strongly suggest that there exist several cross-neutralization epitopes in the HPV16 L2 region from aa 17 to 115. Amino acids sequences of the L2 regions corresponding to the HPV16 L2 region are highly conserved between the high-risk HPVs.

Several animal experiments have indicated that L2-vaccines can prevent immunized animals from papilloma formation by not only homologous papillomaviruses but also heterologous papillomaviruses. Vaccination of rabbits with a synthetic peptide with an aa sequence of L2 aa 94–112 or 107–122 of cottontail rabbit papillomavirus (CRPV) protects them against challenge with CRPV.[19] Vaccination of calves with a bacterially produced peptide with L2 aa 11–200 of bovine papillomavirus type 4 (BPV4) protects them against challenge with BPV4.[20] Vaccination of rabbits with a bacterially produced peptide having HPV16 L2 aa 11–200 efficiently protects them against cutaneous challenge with CRPV and mucosal challenge with rabbit oral papillomavirus.[21]

The activity to neutralize infectious pseudovirions or authentic virions of the papillomaviruses used for the challenge has been measured with the serum samples collected from the protected animals. The neutralizing titers of the serum samples range from an undetectable level to 1:250, which are much lower than those induced by the VLP vaccines. The data suggest that despite their lower ability to elicit neutralizing titers, the L2 vaccines are capable of effectively protecting animals against challenge with papillomaviruses.

Chimera VLP as a Vaccine Candidate for Multiple HPVs

It is an attractive idea to add the cross-neutralization L2-epitopes to the current VLP vaccine, whose safety has been well established, to produce a modified vaccine effective against a broader spectrum of the high-risk HPVs. We have inserted the peptides containing the cross neutralizing L2-epitopes between aa 430 and 433 of HPV16 L1.[22] The chimeric L1s are able to assemble themselves into the chimeric VLPs. Although the sizes of the chimeric VLPs are not identical with that of HPV16VLP, immunization of rabbits with the chimeric VLPs has induced neutralizing antibody against HPV16, as HPV16 VLP does, indicating that the chimeric VLPs retained neutralization epitope of HPV16 VLP. The antisera induced by the chimeric VLP with the peptide having HPV16 L2 aa 56–75 (Ch56/75VLP) have cross-neutralized HPV18, 31, 35, 52, 58 (Kondo K et al.[22] and our unpublished data). The level of the anti-L2 neutralizing antibody induced by Ch56/75VLP is comparable to those protecting the rabbits or the calves against the experimental challenge with the papillomaviruses in the previous animal experiments.[19-21] Therefore, the chimera VLP, Ch56/75VLP, may serve as a vaccine candidate for a broad spectrum of high-risk HPVs.

Future Issues to be Addressed

The current HPV vaccine is expected to inhibit latent infection with HPV, whereas the other successful vaccines, such as polio vaccine and measles vaccine, inhibit the onset of symptoms through the suppression of large scale viral propagation by quick secondary immune reaction after the early propagation of the infecting viruses. For effective protection, the anti-HPV neutralizing antibody should be present continuously. At present we do not know the minimum serum antibody level required for the protection. Data should be collected on the serum neutralizing antibody level required for preventing the recipients from infection with HPV in large-scale post marketing surveillance studies. The kinetics of the antibody levels of the recipients over time will provide a basis to predict the plateau level from the peak level and to consider an appropriate dose regimen and the need for boosting.

Although HPV vaccine is expected to prevent cervical cancer, some practical virological surrogate indicators should be developed to evaluate the vaccine efficacy. One candidate is the prevention of persistent infection with HPV targeted by the vaccine. The HPV

persistent infection may be detected by continuous monitoring of HPV DNA in cervical and vaginal specimens. PCR amplification of HPV DNA is the most sensitive and accurate method for HPV detection. Unfortunately, however, we have little knowledge on the fate of basal cells latently infected with HPV, the frequency and the burst size of HPV propagation starting from one latently infected cell, and so on. Because HPV DNA in the latently infected basal cells may be undetectable, the HPV negative state should be carefully assessed. It is necessary to establish the relation between the latent infection and PCR-detection of HPV DNA.

It is possible that natural infection with HPV does not induce protective immunity, because HPV DNA is repeatedly detected with cervical and vaginal specimens collected at different times from single individuals who have neutralizing antibody at a low level against cognate HPV type (our unpublished data). The number of the latently infected basal cells may increase through repeated infection with HPV propagated in differentiating epithelial cells in the genital tract. The spread of HPV would increase the risk of the HPV DNA integration into cellular DNA, the key event to produce immortalized cells. Thus, the HPV vaccine may reduce the cancer risk, by inhibiting repeated infection, for women who have been infected with HPV. Further studies are needed on the life cycle of HPV in nature and the effect of the current vaccine, to develop an effective next-generation HPV vaccine.

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Neutralizing Antibodies against Human Papillomavirus Types 16, 18, 31, 52, and 58 in Serum Samples from Women in Japan with Low-Grade Cervical Intraepithelial Neoplasia▽

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We have very limited information on serum neutralizing antibody in women naturally infected with the human papillomaviruses (HPVs) that are causally associated with cervical cancer. In this study, serum samples collected from 217 Japanese women with low-grade cervical intraepithelial neoplasia were examined for their neutralizing activities against HPV16, -18, -31, -52, and -58 pseudovirions. Eighty-four patients (39%), 35 patients (16%), 17 patients (8%), and 1 patient were positive for neutralizing antibodies against one, two, three, and four of these types, respectively. Presence of neutralizing antibody did not always correlate with detection of HPV DNA in cervical swabs collected at the time of blood collection. The neutralizing titers of the majority of sera, ranging between 40 and 640, were found to be conserved in the second sera, collected 24 months later, independently of emergence of HPV DNA in the second cervical swabs. The data strongly suggest that HPV infection induces anti-HPV neutralizing antibody at low levels, which are maintained for a long period of time.

Human papillomavirus (HPV) is a small, nonenveloped virus with a circular, double-stranded DNA of 8 kbp packaged in icosahedral capsids composed of two capsid proteins, L1 (major) and L2 (minor) (2, 3). Of the more than the 100 genotypes classified for DNA homology (4), 15 types (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68, and -73) are called high-risk HPVs because they are causally associated with cervical cancer (15), the second most frequent gynecological cancer in the world (17).

HPV infects the basal cells of the stratified epithelia through small epithelial lesions. In the infected cells, the viral DNA is maintained as episomes, which start to be transcribed and replicate with the onset of terminal cell differentiation (5). HPV progeny virions are produced in the upper layers of the epidermis or mucosa and released from them. The HPV DNA detectable in cervical swabs is likely to originate from these virions and thus to be considered a sign of HPV propagation, probably because it seems difficult to collect HPV DNA by swabbing the episomal HPV DNA maintained in the infected basal cells. The high-risk HPVs occasionally induce low-grade cervical intraepithelial neoplasia types 1 and 2 (CIN1 and CIN2), which usually regress spontaneously (18). A small fraction of the lesions progress to high-grade CIN (CIN3), the precursor of cervical cancer (18).

Expression of L1 in insect cells or in *Saccharomyces cerevisiae* results in the spontaneous formation of virus-like particles (VLPs). VLPs are highly immunogenic in animals and humans (6, 8, 9, 11) and induce predominantly type-specific neutralizing antibodies (7, 19). A prophylactic vaccine using HPV6, -11, -16, and -18 VLPs as antigens has been developed and shown to successfully induce type-specific neutralizing antibodies in recipients in large-scale clinical trials (8, 12, 21). These studies strongly suggest that type-specific neutralizing antibody may be induced in women naturally infected with an HPV.

Although cell cultures supporting efficient HPV replication are not available, coexpression of L1 and L2 from codon-modified L1 and L2 genes in cultured animal cells harboring episomal DNA with a reporter results in packaging of the reporter DNA into L1/L2 capsids to produce infectious pseudovirions (PVs) (1). PVs are used as a surrogate virus to detect the neutralizing activities of anti-HPV antibodies (14, 16).

In this study, we collected serum samples and cervical swabs from 217 Japanese women with CIN1 or CIN2 at diagnosis (first samples) and 24 months later (second samples) and examined these samples for the presence of anti-HPV neutralizing antibody and HPV DNA, respectively. The results suggest that even at low levels, HPV infection induces persisting neutralizing antibody.

MATERIALS AND METHODS

Patients and samples. Serum samples and cervical swabs were collected from 217 Japanese women (20 to 49 years old) with CIN1 or CIN2 with informed consent. The first samples were obtained at diagnosis, and the second ones were obtained 24 months later. Three of the second samples were collected at 10, 14,

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