C 型慢性肝炎患者 MDC の機能低下に TLR3-TRIF-TRAF6 の系が関与しており、HCV に対する新たな免疫療法の治療標的になり得る可能性が示された。

F. 健康危険情報　なし

G. 研究発表

1. 論文発表

1. Miyazaki, M., et al. Impaired cytokine response in myeloid dendritic cells in chronic hepatitis C virus infection regardless of enhanced expression of Toll-like receptors and retinoic acid inducible gene-I. ***J Med Virol*** 2008. 80: 980-988.

2. Kanto T. Virus-associated innate immunity in liver. ***Front Bioscience*** 2008. 13. 6183-6192

3. Yamaguchi, S., et al. Dendritic cell-based vaccines suppress metastatic liver tumor via activation of local innate and acquired immunity. ***Cancer Immunol Immunother*** 2008. 57: 1861-1869.

4. Tanaka, H., et al. Declining incidence of hepatocellular carcinoma in Osaka, Japan, from 1990 to 2003. ***Ann Intern Med*** 2008. 148: 820-826.

5. Kohga, K., et al. Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver diseases and changes during transcatheter arterial embolization for hepatocellular carcinoma. ***Cancer Sci*** 2008. 99: 1643-1649.

6. Tatsumi, T., et al. Decreased expressions of CD1d molecule on liver dendritic cells in subcutaneous tumor bearing mice. ***J Hepatol*** 2008. **49**: 779-786.

7. Hiramatsu, N., et al. Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C. ***Hepatol Res*** 2008. 38: 52-59.

8. Kanada, A., et al. Early emergence of entecavir-resistant hepatitis B virus in a patient with hepatitis B virus/human immunodeficiency virus coinfection. ***Hepatol Res*** 2008. 38: 622-628.

H. 知的財産権の出願・登録状況

特に予定なし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

初回献血者におけるがんウイルスマーカー陽性率の調査と輸血によるがんウイルス感染予防に関する研究

分担研究者　　内田　茂治　　日本赤十字社　中央血液研究所

研究要旨：われわれは１９９５年から東京都、茨城県、栃木県、神奈川県および福岡県の初回献血者を対象として、ＨＢｓ抗原、ＨＢｃ抗体、ＨＣＶ抗体およびＨＴＬＶ－１抗体の陽性率の調査を行っている。初回献血者は陽性通知による選択を受けないため、陽性率はその地域住民の陽性率を反映するものと考えられる。これらの調査により、１）ＨＢｓ抗原、ＨＢｃ抗体ならびにＨＣＶ抗体の陽性率は年毎に低下傾向を示しており、使い捨て注射器・注射針が一般化する以前の感染状態と比べると、Ｂ型肝炎ウイルス（ＨＢＶ）、Ｃ型肝炎ウイルス（ＨＣＶ）の新たな感染は限定的であると考えられる、２）１６歳初回献血者のＨＢｓ抗原陽性率の調査から、１９８６年から開始された「ＨＢＶの母子感染防止対策事業」は非常に効果が高かったことが確認された、３）ＨＴＬＶ－１抗体の陽性率は、福岡県では調査開始から低下傾向が認められていたが、関東地方の１都３県では明確な低下傾向が認められていない、などが明らかとなった。

また、献血者からいただいた血液から輸血を介してこれらがんウイルス感染がどの程度発生しているかの調査を併せて行っている。輸血によるがんウイルス感染は検査試薬の改良や自動検査機器の導入による検査精度向上によって減少してきたが、ＨＢＶ、ＨＣＶ、後天性ヒト免疫不全ウイルスを対象に１９９９年に導入された核酸増幅検査（ＮＡＴ）によって輸血用血液の安全性は飛躍的に高まった。しかしながら、それでも毎年１０例程度の輸血後ＨＢＶ感染と数年に１例の輸血後ＨＣＶ感染が確認されていた。今年度新たに導入されたＮＡＴ装置・試薬は従来のものより検出感度が高く、輸血後ＨＢＶ・ＨＣＶ感染は更に減少すると考えられる。今後も調査を継続してこれらＮＡＴ装置・試薬の評価を行っていきたい。

Ａ．研究目的

献血者のウイルスマーカー陽性率の動向を調査することは、輸血用血液の安全性や献血者スクリーニングの有効性をモニタリングする上で重要である。とくに初回献血者は感染症陽性通知による選択を受けないため、その陽性率は地域住民の陽性率を反映していると考えられ、そのウイルスの疫学調査が可能となる。また、年齢別の調査を行うことにより、過去の感染原因の推定や将来の発がん件数を算定することが可能と

なる。１９８５年から一部の医療機関で、１９８６年からは全国の医療機関で行われ「ＨＴＬＶ－１の母子感染予防対策」の成果を確認するためにもこれら調査は重要である。

また、献血者からいただいた血液から輸血を介してこれらがんウイルス感染がどの程度発生しているかの調査は、輸血用血液の安全性の評価とそれに対する対策を考慮するための重要な指標となりうる。１９６０年代には売買血の横行により輸血を受けた患者の約半数が肝炎を発症していたと報告されている。１９７０年代に入り完全な献血への移行がなされ、ＨＢＶの特異的な診断法の確立、更には１９８９年にＨＣＶの遺伝子断片が見出され、これら検査が輸血用血液のスクリーニング検査に取り入れられたため、輸血に伴う肝炎ウイルス感染は激減した。その後も検査試薬の改良や自動検査機器の導入による検査精度向上によって輸血後感染は減少したが、年間数十例の感染が報告されてきた。１９９９年に導入されたＮＡＴにより感染直後のいわゆるウインドウ期の血液が輸血用から排除されるようになり、輸血用血液の安全性は飛躍的に高まった。しかしながら、それでも毎年１０例程度の輸血後ＨＢＶ感染と数年に１例の輸血後ＨＣＶ感染が確認されていた。今年度新たに導入されたＮＡＴ装置・試薬は従来のものより検出感度が高く、輸血後ＨＢＶ・ＨＣＶ感染は更に減少すると考えられる。今後も調査を継続してこれらＮＡＴ装置・試薬の評価を行っていきたい。

Ｂ．研究方法

１）初回献血者のがんウイルスマーカー陽性率の調査

東京都、茨城県、栃木県、神奈川県および福岡県の初回献血者を対象としてＨＢｓ抗原、ＨＢｃ抗体、ＨＣＶ抗体およびＨＴＬＶ－Ｉ抗体の陽性率の調査を行った。従来、ＨＢｓ抗原検査は逆受身赤血球凝集反応（ＲＰＨＡ法）、ＨＢｃ抗体検査は逆受身赤血球凝集阻止法（ＨＩ法）、ＨＣＶ抗体検査は血球凝集反応（ＰＨＡ法）または粒子凝集反応（ＰＡ法）、ＨＴＬＶ－Ｉ抗体は粒子凝集反応（ＰＡ法）により行ってきたが、福岡県は平成２０年１月から、栃木県は同２月、東京都、茨城県、神奈川県は同４月から化学発光酵素免疫法（ＣＬＥＩＡ法）に検査方法を変更した。これらの検査結果を日本赤十字社血液事業統一システムから抽出し、性別、年齢ごとに陽性率を集計した。

２）輸血によるがんウイルス感染に関する調査

輸血によるがんウイルス感染に関する調査は、①輸血を受けた患者の感染症マーカーが陽転化して医療機関から報告のある「自発報告例」、②前回献血時には陰性であった献血者が今回献血時に陽転化した場合や、献血後に体調不良等で受診して感染が確認された等の「献血後情報例」が発生した場合に、献血時に同時採血して冷凍保管してある「保管検体」を融解し、ウイルスの有無や患者血液から検出されたウイルスとの遺伝子型や多変領域遺伝子配列を調べ、両ウイルスの相同性の検討を行った。ＨＢＶの遺伝子型はｓ領域のダイレクトシークエンス法、ＨＣＶの遺伝子型はcore 領域のダ

イレクトシークエンス法により行った。
3）核酸増幅スクリーニング検査で検出されたＨＢＶ、ＨＣＶの解析
トシークエンス法、ＨＣＶの遺伝子型はcore領域のダイレクトシークエンス法により行った。

C．研究結果
1）初回献血者のがんウイルスマーカー陽性率の調査
平成２１年中に献血者の感染症血清学検査の方法が従来の血球・粒子凝集法からＣＬＥＩＡ法に変更となった。このため非特異的反応による擬陽性が多く検出され、ＨＢｃ抗体検査以外の項目では陽性率が２～３倍上昇してしまった。そのためＨＢｓ抗原検査ではほぼ全年齢で平成１７年の陽性率を大きく上回ってしまい、参考となるデータも得られなかった。しかしながら、１６歳初回献血者では幸運にも擬陽性が出現せず、陽性の献血者も水平感染であることが確認されており、国による「ＨＢＶの母子感染防止対策事業」実施後に出生した児が献血可能年齢となる平成１５年以降、平成１８年に「ＨＢＶの母子感染防止対策事業」によるワクチンとガンマグロブリンを投与したにもかかわらずキャリア化してしまった１例を除き、ＨＢＶキャリアは確認されていない。ＨＣＶ抗体検査も擬陽性が出現し平成２０年の陽性率は平成１７年の陽性率とほぼ同様の結果となったが、実際には更に低いものと考えられる。しかし、かつては４％を超えていた６０歳代での陽性率も１％を下回っており、ＨＣＶ抗体陽性者のほとんどが献血可能年齢を超えてしまったと考えられる。ＨＴＬＶ－１抗体検査も擬陽性が出現したが、平成２０年の陽性率は平成１７年よりも減少した。これはもともと陽性率が他の地域より高かった福岡県での低下が寄与しており、関東地方の１都３県では明確な低下傾向を示していない。しかし、平成１４年には９％を超えていた６０歳代でも１．２％と低下し、ＨＣＶ抗体と同様に陽性率の高かった年代層が献血年齢を超えてしまったと考えられる。ＣＬＥＩＡ法でのＨＢｃ抗体検査の陽性率は、従来の凝集法とほぼ同等で、平成２０年のＨＢｃ抗体陽性率は３０歳未満では平成１７年とほぼ同様であったが、３０歳以上では若干の低下が認められた。したがって、ＨＢｃ抗体の陽性率は今年度以降も継続可能であると考えられる。
2）輸血によるがんウイルス感染に関する調査
２００８年の確認された輸血感染例はＨＢＶの４例（自発報告例３例、献血後情報例１例）であった。これらの感染の原因となった血液はいづれも従来のＮＡＴシステムでスクリーニングされたもので、新たなＮＡＴシステム導入後のスクリーニング血液での感染は現在までのところ確認されていない。
3）核酸増幅スクリーニング検査で検出されたＨＢＶ、ＨＣＶの解析
１９９９年７月のスクリーニングＮＡＴ開始から２００８年３月までに、ＨＢＶ－ＤＮＡが８０９例検出された。その遺伝子型分布はＡ：１３７例（１６．９％）、Ｂ：１１７例（１４．５％）、Ｃ：５４３例（６７．

1%)、D：8例（1．0%)、E：1例（0．1%)、H：3例（0．4%）であった。もともと日本では稀といわれていた遺伝子型Aは2000年に2例が見出され、2001年16例、2002年18例、2003年27例と増加した。また、137例中117例（85．4%）のsubgenotypeは欧米型のAeで、アジア型のAaは17例（12．4%)、3例（2．2%）は判定不能であった。このように欧米型のHBVが日本に入り込み、感染が拡大していることがうかがえる。感染者は20歳代71人（51．8%)、30歳代45人（32．8%）と若年者がおもであるが、遺伝子型Aは成人での感染でもその約10%がキャリア化するといわれており、キャリア化した感染者からその周囲へと感染が広がっていると考えられる。一方、HCV－RNAはスクリーニングNAT開始以降113例が検出され、その遺伝子型分布は1a：1例（0．9%)、1b：30例（26．5%)、2a：53例（46．9%)、2b：29例（25．7%）であった。慢性肝疾患患者で報告されている遺伝子型は、1b：70%、2a：20%、2b：10%であり、NAT検出例とは分布が大きく異なる。慢性肝疾患患者は現在より20～40年前に感染したと考えられ、NAT検出例はすべてHCV抗体陰性の感染初期例である。NAT検出例の献血者は男性71人（62．8%)、女性42人（37．2%）と男性の方が多かったが、この比率は全献血者の男女比とほぼ一致しており、性別による差はみられなかった。遺伝子型別の男女比は1aが0：1、1bが15：15、2aが33：20、2bが23：6で、2bで男性の比率が有意に高かった。年代別では10歳代：13例（11．5%)、20歳代：48例（42．5%)、30歳代：29例（25．7%)、40歳代：13例（11．5%)、50歳代：5例（4．4%)、60歳代：5例（4．4%）であった。これを献血者年代別構成比と比較すると10歳代・20歳代の若い世代でHCV－RNA陽性者の割合が高かった。遺伝子型別に採血地域を調べたところ、1bは関東以北に比べ中部地方より西の地域で多く、2aは中部地方が一番多く、北海道を除くその他の地域であまり差はみられなかった。2bは関東で一番多く次いで近畿、中国、北海道の順で、逆に中部地方と東北地方ではみられなかった。

D．考察

初回献血者のがんウイルスマーカー陽性率の調査は検査法の変更による非特異反応でHBc抗体以外のマーカーは信頼性が乏しかった。そこで平成20年からは、①HBs抗原検査は吸収試験陽性例のみを集計する、②HCV抗体検査はHCV－RNA陽性例のみを集計する、③HTLV－1抗体検査は間接蛍光抗体法またはウエスタンブロット法陽性例のみを集計する、に変更して調査を継続する予定である。また、今回の結果からHCV抗体・HTLV－1抗体の陽性率は非特異反応を考慮しても明らかに低下傾向が確認されている。特にかつては陽性率の高かった60歳代での陽性率低下は著しく、現在から10～20年経過すればHCVによる肝臓がん発症や、HTLV－1関連疾病の発症も大幅に減少すると考えられた。

輸血によるがんウイルス感染に関する調査

ではＨＢＶ感染が平成１９年の１３例から、平成２０年は４例と減少した。平成２０年中に輸血を実施し、未だ発症していない感染例も存在する可能性はあるものの、平成２０年に新たに導入されたＮＡＴシステムの感度向上による効果と考えられた。今後数年をかけて新ＮＡＴシステムの評価を行っていきたい。

核酸増幅スクリーニング検査で検出されたＨＢＶ、ＨＣＶの解析により、ＨＢＶは欧米型の遺伝子型Ａが２０００年頃より急速に国内で拡がったと推定され、１３７例中女性は４人（Ａａ：２、Ａｅ：２）のみで、若年男性を中心とした性感染症として拡大したと考えられる。ＨＢＶ－ＤＮＡが検出された８０７例の約７０％はＨＢｃ抗体陰性であり、日本人のＨＢｓ抗原陽性率が低下してきたとはいえ、新たな感染が無くなったわけではない。

ＨＣＶ－ＮＡＴ陽性例の解析から従来日本で多いと考えられていた遺伝子型１ｂは全体の約４分の１で、２ａが感染の主体となってきている。感染は若年層を中心とした違法薬物の静脈内投与が原因と考えられている。今後同一地域での陽性例の遺伝子配列を比較して、感染実態の解明を検討したい。

Ｅ．論文・学会発表

＜学会発表＞

１）内田茂治：ＮＡＴ検査と今後の検査. 第１５回日本輸血・細胞治療学会秋季シンポジウム（２００８年１０月　大阪）

２）古居保美、五十嵐正志、蕎麦田理英子、猪俣尋史、星友二、福田俊洋、松本千恵子、鈴木光、柚木久雄、内田茂治、三根英子、田所憲治：ＮＡＴスクリーニング検査で検出されたＨＣＶ－ＲＮＡ陽性検体の解析. 第３２回日本血液事業学会総会　（２００８年１０月　大阪）

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ochi H, Kondo K, Matsumoto K, Oki A, Yasugi T, Furuta R, Hirai Y, Yoshikawa H, Kanda T.	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.	Clin Vaccine Immunol.	15	1536-1540	2008
Kanda T, Kondo K.	Development of an HPV vaccine for a broad spectrum of high-risk types.	Hum Vaccin.	5	43-45	2009
Kukimoto I, Mori S, Sato H, Takeuchi T, Kanda T.	Transcription factor human Skn-1a enhances replication of human papillomavirus DNA through the direct binding to two sites near the viral replication origin.	FEBS J.	275	3123-3135	2008
Kawana K, Matsumoto J, Miura S, Shen L, Kawana Y, Nagamatsu T, Yasugi T, Fujii T, Yang H, Quayle AJ, Taketani Y, Schust DJ.	Expression of CD1d and ligand-induced cytokine production are tissue specific in mucosal epithelia of the human lower reproductive tract.	Infect Immun.	76	3011-3018	2008
Matsumoto J, Kawana K, Nagamatsu T, Schust DJ, Fujii T, Sato H, Hyodo H, Yasugi T, Kozuma S, Taketani Y.	Expression of surface CD1d in the extravillous trophoblast cells of early gestational placenta is downregulated in a manner dependent on trophoblast differentiation.	Biochem Biophys Res Commun.	371	236-241	2008
Tomio A, Schust DJ, Kawana K, Yasugi T, Kawana Y, Mahalingaiah S, Fujii T, Taketani Y.	Prolactin can modulate CD4+ T-cell response through receptor-mediated alterations in the expression of T-bet.	Immunol Cell Biol.	86	616-621	2008
Yoshida S, Kajitani N, Satsuka A, Nakamura H, Sakai H.	Ras modifies proliferation and invasiveness of cells expressing human papillomavirus oncoproteins.	J Virol.	82	8820-8827	2008

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Okamoto K, Mori Y, Komoda Y, Okamoto T, Okochi M, Takeda M, Suzuki T, Moriishi K, Matsuura Y.	Intramembrane processing by signal peptide peptidase regulates the membrane localization of hepatitis C virus core protein and viral propagation.	J Virol.	82	8349-8361	2008
Masaki T, Suzuki R, Murakami K, Aizaki H, Ishii K, Murayama A, Date T, Matsuura Y, Miyamura T, Wakita T, Suzuki T.	Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles.	J Virol.	82	7964-7976	2008
Aizaki H, Morikawa K, Fukasawa M, Hara H, Inoue Y, Tani H, Saito K, Nishijima M, Hanada K, Matsuura Y, Lai MM, Miyamura T, Wakita T, Suzuki T.	Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection.	J Virol.	82	5715-5724	2008
Okamoto T, Omori H, Kaname Y, Abe T, Nishimura Y, Suzuki T, Miyamura T, Yoshimori T, Moriishi K, Matsuura Y.	A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication.	J Virol.	82	3480-3489	2008
Taguwa S, Okamoto T, Abe T, Mori Y, Suzuki T, Moriishi K, Matsuura Y.	Human butyrate-induced transcript 1 interacts with hepatitis C virus NS5A and regulates viral replication.	J Virol.	82	2631-2641	2008
Suzuki R, Moriishi K, Fukuda K, Shirakura M, Ishii K, Shoji I, Wakita T, Miyamura T, Matsuura Y, Suzuki T.	Proteasomal turnover of hepatitis C virus core protein is regulated by two distinct mechanisms: a ubiquitin-dependent mechanism and a ubiquitin-independent but PA28gamma-dependent mechanism.	J Virol.	83	2389-2392	2009
Nitahara-Kasahara Y, Fukasawa M, Shinkai-Ouchi F, Sato S, Suzuki T, Murakami K, Wakita T, Hanada K, Miyamura T, Nishijima M.	Cellular vimentin content regulates the protein level of hepatitis C virus core protein and the hepatitis C virus production in cultured cells.	Virology	383	319-327	2009
Akazawa D, Date T, Morikawa K, Murayama A, Omi N, Takahashi H, Nakamura N, Ishii K, Suzuki T, Mizokami M, Mochizuki H, Wakita T.	Characterization of infectious hepatitis C virus from liver-derived cell lines.	Biochem Biophys Res Commun.	377	747-751	2008
Murakami K, Kimura T, Osaki M, Ishii K, Miyamura T, Suzuki T, Wakita T, Shoji I.	Virological characterization of the hepatitis C virus JFH-1 strain in lymphocytic cell lines.	J Gen Virol.	89	1587-1592	2008
Ishii K, Murakami K, Hmwe SS, Zhang B, Li J, Shirakura M, Morikawa K, Suzuki R, Miyamura T, Wakita T, Suzuki T.	Trans-encapsidation of hepatitis C virus subgenomic replicon RNA with viral structure proteins.	Biochem Biophys Res Commun.	371	446-450	2008

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Omata K, Suzuki R, Masaki T, Miyamura T, Satoh T, Suzuki T.	Identification and characterization of the human inhibitor of caspase-activated DNase gene promoter.	Apoptosis.	13	929-937	2008
Kuroki M, Ariumi Y, Ikeda M, Dansako H, Wakita T, Kato N.	Arsenic trioxide inhibits hepatitis C virus RNA replication through modulation of the glutathione redox system and oxidative stress.	J Virol.	83	2338-2348	2009
Ariumi Y, Kuroki M, Dansako H, Abe K, Ikeda M, Wakita T, Kato N.	The DNA damage sensors ataxia-telangiectasia mutated kinase and checkpoint kinase 2 are required for hepatitis C virus RNA replication.	J Virol.	82	9639-9646	2008
Dansako H, Ikeda M, Abe K, Mori K, Takemoto K, Ariumi Y, Kato N.	A new living cell-based assay system for monitoring genome-length hepatitis C virus RNA replication.	Virus Res.	137	72-79	2008
Mori K, Abe K, Dansako H, Ariumi Y, Ikeda M, Kato N.	New efficient replication system with hepatitis C virus genome derived from a patient with acute hepatitis C.	Biochem Biophys Res Commun.	371	104-109	2008
Ando M, Korenaga M, Hino K, Ikeda M, Kato N, Nishina S, Hidaka I, Sakaida I.	Mitochondrial electron transport inhibition in full genomic hepatitis C virus replicon cells is restored by reducing viral replication.	Liver Int.	28	1158-1166	2008
Hirano K, Ichikawa T, Nakao K, Matsumoto A, Miyaaki H, Shibata H, Eguchi S, Takatsuki M, Ikeda M, Yamasaki H, Kato N, Kanematsu T, Ishii N, Eguchi K.	Differential effects of calcineurin inhibitors, tacrolimus and cyclosporin a, on interferon-induced antiviral protein in human hepatocyte cells.	Liver Transpl.	14	292-298	2008
Nakamura M, Saito H, Ikeda M, Tada S, Kumagai N, Kato N, Shimotohno K, Hibi T.	Possible molecular mechanism of the relationship between NS5B polymorphisms and early clearance of hepatitis C virus during interferon plus ribavirin treatment.	J Med Virol.	80	632-639	2008
Miyazaki M, Kanto T, Inoue M, Itose I, Miyatake H, Sakakibara M, Yakushijin T, Kakita N, Hiramatsu N, Takehara T, Kasahara A, Hayashi N.	Impaired cytokine response in myeloid dendritic cells in chronic hepatitis C virus infection regardless of enhanced expression of Toll-like receptors and retinoic acid inducible gene-I.	J Med Virol.	80	980-988	2008
Yamaguchi S, Tatsumi T, Takehara T, Sasakawa A, Hikita H, Kohga K, Uemura A, Sakamori R, Ohkawa K, Hayashi N.	Dendritic cell-based vaccines suppress metastatic liver tumor via activation of local innate and acquired immunity.	Cancer Immunol Immunother.	57	1861-1869	2008

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tanaka H, Imai Y, Hiramatsu N, Ito Y, Imanaka K, Oshita M, Hijioka T, Katayama K, Yabuuchi I, Yoshihara H, Inoue A, Kato M, Takehara T, Tamura S, Kasahara A, Hayashi N, Tsukuma H.	Declining incidence of hepatocellular carcinoma in Osaka, Japan, from 1990 to 2003.	Ann Intern Med.	148	820-826	2008
Kohga K, Takehara T, Tatsumi T, Ohkawa K, Miyagi T, Hiramatsu N, Kanto T, Kasugai T, Katayama K, Kato M, Hayashi N.	Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver diseases and changes during transcatheter arterial embolization for hepatocellular carcinoma.	Cancer Sci.	99	1643-1649	2008
Tatsumi T, Takehara T, Yamaguchi S, Sasakawa A, Yamamoto M, Fujita Y, Miyagi T, Ohkawa K, Hayashi N.	Decreased expressions of CD1d molecule on liver dendritic cells in subcutaneous tumor bearing mice.	J Hepatol.	49	779-786	2008
Hiramatsu N, Kurashige N, Oze T, Takehara T, Tamura S, Kasahara A, Oshita M, Katayama K, Yoshihara H, Imai Y, Kato M, Kawata S, Tsubouchi H, Kumada H, Okanoue T, Kakumu S, Hayashi N.	Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C.	Hepatol Res.	38	52-59	2008
Kanada A, Takehara T, Ohkawa K, Kato M, Tatsumi T, Miyagi T, Sakamori R, Yamaguchi S, Uemura A, Kohga K, Sasakawa A, Hikita H, Kawamura K, Kanto T, Hiramatsu N, Hayashi N.	Early emergence of entecavir-resistant hepatitis B virus in a patient with hepatitis B virus/human immunodeficiency virus coinfection.	Hepatol Res.	38	622-628	2008

CLINICAL AND VACCINE IMMUNOLOGY, Oct. 2008, p. 1536–1540 Vol. 15, No. 10
1556-6811/08/$08.00+0 doi:10.1128/CVI.00197-08

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▽

Hiroyuki Ochi,[1,2]† Kazunari Kondo,[1,2]† Koji Matsumoto,[2] Akinori Oki,[2] Toshiharu Yasugi,[3] Reiko Furuta,[4] Yasuo Hirai,[5] Hiroyuki Yoshikawa,[2] and Tadahito Kanda[1]*

Center for Pathogen Genomics, National Institute of Infectious Diseases, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan[1]; Department of Obstetrics and Gynecology, Graduate School of Comprehensive Human Science, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8575, Japan[2]; Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan[3]; and Department of Pathology, Cancer Institute,[4] and Departments of Gynecology and Cytopathology, Cancer Institute Hospital,[5] Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan

Received 29 May 2008/Returned for modification 28 July 2008/Accepted 18 August 2008

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,

* Corresponding author. Mailing address: Center for Pathogen Genomics, National Institute of Infectious Diseases, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111, ext. 2524. Fax: 81-3-5285-1166. E-mail: kanda@nih.go.jp.
† These authors contributed equally to this work.
▽ Published ahead of print on 27 August 2008.

TABLE 1. Neutralization of HPV16, -18, -31, -52, and -58 PVs with rabbit antisera obtained by immunization with HPV16, -18, -31, -52, and -58 VLPs

Antiserum	Neutralizing titer of antibody against:				
	HPV16	HPV18	HPV31	HPV52	HPV58
Anti-HPV16 VLP	204,800	<40	800	<40	<40
Anti-HPV18 VLP	<40	1,638,400	<40	<40	<40
Anti-HPV31 VLP	200	<40	1,638,400	<40	<40
Anti-HPV52 VLP	<40	<40	<40	409,600	400
Anti-HPV58 VLP	<40	<40	<40	25,600	409,600

TABLE 2. Numbers of CIN-afflicted women negative and positive for neutralizing antibody

HPV type	No. of women							
	Neutralization		Neutralizing titer					
	−	+	40	80	160	320	640	≥1,280
HPV16	167	50	11	14	8	8	6	3
HPV18	194	23	8	4	5	2	2	2
HPV31	187	30	9	6	4	6	4	1
HPV52	174	43	12	18	6	3	2	2
HPV58	155	62	10	11	13	9	12	7

and 11 months, however, because the lesions that progressed to CIN3 were surgically removed.

Rabbit antisera against HPV16, -18, -31, -52, and -58 VLPs. The recombinant baculovirus expressing HPV16, -18, -31, -52, or -58 L1 was inoculated into Sf9 cells (five flasks containing 175 cm^2 culture) and incubated for 72 h at 27°C. The cells were collected and suspended in 5 ml of phosphate-buffered saline (PBS) containing 0.5% NP-40. After incubation for 10 min at room temperature, the cells were centrifuged at 10,000 × *g* at 4°C for 15 min to precipitate nuclei. The nuclei were suspended in PBS containing CsCl (1.28 g/ml) and lysed with brief sonication (Sonifier250; Branson, Germany). The solution was centrifuged at 34,000 rpm for 20 h at 20°C in an SW50.1 rotor (Beckman Coulter Inc., Fullrerion, CA). The fractions with buoyant densities around 1.28 g/ml were pooled and dialyzed against PBS supplemented with 0.5 M NaCl at 4°C to remove CsCl. Then, the solution was layered on the top of a discontinuous sucrose density gradient (5% and 60%) in PBS and centrifuged at 31,000 rpm for 2 h at 4°C in an SW50.1 rotor. The particles between the 5% and 60% sucrose layers were collected and dialyzed against PBS supplemented with 0.5 M NaCl at 4°C to obtain purified VLPs.

Japanese white rabbits (2.3 to 3.0 kg in weight) were subcutaneously injected with a mixture of each purified VLP and Freund's complete adjuvant (injection performed by Scrum Inc., Tokyo, Japan). Immunization was repeated four times at 2-week intervals, and serum samples were obtained 1 week after the last immunization. The antisera were filtered (Steradisc25; Kurabo Inc., Osaka, Japan) before use for the assays in this study.

Preparation of PVs. Five plasmids (pYSEAP, expressing secreted alkaline phosphatase [SEAP]; p16L1h, expressing HPV16 L1; p16L2h, expressing HPV16 L2; peL1fB, expressing HPV18 L1; and peL2bhb, expressing HPV18 L2) were gifts from J. T. Schiller. Six plasmids (p31L1h, expressing HPV31 L1; p31L2h, expressing HPV31 L2; p58L1h, expressing HPV58 L1; p58L2h, expressing HPV58 L2; p52L1h, expressing HPV52 L1; and p52L2h, expressing HPV52 L2) had previously been constructed (10, 11).

293FT cells (Invitrogen Corp., Carlsbad, CA), which had been seeded in two 10-cm culture dishes (6×10^6 cells/dish) 16 h before transfection, were transfected with a mixture of an L1 plasmid (13.5 μg), an L2 plasmid (3 μg), and pYSEAP (13.5 μg) by using Fugene HD (Rosch Diagnostics GmbH, Mannheim, Germany). Sixty hours later, PVs were extracted and purified from the cells as described previously (11). The infectivity of the purified PV was estimated from the SEAP activity of the culture medium of cells infected with PVs by a colorimetric SEAP assay (http://home.ccr.cancer.gov/lco/ColorimetricSEAP.htm).

Neutralization test. The serum samples were diluted with a neutralization medium (Dulbecco's modified Eagle's medium [without phenol red] containing 10% FBS, 1% nonessential amino acids, and 1% GlutaMax-I). Fifty microliters of a serum sample was mixed with 50 μl of neutralization medium containing an aliquot of the PV stock sufficient to induce an optical density at 405 nm of approximately 1.0 of SEAP under our assay conditions (400 pg L1 for HPV16, 800 pg L1 for HPV18 and HPV52, 200 pg L1 for HPV31, and 125 pg L1 for HPV58) and incubated for 1 h at 4°C. The numbers of PVs used were in the linear range of dose-response relation. Then, the mixture was inoculated into 293FT cells, which had been seeded in a well of a 96-well plate (1×10^4 cells) 16 h before the inoculation. The culture medium was harvested after incubation of the cells for 4.5 days at 37°C, and the SEAP activity of the culture medium was measured. The neutralization titer was presented as the reciprocal of the maximum dilution of serum that reduced the SEAP level to half of that for the sample not treated with serum.

HPV DNA detection and typing. HPV DNA was detected in the cervical swabs by a PCR-based method as described previously (23). In brief, total cellular DNA was extracted from each specimen by the standard procedure. HPV DNA was amplified by PCR using the consensus primers for the HPV L1 region. HPV types were identified on the basis of restriction fragment length polymorphism, which has been shown to identify at least 26 types of genital HPVs (23).

RESULTS

Neutralization of HPV16, -18, -31, -52, and -58 PVs with rabbit antisera obtained by immunization with HPV16, -18, -31, -52, or -58 VLPs. The rabbit anti-VLP serum prepared in this study was found to type-specifically and efficiently neutralize the PV used for immunization (Table 1). The neutralizing activity of the antiserum was evaluated from the reduction of infectivity of the PV that had been incubated with the diluted serum. Although anti-HPV18 VLP did not cross-neutralize the PVs of the heterologous types tested, the other antisera showed very limited cross-neutralization between phylogenetically closely related HPV16, -31, -52, and -58 PVs (4). The cross-neutralizing titers of anti-HPV16 VLP against HPV31 PV, anti-HPV31 VLP against HPV16 PV, anti-HPV52 VLP against HPV58 PV, and anti-HPV58 VLP against HPV52 PV were 1/256, 1/8,192, 1/1,024, and 1/16, respectively, of the titers neutralizing the homologous PVs. The data strongly suggest that, like VLPs, natural HPV virions have highly type-specific dominant neutralization epitopes (as revealed in the rabbit experiments), which are likely to be recognized by humans in the natural infection.

Neutralization of HPV16, -18, -31, -52, and -58 PVs with serum samples from women with CIN1 or CIN2. Diluted serum samples from 217 women with CIN1 or CIN2 were each mixed with one of the PVs, and the resulting reductions of infectivity were measured. Serum samples that did not reduce the infectivity to less than half at a dilution of 1:40 were tentatively defined as negative for neutralization. Under this cutoff condition, 137 samples (63%) were found to neutralize PVs of one or more of the following: HPV16, -18, -31, -52, and -58.

Table 2 shows the numbers of women negative and positive for neutralization. Neutralizing antibodies against HPV16, -18, -31, -52, and -58 were detected in 50 (24%), 23 (11%), 30 (19%), 43 (21%), and 62 (30%) samples, respectively. The titers of the majority (94% for HPV16, 91% for HPV18, 98% for HPV31, 95% for HPV52, and 90% for HPV58) of the neutralizing sera ranged between 40 and 640.

Table 3 shows the numbers of women positive for neutralizing antibodies against one or multiple HPV types. One woman had antibodies against four types (HPV16, -18, -31, and -58). Seventeen women had antibodies against three types, and 35 women had antibodies against two types. Titers of antibodies against HPV16 and 52 did not correlate with titers of

TABLE 3. Numbers of CIN-afflicted women positive for neutralizing antibody

HPV type(s)	No. of women
16, 18, 31, 52, 58	0
18, 31, 52, 58	0
16, 31, 52, 58	0
16, 18, 52, 58	0
16, 18, 31, 58	1
16, 18, 31, 52	0
16, 18, 31	2
16, 18, 52	2
16, 18, 58	0
16, 31, 52	1
16, 31, 58	4
16, 52, 58	3
18, 31, 52	1
18, 31, 58	0
31, 52, 58	4
16, 18	3
16, 31	5
16, 52	3
16, 58	6
18, 31	1
18, 52	3
18, 58	3
31, 52	2
31, 58	3
52, 58	6
16	21
18	6
31	6
52	18
58	33
None	80

antibodies against HPV31 and -58, respectively (data not shown), strongly suggesting that the titers were not influenced by the cross-neutralization found by the hyperimmune rabbit antisera (Table 1).

Table 4 shows the numbers of women positive for both serum neutralizing antibody and corresponding HPV DNA by the cervical swabbing assay, positive for neutralizing antibody but negative for HPV DNA, and negative for neutralizing antibody but positive for HPV DNA. The cervical swabs, which

TABLE 4. Numbers of CIN-afflicted women with and without neutralizing antibody and HPV DNA

HPV type	Presence of HPV DNA	No. of women	
		With neutralizing antibody	Without neutralizing antibody
HPV16	+	24	14
	−	23	
HPV18	+	5	4
	−	15	
HPV31	+	4	3
	−	26	
HPV52	+	24	16
	−	19	
HPV58	+	23	7
	−	39	

TABLE 5. Fluctuation of HPV-neutralizing titers between serum samples collected after a 24-month interval

Antibody	Neutralizing titer for indicated group		
	Descent[a]	Conservation[b]	Ascent[c]
Anti-HPV16	12	37	10
Anti-HPV18	5	17	8
Anti-HPV31	10	20	4
Anti-HPV52	7	34	12
Anti-HPV58	9	51	11

[a] For the descent group, neutralizing titers decreased by more than half or converted from positive to negative.

[b] For the conservation group, neutralizing titers fluctuated between half and twofold.

[c] For the ascent group, neutralizing titers increased more than twofold or converted from negative to positive.

were collected at the time of serum collection, were examined for the presence of HPV DNA by PCR amplifying the L1 region with the consensus primers. Although there were women positive for both neutralizing antibody and HPV DNA, approximately half (HPV16 and -52) or more than half (HPV18, -31, and -58) of the women were positive for neutralizing antibody without detection of HPV DNA. The neutralizing titers of the serum samples from the DNA-positive women were not statistically different from those from the DNA-negative women. Furthermore, some serum samples from the women positive for HPV DNA were not neutralizing. The data indicate that the presence of HPV DNA, which is clear evidence of HPV replication shortly prior to serum collection, did not always correlate with the presence of neutralizing antibody or with a boost of the level of neutralizing antibody.

Neutralizing titers of serum samples collected 24 months later. The majority of women conserved their levels of serum neutralizing antibodies for 24 months. The second samples were collected 24 months after the first collection. Nine, 7, 4, 10, and 9 women converted from negative to positive for serum neutralizing antibodies against HPV16, -18, -31, -52, and -58, respectively. Eight, three, six, seven, and three women lost neutralizing activity against HPV16, -18, -31, -52, and -58, respectively. The titers of the majority of the serum samples fluctuated between half and twofold (Table 5).

The ascent of the neutralizing titer was not associated with detection of HPV DNA in the cervical swabs. Table 6 shows the neutralizing titers of the 21 paired serum samples collected from women whose first cervical swabs were negative for HPV DNA and whose second swabs were positive for HPV DNA. Although the neutralizing titers for 6 women rose slightly, those for 15 women did not. The data, in agreement with the data shown in Table 3, indicate that the presence of cervical HPV DNA does not necessarily boost the level of serum neutralizing antibody.

DISCUSSION

Accumulating data indicate that HPV infection induces highly type-specific neutralizing antibody. The recipients injected with the HPV vaccine containing HPV16 and -18 VLPs develop antibodies neutralizing HPV16 and -18 exclusively (7). The rabbits immunized with HPV16, -18, -31, -52, and -58

TABLE 6. Neutralizing titers of pairs of serum samples from women negative for HPV DNA at the first blood collection and positive for HPV DNA at the second blood collection

Neutralizing antibody	Neutralizing titer for indicated serum sample		No. of patients
	First (HPV DNA$^-$)	Second (HPV DNA$^+$)	
Anti-HPV16	<40	<40	3
	320	160	1
Anti-HPV18	<40	<40	1
	<40	40	2
	320	320	1
Anti-HPV52	<40	<40	4
	<40	80	1
	<40	160	1
	<40	640	1
	160	80	1
	80	40	1
Anti-HPV58	<40	320	1
	40	40	1
	80	80	1
	160	160	1

VLPs developed antibodies neutralizing homologous HPV efficiently with very limited cross-neutralization (Table 1). Thus, the past and the persistent infections of women with HPV could be traced by detection of anti-HPV type-specific neutralizing antibody. In this study, we examined serum samples from 217 Japanese women with CIN1 or CIN2 for neutralizing antibodies against HPV16, -18, -31, -52, and -58, the types common in CIN1 and -2 in Japan (13).

One hundred thirty-seven (63%) serum samples were positive for neutralizing antibodies against one or more HPV types tested (Table 2). The neutralizing titers of the majority of the serum samples, which ranged between 40 and 640 (Table 2), fluctuated within the narrow range, between half and twofold, after 24 months (Table 5). The data strongly suggest that women infected with an HPV develop a low-level anti-HPV neutralizing antibody and keep the level for a long period.

The titers for the serum samples from women positive for HPV DNA by the cervical swabbing assay were similar to those for women negative for HPV DNA. Detection of HPV DNA in the second swabbing did not correlate with a marked rise in neutralizing titers in the second serum samples (Table 6). The cervical swabs probably did not contain HPV propagated in the lower genital tract (22). Low-level propagation of HPV usually continues in CIN1 and -2 lesions. Therefore, a negative result for HPV DNA may be due, at least partly, to inappropriate sampling and to low sensitivity in the detection method and must be carefully interpreted. But a positive result for HPV DNA indicates that HPV propagated at a detectable level. The data strongly suggest that the level of anti-HPV neutralizing antibody is not boosted by HPV propagation.

The inefficient immune response of the women to the infecting HPV may be associated with the HPV life cycle: propagation in the terminally differentiating cells with a limited burst size. During the long history of virus and human, the life cycle may have evolved to escape the host immune systems. A low level of neutralizing antibody may not be protective against repeated infection with the homologous HPV type. Repeated infection may raise the risk of integration of the HPV E6/E7 oncogenes into cell DNA, leading to the initial step for cell immortalization by HPV.

The serum samples from 53 (24%) women were positive for neutralization against two or more HPV types tested (Table 3). Cross-neutralization does not seem to contribute to neutralization of multiple HPV types, because the anti-HPV16 titers did not correlate with the anti-HPV31 titers and the anti-HPV52 titers did not correlate with the anti-HPV58 titers. The data strongly suggest that the women whose sera neutralized multiple types were infected with those multiple HPVs.

The tentative cutoff used in this study probably grouped the serum samples containing very low levels of neutralizing antibody into the negative group, explaining at least partly why the 80 (37%) women with CIN1 and CIN2, the lesions caused by HPV infection, were negative for the neutralizing antibodies against the tested HPV types, which cover the major types detected in CIN1 and -2 in Japan (13). Enhancing the sensitivity of the neutralization assay would be desirable for further studies of natural HPV infection and the host immune response against it.

Recently, Steel et al. (20) measured neutralizing antibody in sera of young women by using HPV16 and -18 PVs produced similarly to those in this study. They found that most of the women seroconverted and kept their neutralizing activity for a long time, while some failed to seroconvert, which is consistent with the data obtained in this study.

ACKNOWLEDGMENTS

We thank Akira Mitsuhashi, Takuma Fujii, Tsuyoshi Iwasaka, Nobuo Yaegashi, Yoh Watanabe, Yutaka Nagai, and Tomoyuki Kitagawa for the sample collection and Kunito Yoshiike for critical reading of the manuscript.

This work was supported by grants-in-aid from the Ministry of Health, Labor and Welfare for the Third-Term Comprehensive 10-year Strategy for Cancer Control; from the Ministry of Education, Science, Sports and Culture of Japan (number 12218102); and from the Smoking Research Foundation.

REFERENCES

1. **Buck, C. B., D. V. Pastrana, D. R. Lowy, and J. T. Schiller.** 2004. Efficient intracellular assembly of papillomaviral vectors. J. Virol. **78:**751–757.
2. **Buck, C. B., N. Cheng, C. D. Thompson, D. R. Lowy, A. C. Steven, J. T. Schiller, and B. L. Trus.** 2008. Arrangement of L2 within the papillomavirus capsid. J. Virol. **82:**5190–5197.
3. **Chen, X. S., R. L. Garcea, I. Goldberg, G. Casini, and S. C. Harrison.** 2000. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. Mol. Cell **5:**557–567.
4. **de Villiers, E. M., C. Fauquet, T. R. Broker, H. U. Bernard, and H. zur Hausen.** 2004. Classification of papillomaviruses. Virology **324:**17–27.
5. **Doorbar, J.** 2006. Molecular biology of human papillomavirus infection and cervical cancer. Clin. Sci. **110:**525–541.
6. **Giannini, S. L., E. Hanon, P. Moris, M. Van Mechelen, S. Morel, F. Dessy, M. A. Fourneau, B. Colau, J. Suzich, G. Losonksy, M. T. Martin, G. Dubin, and M. A. Wettendorf.** 2006. Enhanced humoral and memory B cellular using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only. Vaccine **24:**5937–5949.
7. **Giroglou, T., M. Sapp, C. Lane, C. Fligge, N. D. Christensen, R. E. Streeck, and R. C. Rose.** 2001. Immunological analyses of human papillomavirus capsids. Vaccine **19:**1783–1793.
8. **Harper, D. M., E. L. Franco, C. M. Wheeler, A. B. Moscicki, B. Romanowski, C. M. Roteli-Martins, D. Jenkins, A. Schuind, S. A. Costa Clemens, G. Dubin, et al.** 2006. Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papilomavirus types 16 and 18: follow-up from a randomized control trial. Lancet **367:**1247–1255.

9. **Kirnbauer, R., F. Booy, N. Cheng, D. R. Lowy, and J. T. Schiller.** 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc. Natl. Acad. Sci. USA **89:**12180–12184.
10. **Kondo, K., Y. Ishii, H. Ochi, T. Matsumoto, H. Yoshikawa, and T. Kanda.** 2007. Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. Virology **358:**266–272.
11. **Kondo, K., H. Ochi, T. Matsumoto, H. Yoshikawa, and T. Kanda.** 2008. Modification of human papillomavirus-like particle vaccine by insertion of the cross-reactive L2-epitopes. J. Med. Virol. **80:**841–846.
12. **Koutsky, L. A., K. A. Ault, C. M. Wheeler, D. R. Brown, E. Barr, F. B. Alvarez, L. M. Chiacchierini, and K. U. Jansen.** 2002. A controlled trial of a human papillomavirus type 16 vaccine. N. Engl. J. Med. **347:**1645–1651.
13. **Maehama, T.** 2005. Epidemiological study in Okinawa, Japan, of human papillomavirus infection of the uterin cervix. Infect. Dis. Obstet. Gynecol. **13:**77–80.
14. **Mbulawa, Z. Z., A. L. Williamson, D. Stewart, J. A. Passmore, L. Denny, B. Allan, and D. J. Marais.** 2008. Association of serum and mucosal neutralizing antibodies to human papillomavirus type 16 (HPV-16) with HPV-16 infection and cervical disease. J. Gen. Virol. **89:**910–914.
15. **Muñoz, N., F. X. Bosch, X. Castellsague, M. Diaz, S. Sanjose, D. Hammouda, K. V. Shah, and C. J. L. M. Meijer.** 2004. Against which human papillomavirus types shall we vaccinate and screen? Int. J. Cancer **111:**278–285.
16. **Pastrana, D. V., C. B. Buck, Y. Y. Pang, C. D. Thompson, P. E. Castle, D. C. FitzGerald, S. Kruger Kjaer, D. R. Lowy, and J. T. Schiller.** 2004. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. Virology **321:**205–216.
17. **Pisani, P., F. Bray, and M. Parkin.** 2002. Estimates of the world-wide prevalence of cancer for 25 sites in the adult population. Int. J. Cancer **97:**72–81.
18. **Schiffman, M., P. E. Castle, J. Jeronimo, A. C. Rodriguez, and S. Wacholder.** 2007. Human papillomavirus and cervical cancer. Lancet **370:**890–907.
19. **Smith, J. F., M. Brownlow, M. Brown, R. Kowalski, M. T. Esser, W. Ruiz, E. Barr, D. R. Brown, and J. T. Bryan.** 2007. Antibodies from women immunized with Gardasil cross-neutralize HPV 45 pseudovirions. Hum. Vaccin. **3:**109–115.
20. **Steele, J., S. Collins, K. Wen, G. Ryan, C. Constandinou-Williams, and C. B. J. Woodman.** 2008. Measurement of the humoral immune response following an incident HPV16 or HPV18 infection in young women by a pseudovirion-based neutralizing antibody assay. Clin. Vaccine Immunol. **15:**1387–1390.
21. **Villa, L. L., R. L. Costa, C. A. Petta, R. P. Andrade, K. A. Alut, A. R. Giuliano, C. M. Wheeler, L. A. Koutsky, C. Malm, M. Lehtinen, F. F. Skjeldestad, S. E. Olsson, M. Steinwall, D. R. Brown, R. J. Kurman, B. M. Ronnett, M. H. Stoler, A. Frenczy, D. M. Harper, G. M. Tamms, J. Yu, L. Lupinacci, R. Railkar, F. J. Taddeo, K. U. Jansen, M. T. Esser, H. L. Sings, A. J. Saah, and E. Barr.** 2005. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomized double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncol. **6:**271–278.
22. **Winer, R. L., Q. Feng, J. P. Hughes, M. Yu, N. B. Kiviat, S. O'Reilly, and L. A. Koutsky.** 2007. Concordance of self-collected and clinician-collected swab samples for detecting human papillomavirus DNA in women 18 to 32 years of age. Sex. Transm. Dis. **34:**371–377.
23. **Yoshikawa, H., T. Kawana, K. Kitagawa, M. Mizuno, H. Yoshikura, and A. Iwamoto.** 1991. Detection and typing of multiple genital papillomaviruses by DNA amplification with consensus primers. Jpn. J. Cancer Res. **82:**524–531.

[Human Vaccines 5:1, 43-45; January 2009]; ©2009 Landes Bioscience

Commentary

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

Tadahito Kanda* and Kazunari Kondo

Center for Pathogen Genomics; National Institute of Infectious Diseases; Shinjuku-ku, Tokyo, Japan

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.

*Correspondence to: Tadahito Kanda; Center for Pathogen Genomics; National Institute of Infectious Diseases; 1-23-1 Toyama; Shinjuku-ku, Tokyo 162-8640 Japan; Tel.: +81.3.5285.1111 ext. 2524; Fax: +81.3.5285.1166; Email: kanda@nih.go.jp

Submitted: 04/25/08; Accepted: 07/07/08

Previously published online as a *Human Vaccines* E-publication: http://www.landesbioscience.com/journals/vaccines/article/6554

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.

References

1. Ferlay J, Bray F, Pisani P, Parkin DM. GLOBOCAN 2002 cancer incidence. Mortality and prevalence worldwide. IARC Press 2004.
2. Munoz N, Bosch FX, Castellsague X, Diaz M, Sanjose S, Hammouda D, Shah KV, Meijer CJLM. Against which human papillomavirus types shall we vaccinate and screen? Int J Cancer 2004; 111:278-85.
3. Clifford G, Franceschi S, Diaz M, Munoz N, Villa LL. Chapter 3: HPV type-distribution in women with and without cervical neoplastic diseases. Vaccine 2006; 24:26-34.
4. Zur Hausen H. Papillomavirus infections-A major cause of human cancers. Biochem Biopys Acta 1996; 1288:55-78.
5. Zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 2000; 92:690-8.
6. Buck CB, Cheng N, Thompson CD, Lowy DR, Steven AC, Schiller JT, Trus BL. Arrangement of L2 within the papillomavirus capsid. J Virol 2008; 82:5190-7.
7. Kirnbauer R, Taub J, Greenstone H, Roden R, Duerst M, Gissmann L, Lowy DR, Schiller JT. Efficient self-assembly of human papillomavirus type 16 and L1–L2 into virus-like particles. J Virol 1993; 67:6929-36.
8. Volpers C, Schirmacher P, Streeck R, Sapp M. Assembly of the major and the minor capsid protein of human papillomavirus type 33 into virus-like particles and tubular structures in insect cells. Virology 1994; 200:504-12.
9. Buck CB, Pastrana DV, Lowy DR, Schiller JT. Efficient intracellular assembly of papillomaviral vectors. J Virol 2004; 78:751-7.
10. Pastrana DV, Buck CB, Pang YY, Thompson CD, Castle PE, FitzGerald DC, Kruger Kjaer S, Lowy DR, Schiller JT. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. Virology 2004; 321:205-16.
11. Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc Natl Acad Sci USA 1992; 89:12180-4.
12. Koutsky LA, Ault KA, Wheeler CM, Brown DR, Barr E, Alvarez FB, Chiacchierini LM, Jansen KU. A controlled trial of a human papillomavirus type16 vaccine. N Engl J Med 2004; 347:1645-51.
13. Giroglou T, Sapp M, Lane C, Fligge C, Christensen ND, Streeck RE, Rose RC. Immunological analyses of human papillomavirus capsids. Vaccine 2001; 19:1783-93.
14. Kirnbauer R, Chandrachud LM, O'Neil BW, Wagner ER, Grindlay GJ, Armstrong A, McGarvie GM, Schiller JT, Lowy DR, Campo MS. Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization. Virology 1996; 219:37-44.
15. Villa LL, Costa RL, Petta CA, Andrade RP, Ault KA, Giuliano AR, Wheeler CM, Koutsky LA, Malm C, Lehtinen M, Skjeldestad FE, Olsson SE, Steinwall M, Brown DR, Kurman RJ, Ronnett BM, Stoler MH, Ferenczy A, Harper DM, Tamms GM, Yu J, Lupinacci L, Railkar R, Taddeo FJ, Jansen KU, Esser MT, Sings HL, Saah AJ, Barr E. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16 and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncology 2005; 6:271-8.
16. Harper DM, Franco DM, Wheeler CM, Moscicki AB, Romanowski B, Roteli-Martins CM, Jenkins D, Schuind A, Costa Clemens SA, Dubin G. HPV vaccine study group. 2006. Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. Lancet 367:1247-55.
17. Kondo K, Ishii Y, Ochi H, Matsumoto T, Yoshikawa H, Kanda T. Neutralization of HPV16, 18, 31 and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. Virology 2007; 358:266-72.
18. Gambhira R, Karanam B, Jagu S, Roberts JN, Buck CB, Bossis I, Alphs H, Culp T, Christensen ND, Roden RB. A protective and broadly cross-neutralizing epitope of human papillomavirus L2. J Virol 2007; 81:13927-31.
19. Embers ME, Budgeon LR, Pickel M, Christensen ND. Protective immunity to rabbit oral and cutaneous papillomaviruses immunization with short peptides of L2, the minor capsid protein. J Virol 2002; 76:9798-805.
20. Gaukroger JM, Chandrachud LM, O'Neil BW, Grindlay GJ, Knowles G, Campo MS. Vaccination of cattle with bovine papillomavirus type 4 L2 elicits the production of virus-neutralizing antibodies. J Gen Virol 1996; 77:1577-83.
21. Gambhira R, Jagu S, Karanam B, Gravitt PE, Culp TD, Christensen ND, Roden RBS. Protection of rabbits against challenge with rabbit papillomaviruses by immunization with the N-terminal of minor antigen L2. J Virol 2007; 81:11585-92.
22. Kondo K, Ochi H, Matsumoto T, Yoshikawa H, Kanda T. Modification of human papillomavirus-like particle vaccine by insertion of the cross-reactive L2-epitope. J Med Virol 2008; 80:841-6.

the FEBS Journal

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

Iwao Kukimoto, Seiichiro Mori, Hidetaka Sato, Takamasa Takeuchi and Tadahito Kanda

Center for Pathogen Genomics, National Institute of Infectious Diseases, Tokyo, Japan

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

Correspondence
I. Kukimoto, Center for Pathogen Genomics, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
Fax: +81 3 5285 1166
Tel: +81 3 5285 1111
E-mail: ikuki@nih.go.jp

(Received 10 December 2007, revised 19 March 2008, accepted 15 April 2008)

doi:10.1111/j.1742-4658.2008.06468.x

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.