EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 39, No.5, 621-628, October 2007

Immunocytochemical detection of HPV16 E7 in cervical smear

Ju-Hong Jeon[1]*, Dong-Myung Shin[2]*, Sung-Yup Cho[2], Kye-Yong Song[3], Noh-Hyun Park[4], Heun-Soo Kang[5], Yung Dai Kim[5] and In-Gyu Kim[2,6]

[1]Department of Physiology
[2]Department of Biochemistry and Molecular Biology
Aging and Apoptosis Research Center
[4]Department of Obstetrics and Gynecology
Seoul National University College of Medicine
Seoul 110-799, Korea
[3]Department of Pathology
Chung-Ang University College of Medicine
Seoul 156-756, Korea
[5]Metabolic Engineering Laboratories
Seoul 110-510, Korea
[6]Corresponding author: Tel, 82-2-740-8248;
Fax, 82-2-744-4534; E-mail, igkim@plaza.snu.ac.kr
*These authors contributed equally to this work.

Accepted 6 August 2007

Abbreviations: HPV, human papillomavirus; Pap, Papanicolaou

Abstract

Cervical cancer is characterized by a long period of preclinical dysplasia or carcinoma *in situ* progressing into invasive cancer. Although Papanicolaou (Pap) smear test has contributed significantly to the early detection of precursor lesions, the cytological screening has inherent problems that produce considerable false negative/positive results. Since the infection of high-risk type of human papillomavirus (HPV) is strongly associated with cervical cancer, we investigated the feasibility of an immunostaining test to detect cells infected by HPV in cervical smear. We produced monoclonal antibodies against HPV16 E7 in mice by repeated injections with the recombinant HPV16 E7. Western blot analysis and immunocytochemical assay demonstrated that the selected monoclonal antibody, mAb (130-9-7), reacts specifically with cultured cervical cancer cell lines infected by HPV16. Specific staining was observable with the HPV16-positive smear specimens obtained from the cervical cancer patients, whereas no staining was detected with the HPV-negative smear specimens. To achieve the desired sensitivity, specificity and reproducibility, we modified and optimized the conventional immunocytochemical procedure for cervical smear specimens. Our results suggest that this immunostaining method for detecting high-risk HPV in cervical smear may be used as a strategy to distinguish a high-risk group, especially those patients with low grade cytological abnormality.

Keywords: antibodies, monoclonal; human papillomavirus 16; immunologic tests; papillomavirus E7 proteins; uterine cervical neoplasms

Introduction

Human papillomaviruses (HPVs) are small, double-stranded DNA viruses which infect epithelia of the skin and mucosa. Although they mostly cause a variety of benign lesions such as warts and verrucae, HPV infection is the most important risk factor associated with cervical cancer (Walboomers *et al.,* 1999). Among more than 120 HPV types identified so far, HPV16 is regarded as the most common high-risk type. E7 is one of 8 viral proteins encoded by HPV and recognized as the most important oncogenic protein that plays a key role in cellular transformation in the HPV-infected cervical cells (Munger and Phelps, 1993; Munoz *et al.,* 2003). Thus, E7 is becoming a valuable diagnostic marker for HPV infection and assessment of the risk of progression to cervical cancer (Senior, 2002).

Papanicolaou (Pap) smear test, designed to detect abnormal or cancerous cervical cells in cervical smear specimens, has contributed to reduce the mortality rate of cervical cancer by 70% between 1950 and 1970 (National Cancer Institute, Women's Health Report, Fiscal Year 2001-2002). Despite the phenomenal success, the Pap smear test has inherent problems that may produce false negative or false positive results, so that between 10 to 50 percent of all Pap smears are incorrectly analyzed. The difficulty involved in identifying as few as a dozen abnormal/cancerous cells among 50,000 to 300,000 cervical cells, the large number of specimens that each trained cytotechnologist has to examine in a given time, and the sampling inadequacy are the factors associated with the problems. Recently, new technologies have contributed to improve the clinical sensitivity of the conventional Pap smear test by liquid-based preparation (Wilbur *et al.*, 1994), pre-screening smears before a technologist's analysis (Wertlake, 1999), and confirmatory, reevaluation of a technologist's decisions (Koss *et al.,* 1994). However,

these modified Pap smear tests require elaborate apparatus and expensive reagents for routine operations.

The close association of HPV infection with cervical carcinoma provides the rationale for HPV testing, in addition to cervical cytology, in routine screening. The HPV tests identify the strains of high-risk HPV and clarify uncertain Pap smear results, eliminating the need for repeated screening and colposcopy. The DNA-based tests, such as Hybrid Capture (Vince *et al.,* 2002), PCR (Venturoli *et al.,* 2002) and DNA Chip (Cho *et al.,* 2003) test for HPV detection, can supplement Pap smear test. Nonetheless, these tests require expensive instruments, reagents and skilled technical personnel. It is desirable, therefore, to develop a new HPV test that is accurate, user-friendly, inexpensive, and compatible to Pap smear test.

In this study, we have generated mAb specific for HPV16 E7. Using a selected mAb, we have shown the feasibility of developing a practical immunostaining test of cervical smear to detect cervical cells infected by HPV16.

Materials and Methods

Cell lines and clinical specimens

Caski; SiHa; HeLa; PC3M; MCF7; L-132; WI-26VA4; C33A; A549; Saos2; U937; HL60; HaCaT; HepG2; RHEK; HT-29; SK-N-SH; HEK293; NIH 3T3; and HDF cell lines were obtained from American Type Culture Collection (Rockville, MD). Snu-17; Snu-703; and Snu-1299, were supplied by Seoul National University Type Culture Collection (Seoul, Korea). The cells were grown in the media as recommended by the suppliers. Cervical smear specimens from normal individuals as well as from patients diagnosed to have cervical carcinoma were collected at Seoul National University Hospital. Cervical smear for immunostaining was collected on lysine-coated microscope slide, fixed and transported in 4% formaldehyde (Sigma, St. Louis, MO) in PBS and stored in PBS at 4°C. The smear specimen collected for PCR test was suspended in PBS and stored at 4°C until tested. Consent from each patient was obtained for research use of the clinical specimen, and the protocols were in accord with the guidelines approved by the committee at Seoul National University.

Generation and purification of monoclonal antibodies

His-tagged HPV16 E7 protein was expressed in BL21(DE3) and purified by Ni-affinity chromatography as previously described (Jeon *et al.,* 2002). Female BALB/c mice (6-8 weeks old) were injected intra-peritoneally with His-tagged E7 (10 μg) mixed with an equal volume of Freund's complete adjuvant. The serum sample was monitored for the production of antibody to E7 using a 96-well microtiter plate coated with His-tagged E7, and HRP-conjugated anti-mouse immunoglobulins (Sigma). The injection was repeated 2 times until the antibody titer reached $OD_{490} > 2$ at 100-fold dilution of the mouse serum. The splenocytes were fused with mouse myeloma cell line SP2/0-Ag14 cells (de St Groth and Scheidegger, 1980) and hybridoma clones exhibiting a high titer to E7 were selected by ELISA.

Pristane-primed female BALB/c mice were injected with 4.5×10^7 cells of the selected hybridoma. Mouse ascitic fluid was fractionated by precipitation in 50% saturated ammonium sulfate. The precipitate was dissolved in PBS, dialyzed against the same buffer and subjected to a Protein G-coupled Sepharose 4B (Pharmacia, Peapack, NJ) column chromatography. The antibody concentration was determined by the absorbance measurement at 280 nm. The isotype of mAbs was determined by using mouse monoclonal isotyping kit (Sigma).

Western blot analysis

Western blot analysis was performed as previously described (Jeon *et al.,* 2002). Briefly, the cells were harvested and resuspended with lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM DTT, 2 μg/ml PMSF, protease inhibitor cocktail). The cell lysates were allowed to incubate on ice for 30 min, and centrifuged at 12,000 *g* for 10 min at 4°C. The crude extract (80 μg) was boiled for 10 min with 2 × sample buffer (120 mM Tris, pH 7.9, 4% SDS, 0.02% bromophenol blue, 20% glycerol, 2% 2-mercaptoethanol) and resolved in 15% SDS-PAGE. Transferred membranes were treated with 0.5% glutaldehyde (Sigma) at room temperature for 30 min and probed by mAb (130-9-7) and HRP-conjugated anti-mouse IgG antibody (Zymed).

Transfection experiments

Transient transfection experiments with HPV16 E7 or E6 were performed as previously described (Jeon *et al.,* 2003).

Immunostaining with cultured cells and tissue specimens from cervical cancer

Cells grown on the glass slip were fixed with 4% formaldehyde in PBS for 15 min, and then permeabilized by treating with 0.1% Triton X-100 in PBS for 8 min at room temperature (RT). For immunohistochemistry, cervical cancer tissue specimens on a lysine-coated slide were incubated in 3% H_2O_2 solution for 30 min. After treating the cells with blocking solution (5% BSA, 0.1% gelatin and 5% goat normal serum in PBS) for 30 min at RT, mAb to HPV16 E7 in 3% BSA-PBST (PBS with 0.1% Tween 20) was added and incubated for 1 h at RT. The cells were washed 5 times with PBST and incubated with the secondary antibody (PicTure Plus, Zymed, CA) for 30 min. After addition of substrate solution (AEC solution, Zymed) with gentle rocking for 5 min, the cells were mounted using Aqueous mounting medium (Dako, CA) and examined by microscope (Nikon TS100, Japan). Caski and MCF7 cell lines were used as controls in each experiment. The procedure, in which the incubation with the primary mAb to E7 was omitted, was employed as a negative control.

Immunostaining with cervical smear specimens

Cervical smear fixed with 4% formaldehyde was washed 2 times with PBS and stored at 4°C until tested. The entire procedure was carried out at RT. The specimen was treated with 0.1% Triton X-100 for 30 min for permeabilization, followed by a reaction with 6.5% H_2O_2 solution for 30 min. To effectively block nonspecific interaction between mAb and extraneous proteins in cervical smear, including mucinous macromolecules, a special blocking solution was formulated, which comprises 5% BSA, 0.1% gelatin and 5% normal goat serum in PBS. The specimen was soaked in the blocking solution for 30 min, and allowed to react with primary mAb in blocking solution contained 0.1% Tween 20 for 2 h. The specimen was then reacted with the secondary antibody solution for 1 h which was prepared by diluting PicTure Plus solution (40 fold) with blocking solution containing 0.1% Tween 20. After processing for color development with AEC solution, the specimen was mounted for examination under microscope. The control experiments were performed in the same manner as described.

PCR analysis with cervical smear specimens

Genomic DNAs were extracted from cervical smear specimen and analyzed by PCR method using BioCore HPV PCR kit (Sydney, Australia). Briefly, cervical smear was collected using CytobrushPlus GT (Medscand Medical, Sweden) into a tube containing PBS. The cells were separated by centrifugation, suspended in PBS and sonicated. Following centrifugation, the cell pellet was suspended in Extraction Buffer and boiled for 20 min. The supernatant separated by centrifugation was subjected to PCR to amplify the DNA specific for either all types of HPV (Jacobs *et al.,* 1995) or subtype such as HPV16 or 18 (van den Brule *et al.,* 1990; de Roda Husman *et al.,* 1994). The amplified products were resolved in agarose gel electrophoresis, and identified according to their molecular size corresponding to the standards included in the kit.

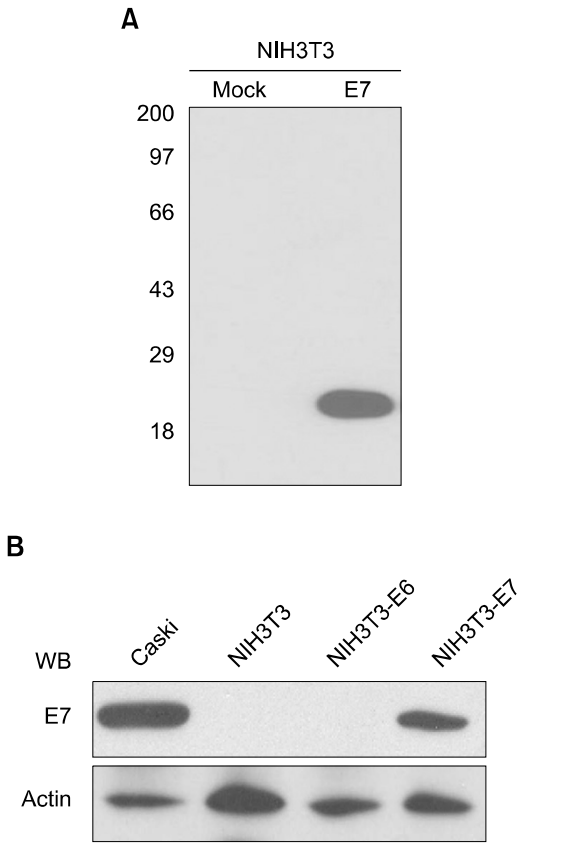

Figure 1. Characterization of mAb (130-9-7) by Western blot analysis using the crude extracts of NIH3T3 cells expressing either HPV16 E6 or E7. Caski and NIH3T3 cells were employed as positive and negative control, respectively. Actin served as a confirmatory marker for each cell line in the Western blot analysis.

Results

Selection of hybridomas

The fused hybridoma cells were cloned by the limiting-dilution technique, and tested by solid-phase ELISA. Clones producing antibodies of higher titer were selected for subsequent sub-cloning. The subcloned hybridomas produced mAbs with high titer, exhibiting typical dilution patterns in solid-phase ELISA. In this study, mAb (130-9-7) comprising γ2a heavy chain and κ light chain was utilized throughout to demonstrate the feasibility of immunostaining for cervical smear. Specific recognition of antigen by mAb (130-9-7) was evaluated by Western blot analysis (Figure 1). When HPV16 E6 or E7 were transiently expressed in NIH3T3, mAb reacted specifically with HPV16 E7, whereas the mAb did not react with HPV16 E6. The specificity of mAb was further verified by Western blot analysis with a panel of cultured cell lines, comprising cervical cancer cell lines, cancer cells other than cervical carcinoma and non-

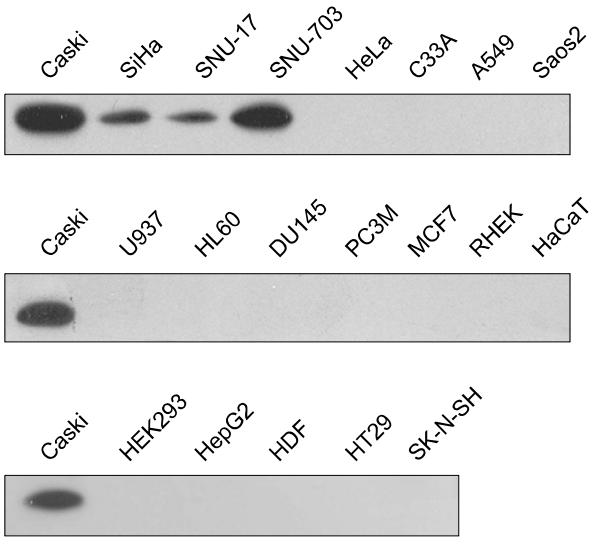

Figure 2. Specificity test of mAb (130-9-7) by Western blot analysis with cultured cell lines. The features of each cell line were described in Table 1.

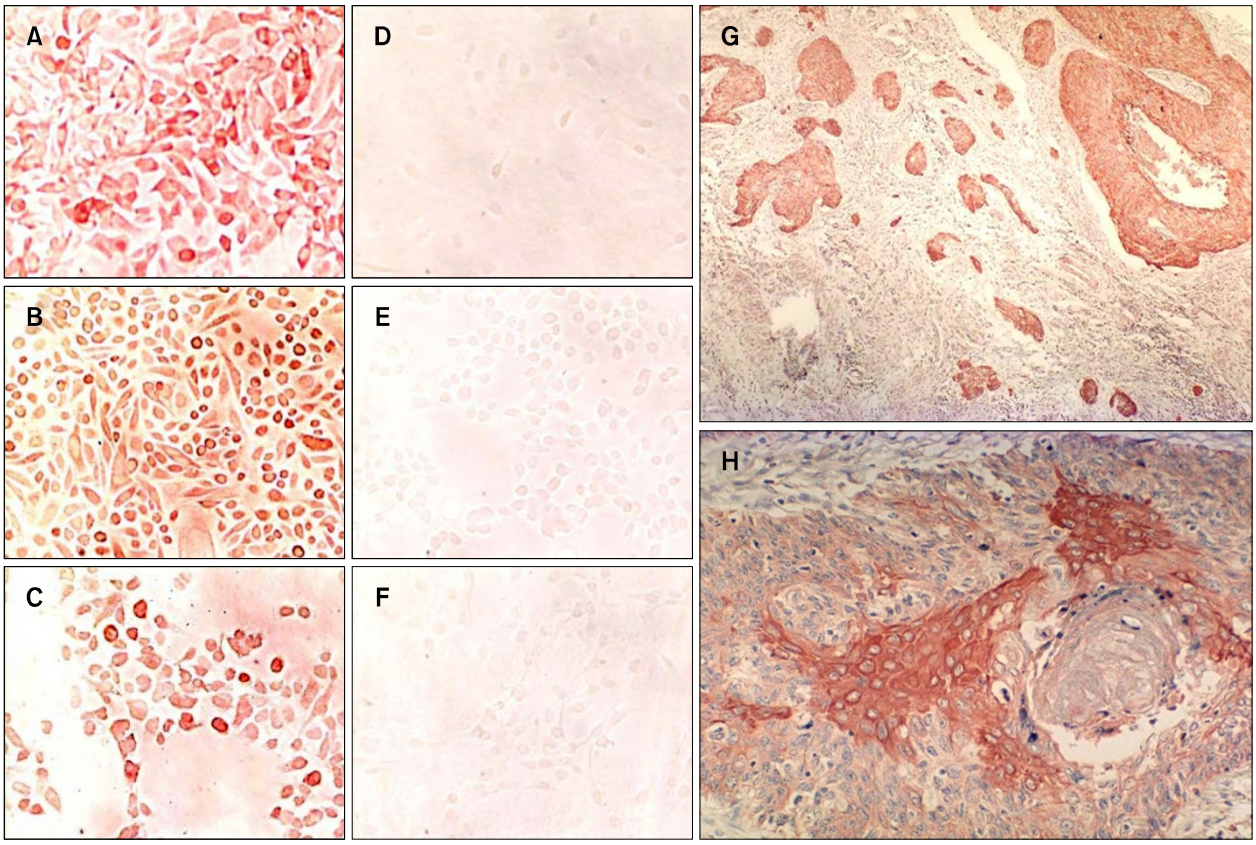

Figure 3. Immunostaining of cultured cells and cervical cancer tissue specimens with mAb (130-9-7). (A)-(F) Immunocytochemical specificity test of mAb (130-9-7) using cultured cell lines. The cultured cell lines were immunostained with mAb according to the protocol outlined in Materials and Methods. (A) Caski, (B) SiHa (C) SNU 703, (D) HeLa, (E) MCF7, (F) WI-26VA4. (G)-(H) Immunohistochemical staining of cervical cancer tissue specimens.

cancerous immortalized cell lines (Figure 2). Among cervical cancer cell lines, Caski, SiHa, SNU17 and SNU703 cells carry HPV16 genes, whereas HeLa cells are infected by HPV18. The mAb reacted only with all the HPV16-infected cervical cancer cell lines, and did not with HPV18-infected HeLa cells, nor any of the HPV-negative cell lines.

Immunostaining of cultured cells and cervical cancer tissue specimens

Application of mAb in immunostaining was initially examined with a panel of cultured cell lines. The results represented in Figure 3 were obtained with mAb (130-9-7) as the primary antibody, following the immunostaining protocol described earlier. The mAb was capable of positively staining all the HPV16-infected cervical cancer cell lines, and did not stain HPV18-infected HeLa cells, nor any of the HPV-negative cell lines. The results are summarized in Table 1, including the status of HPV-infection as well as the HPV type involved. Thus, mAb (130-9-7) specifically identified all the cervical cancer cell lines infected by HPV16. In the positively stained cells, HPV16 E7 was localized in cytosol as well as in the nucleus of the cells.

We next examine whether mAb (130-9-7) could react with HPV16 E7 protein in the cervical cancer tissue. The mAb (130-9-7) positively stained the paraffin-embedded specimens obtained from patients with cervical squamous cell carcinoma (Figure 3G and 3H). HPV 16 infection in the specimens was confirmed by *in situ* hybridization with HPV-specific oligonucleotide (data not shown). The specificity of immunostaining was confirmed by omitting the incubation step with primary antibody (data not shown), indicating that mAb (130-9-7) specifically reacts with HPV 16 E7 in tissue specimens.

Table 1. Immunostaining and PCR analysis of cultured human cell lines.

Cell	Origin	Immunostaining*	PCR analysis**
Caski	Cervical cancer	+	HPV16
SiHa	Cervical cancer	+	HPV16
SNU-17	Cervical cancer	+	HPV16
SNU-703	Cervical cancer	+	HPV16
SNU-1299	Cervical cancer	+	HPV16
HeLa	Cervical cancer	−	HPV18
C33A	Cervical cancer	−	HPV (−)
PC3M	Prostate cancer	−	HPV (−)
MCF7	Breast cancer	−	HPV (−)
A549	Lung adenocarcinoma	−	HPV (−)
Saos2	Osteosarcoma	−	HPV (−)
HepG2	Hepatocellular carcinoma	−	HPV (−)
SK-N-SH	Neuroblastoma	−	HPV (−)
HaCaT	Skin keratinocyte	−	HPV (−)
RHEK	Skin keratinocyte	−	HPV (−)
L-132	Lung fibroblast	−	HPV (−)
WI-26VA4	Lung fibroblast	−	HPV (−)
HDF	Skin fibroblast	−	HPV (−)

*Immunostaining was performed using mAb (130-9-7) as primary antibody according to the protocol described in Materials and Methods. **Samples were initially analyzed for the presence of any HPV gene using the primers based on the consensus DNA sequence of L1 gene. The samples manifesting the presence of HPV gene(s) were analyzed further to determine the HPV type involved in the infection.

Immunostaining of cervical smear specimen

Cervical smear specimens were obtained from patients who were to screen for cervical cancer, mostly normal individuals, as well as patients previously diagnosed to have cervical carcinoma. Each cervical smear specimen was analyzed by PCR method to determine if it contained consensus genomic DNA of HPV. The HPV-positive specimens were further analyzed, using the specific sets of primers, to identify the HPV type. An example of PCR analysis of a group of cervical smear specimens is shown in Figure 4. The immunostaining results of 3 specimens (A, B and C) from patients

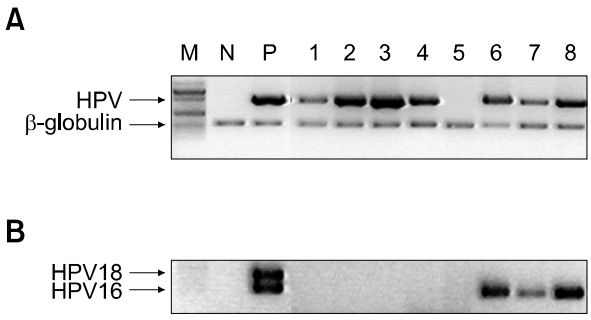

Figure 4. PCR products of cervical smear were analyzed for HPV by agarose gel electrophoresis. (A) PCR analysis for the presence of any HPV type; (B) PCR analysis for HPV16 or 18. The designations in the gel represent: M; molecular weight markers, N; negative control, P; positive control, 1-8; cervical smear specimens. β-globulin was included as the internal control. The band positions corresponding to β-globulin, consensus HPV, HPV16 and HPV18, are indicated, respectively. Among 8 specimens only specimen #5 was HPV-negative. Specimen #1, 2, 3 and 4 are infected by HPV other than type 16 and 18, whereas #6, 7 and 8 are infected by HPV16. BioCore HPV PCR kit was used throughout following the manufacturer's instructions.

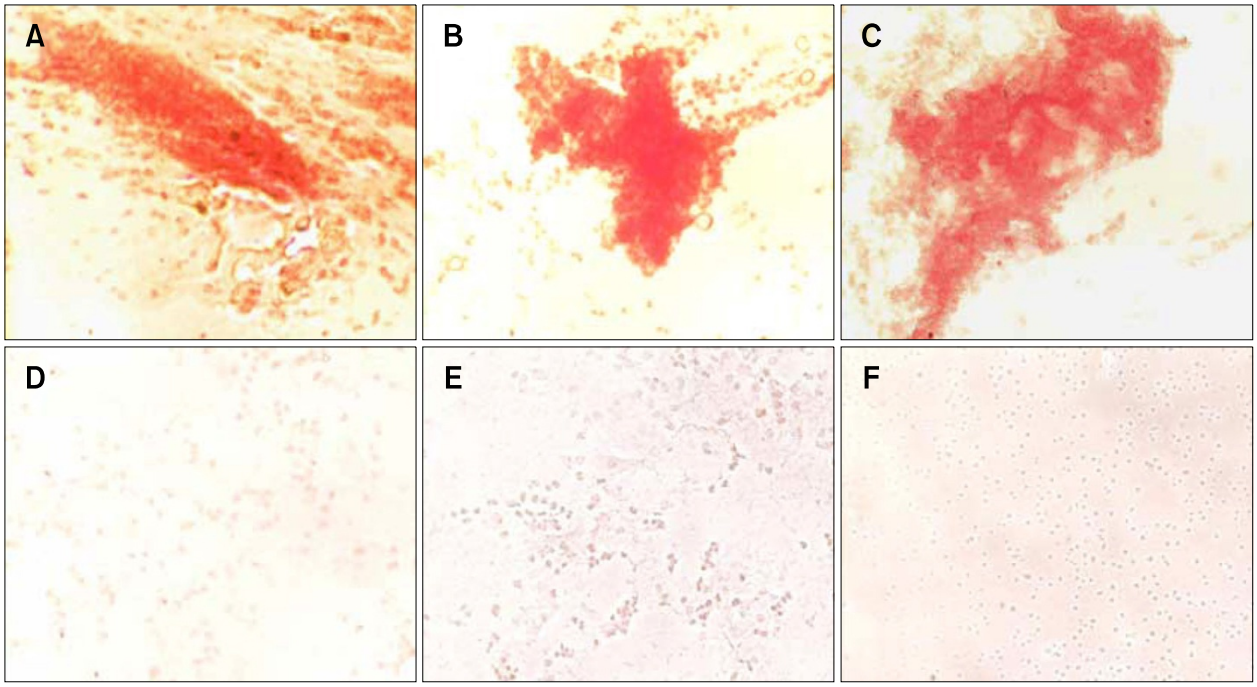

Figure 5. Immunostaining of cervical smear specimens for HPV16 E7 using mAb (130-9-7). Cervical smear specimens (A), (B) and (C) were obtained from 3 patients with cervical cancer (HPV16-positive) and (D), (E) and (F) were from 3 HPV-negative normal donors. The specimens were immunostained using mAb (130-9-7) according to the protocol described in Materials and Methods. HPV16-infected cervical cells appear in red, while other cells were unstained and remain in the background. The photos were taken at original magnification × 20.

with cervical cancer and 3 specimens (D, E and F) from normal donors were illustrated in Figure 5. Specific cell staining is evident in the HPV16-infected smear specimens obtained from the 3 cervical cancer patients, whereas no staining was observable with the 3 HPV-negative smear specimens. The immunostaining results are in agreement with those of the PCR analysis (The PCR data are not shown).

Owing to the nature of cervical smear, the specimen contains a variety of cells including erythrocytes, proteins, carbohydrates, lipids, and cellular components. These substances may produce potentially high nonspecific background in immunostaining, thereby obscuring accurate judgment in the results. Therefore, a special blocking buffer was formulated to suppress/eliminate these nonspecific effects that may interfere with the protocol. By incorporating the new blocking buffer in the primary and the secondary antibody solutions, we were able to minimize the undesirable background staining and, at the same time, preserve the signals originating from the specific interaction between mAb and E7 proteins in a positive smear specimen.

Discussion

In this study, we produced recombinant HPV16 E7 in BL21(DE3) *E. coli* cells for immunization. Because of the relatively small molecular size of HPV16 E7, we have closely monitored the immunological response against E7 protein in mice by measuring the antibodies produced during the course of the immunization. Although other investigators have employed complex vaccination scheme to generate mAbs to HPV16 E7 (Wlazlo *et al.,* 2001), we were able to produce antibodies in high titer in mice by repeated injection of the purified HPV16 E7 protein in complete Freund adjuvant.

The optimization of each step in the protocol is critical in order to achieve the desired sensitivity, specificity and reproducibility. Cervical smears were initially screened by PCR to determine the status of HPV-infection as well as HPV type, so that the PCR result was used as a guide in developing a new immunostaining method. Multiple cervical smears from same individual were obtained on lysine-coated slides, and the slides were cut into 2 parts to test a sample under various immunostaining conditions.

It has been reported that mAb which could

immunoprecipitate the E7 failed to detect E7 in cell lines or in biopsies by immunostaining (Tindle *et al.,* 1990). Greenfield *et al.* (1991) suggested that the masking of epitopes in E7 protein by intracellular components might have interfered with the immunological reactivity of mAb. In the present study, we treated cells with 0.1% Triton X-100 in PBS for 8 min. Using these procedure, we demonstrated positive immunostaining of E7 in all HPV16-infected cervical cancer cell lines examined. Judging from these results, Triton X-100 seemed to eliminate the cellular components masking the epitopes on the E7 protein in addition to permeablizing the antibody into the cells.

We have observed that the mAb to HPV16 E7 protein failed to produce the expected immunostaining with HPV16-infected cervical cancer cells, e.g. Caski cells, if the cells had been initially treated with alcohol (methanol or ethanol) as low as 50% (data not shown). The reason for this phenomenon may be that either E7 protein is extracted from the cells, or denatured in alcohol, resulting in the modification of the epitope for mAb (130-9-7). Thus, cervical smear should be fixed with 4% formaldehyde in PBS for immunodetection.

Unlike other clinical specimens, such as serum and urine, cervical smear contains a variety of cells, proteins, lipids and other intracellular substances. Mucins present particularly difficult problems by forming sticky layers of sheets or disorganized cords, which appear irregularly in the smear specimen. These fixtures tend to contribute non-specific background depending on the status of each specimen. The following optimal conditions were reached after a series of experiments designed to eliminate or minimize the background effects, while preserving the maximum level of specific staining: (a) fixation of smear specimen in 4% formaldehyde in PBS for 15 min, followed by treatment with 0.1% Triton X-100 in PBS for 30 min; (b) inactivation of endogenous peroxidase activity by incubation with 6.5% H_2O_2 for 30 min; (c) special formulation of blocking buffer comprising 5% BSA, 0.1% gelatin and 5% goat normal serum in PBS to block nonspecific effects; (d) dilution of the primary mAb as well as the secondary antibody using the special blocking buffer including 0.1% Tween 20; (e) extension of the reaction time for the primary mAb to 2 h compared with 1 h for the cultured cells; (f) inclusion of Caski cells as the positive control in each set of testing. Counterstaining with hematoxylin is not included in the protocol, because the strong acidity of the solution tends to diminish the sensitivity of the staining.

The results presented in Figure 5 exemplify cervical smear taken from HPV16-positive cervical cancer patients and HPV-negative normal donors, respectively. The adequacy of each cervical smear for the immunostaining was assumed to be equivalent to the status of the similar smear obtained concurrently for Pap test (Bethesda system) from the same individuals (Solomon *et al.,* 2002). Thus, incorporation of these modified conditions enabled cervical smear to yield specific and reproducible results. The immunostaining of HPV developed in this study is not only simple and user-friendly, but also compatible to Pap test. It uses the same type of cervical smear as Pap smear, does not require any special apparatus, and takes 4 h to complete the test. Furthermore, an examiner without an extensive training can judge the results quickly and qualitatively as positive/negative. Therefore, our immunostaining method could help to solve the inherent problems of conventional cytology and the limitation of HPV test. The results with a large number of specimens would establish the clinical sensitivity and specificity of this method.

Although HPV16 is the most prevalent HPV type representing more than 60% of high-risk HPV infection, it is one of 5 high-risk HPV types (16, 18, 33, 52 and 58) associated with cervical cancer (Munoz *et al.,* 2003). To improve clinical sensitivity, the mAb recognizing each high-risk HPV type should be combined and used as the primary mAb in the test. At the present time, the immunostaining of HPV is intended not only to augment or confirm the Pap smear test, but also to use as a new strategy for risk assessment for women with abnormal cytology in Pap smear test.

Acknowledgments

This work was supported by a grant from Korea Science and Engineering Foundation (R11-2002-097-07003-0 and R01-2005-000-10364-0) through the Aging and Apoptosis Research Center. S.Y.C. was supported by the graduate program of BK21 from Ministry of Education and Human Resources Development.

References

Cho NH, An HJ, Jeong JK, Kang S, Kim JW, Kim YT, *et al*. Genotyping of 22 human papillomavirus types by DNA chip in Korean women: comparison with cytologic diagnosis. Am J Obstet Gynecol 2003;188:56-62

de Roda Husman AM, Walboomer JM, Meijer CJ, Risse EK, Schipper ME, Helmerhorst TM, *et al*. Analysis of cytomorphologically abnormal cervical scrapes for the presence of 27 mucosotropic human papillomavirus

genotypes, using polymerase chain reaction. Int J Cancer 1994;56:802-6

de St Groth SF, Scheidegger D. Production of monoclonal antibodies: strategy and tactics. J Immunol Methods 1980; 35:1-21

Greenfield I, Nickerson J, Penman S, Stanley M. Human papillomavirus 16 E7 protein is associated with the nuclear matrix. Proc Natl Acad Sci USA 1991;88:11217-21

Jacobs MV, de Roda Husman AM, van den Brule AJ, Snijders PJ, Meijer CJ, Walboomers JM. Group-specific differentiation between high- and low-risk human papillomavirus genotypes by general primer-mediated PCR and two cocktails of oligonucleotide probes. J Clin Microbiol 1995;33: 901-5

Jeon JH, Cho SY, Kim CW, Shin DM, Kwon JC, Choi KH, *et al*. Improved immunodetection of human papillomavirus E7. Exp Mol Med 2002;34:496-9

Jeon JH, Choi KH, Cho SY, Kim CW, Shin DM, Kwon JC, *et al*. Transglutaminase 2 inhibits Rb binding of human papillomavirus E7 by incorporating polyamine. EMBO J 2003;22:5273-82

Koss LG, Lin E, Schreiber K, Elgert P, Mango L. Evaluation of the PAPNET cytologic screening system for quality control of cervical smears. Am J Clin Pathol 1994;101:220-9

Munger K, Phelps WC. The human papillomavirus E7 protein as a transforming and transactivating factor. Biochim Biophys Acta 1993;1155:111-23

Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003;348:518-27

Senior K. Cervical cancer research focuses on the HPV E7 gene. Lancet 2002;3:585

Solomon D, Davey D, Kurman R, Moriarty A, O'Conner D, Prey M, *et al*. The 2002 Bethesda System: terminology for reporting results of cervical cytology. J Am Med Assoc 2002; 287:2114-9

Tindle RW, Smith JA, Geysen HM, Selvey LA, Frazer IH. Identification of B epitopes in human papillomavirus type 16 E7 open reading frame protein. J Gen Virol 1990;71:1347-54

van den Brule AJ, Meijer CJ, Baakels V, Kenemans P, Walboomers JM. Rapid detection of human papillomavirus in cervical scrapes by combined general primer-mediated type-specific polymerase chain reaction. J Clin Microbiol 1990;28:2739-43

Venturoli S, Cricca M, Bonvicini F, Giosa F, Pulvirenti FR, Galli C, *et al*. Human papillomavirus DNA testing by PCR-ELISA and hybrid capture II from a single cytological specimen: concordance and correlation with cytological results. J Clin Virol 2002;25:177-85

Vince A, Kutela N, Iscic-Bes J, Harni V, Ivanisevic M, Sonicki Z, *et al*. Clinical utility of molecular detection of human papillomavirus in cervical samples by hybrid capture technology. J Clin Virol 2002;25 Suppl 3:S109-12

Waggoner SE. Cervical cancer. Lancet 2003;361:2217-25

Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, *et al*. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999;189:12-9

Wertlake P. Results of AutoPap system-assisted and manual cytologic screening. A comparison. J Reprod Med 1999;44: 11-7

Wilbur DC, Cibas ES, Merritt S, James LP, Berger BM, Bonfiglio TA. ThinPrep processor. Clinical trials demonstrate an increased detection rate of abnormal cytologic specimens. Am J Clin Pathol 1994;101:209-14

Wlazlo AP, Giles-Davis W, Clements A, Struble G, Marmorstein R, Ertl HC. Generation and characterization of monoclonal antibodies against the E6 and E7 oncoproteins of HPV. Hybridoma 2001;20:257-63