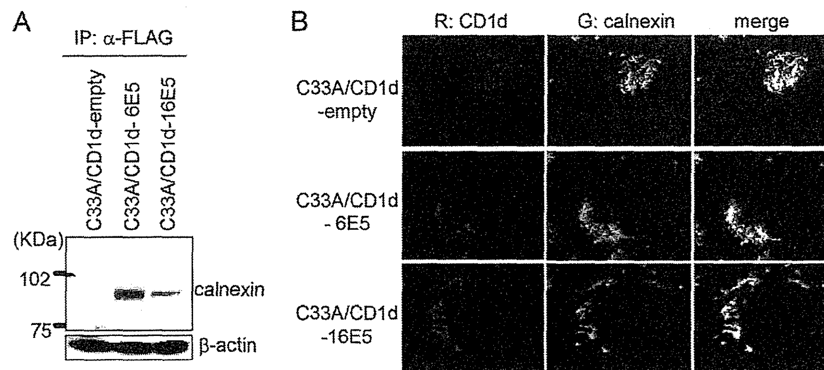

FIG. 8. CD1d and calnexin have direct interactions and colocalize in the perinuclear area in the presence of HPV E5. (A) Protein lysates from C33A/CD1d-empty, C33A/CD1d-6E5, and C33A/CD1d-16E5 cells were immunoprecipitated with an anti-FLAG MAb. Immunoprecipitants were then separated by SDS-PAGE and immunoblotted with an anti-calnexin antibody. (B) C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells were seeded onto coverslips. All cells were exposed to an anti-CD1d MAb (NOR3.2, red) and to an anti-calnexin MAb (green) labeled with Zenon Alexa Fluor 488 using a mouse IgG labeling kit. Cells were then visualized by using fluorescence confocal microscopy. Yellow images represent colocalization of CD1d and calnexin.

CD1d was rescued by treatment of proteasome inhibitor. We have previously demonstrated that surface expression of CD1d in human genital epithelial cells is downregulated by *C. trachomatis* infection and that downregulation involves chlamydial protein-mediated proteasomal pathways (26). We hypothesized that HPV infection could utilize posttranslational cellular proteasomal degradation to inhibit cell surface expression of CD1d HC. To address the role of the cellular proteasome in E5-associated CD1d degradation, C33A/CD1d-empty, -6E5, or -16E5 cells were exposed to the proteasome inhibitor, MG132, and CD1d HC levels in cell lysates compared to those in unexposed cells (Fig. 9). Using the NOR3.2 MAb for immunoblotting, the reduced or abrogated expression of the 48-kDa mature CD1d HC in E5-expressing cells could be rescued by the presence of MG132 (Fig. 9A). To visually replicate this effect, immunofluorescence microscopy was performed with the NOR3.2 MAb and DAPI in MG132 exposed and unexposed E5-expressing and control cells (Fig. 9B). In C33A/CD1d-empty cells, NOR3.2-reactive CD1d was detected throughout the intracellular space (Fig. 9B, upper left image). In contrast, NOR3.2-reactive CD1d was barely detected or undetectable in the majority of unexposed C33A/CD1d-6E5 or -16E5 cells (Fig. 9B, upper, right two images). In the presence of proteasomal inhibition with MG132, E5-expressing cells again show CD1d signals throughout the intracellular space (Fig. 9B, lower panels). HPV E5-expressing cells completely recover their expression mature CD1d molecules upon inhibition of cellular proteasomal degradation.

HPV E5 abrogates CD1d-mediated cytokine production in the epithelial cells. Surface CD1d interacts specifically with iNKT cells bearing an iTCR. The interaction not only activates NKT cells but also induces phosphorylation of CD1d, intracellular signaling, and the release of cytokines from the CD1d-bearing cell. We have previously demonstrated that human reproductive tract epithelial cells expressing CD1d on their cell surfaces have the capacity to produce cytokines, especially IL-12, after CD1d ligation (25). IL-12 is a central mediator in both innate and adaptive immunity and is crucial in the prevention of many infectious diseases and tumors (40). IL-12 induces IFN-γ-producing NK, NKT, T helper, and cytotoxic T cells. Since our investigations had demonstrated a decrease in cell surface expression of CD1d in the presence of HPV and specifically of HPV E5, we next examined whether CD1d-mediated IL-12 production was abrogated in E5-expressing epithelial cells (Fig. 10). An anti-CD1d 51.1 MAb can be used for CD1d cross-linking and represents an *in vitro* model for

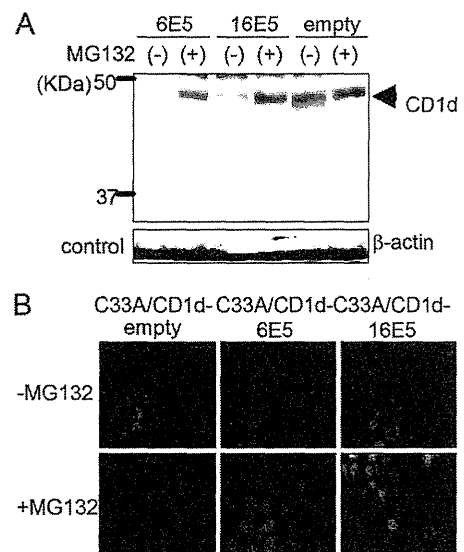

FIG. 9. Proteasome inhibition rescues CD1d from E5-mediated degradation. (A) C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells were cultured for up to 24 h in the presence or absence of the cytosolic proteasome inhibitors MG132 (10 μM) in DMSO. Fifty micrograms of protein lysates from each cell line were analyzed by Western immunoblotting with a peroxidase-labeled anti-CD1d MAb (NOR3.2; 1:200 dilution) and a β-actin loading control. (B) C33A/CD1d-empty, C33A/CD1d-6E5, or C33A/CD1d-16E5 cells were seeded onto coverslips and cultured for up to 24 h in the presence (lower) or absence (upper) of MG132 (10 μM) in DMSO. All coverslips were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton-X, blocked with 6% BSA, and incubated for 1 h at room temperature with an anti-CD1d NOR3.2 MAb (red) directly conjugated with Zenon Alexa Fluor 555 using a mouse IgG1 labeling kit. Cells were then counterstained with a DAPI (blue) nucleic acid stain.

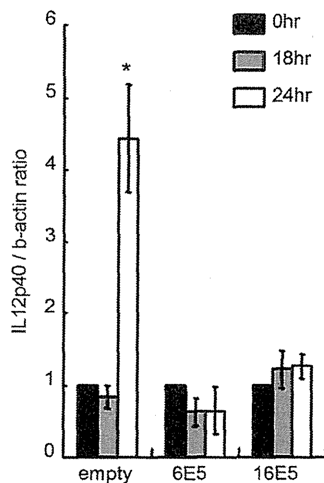

FIG. 10. Autocrine cytokine production upon CD1d cross-linking in C33A/CD1d-empty, C33A/CD1d-6E5, and C33A/CD1d-16E5 cells. An anti-CD1d 51.1 MAb was added at a dosage of 10 μg/ml to cultured epithelial cell monolayers, followed by incubation for 1 h. After being washed with PBS, 10 μg of a goat anti-mouse immunoglobulin antibody/ml was added as a cross-linker for 30 min. The cells were incubated in serum-free growth medium without any antibiotics for 0 to 24 h. cDNA was produced via reverse transcription of 1 μg of total RNA extracted and amplified by PCR with primer pairs for IL-12 p40 and β-actin. IL-12 p40 mRNA levels were normalized to β-actin. Mean values with standard deviations are presented. Asterisks indicate the comparisons (before versus after cross-linking) with statistical significance ($P < 0.05$; $n = 4$).

CD1d ligation (14, 43). C33A/CD1d-empty, -6E5, or -16E5 cells were first exposed to an anti-CD1d 51.1 MAb and then to a secondary anti-mouse IgG cross-linker and then examined for IL-12 production (Fig. 10). IL-12 p40 transcription increased 24 h after cross-linking in the C33A/CD1d-empty cells. This effect was abrogated completely in E5-expressing cells. Decreased cell surface expression of CD1d in E5-expressing cells inhibits the ability of antibodies to cross-link CD1d and thereby halts the downstream signaling that drives IL-12 production.

DISCUSSION

In this study, we attempted to elucidate a mechanism to explain our finding that CD1d was expressed at lower levels in tissues infected with high-risk and low-risk HPV subtypes (16 and 6, respectively). The CD1d protein levels were lower, but the mRNA levels were unaffected in HPV E5-expressing cells, indicating that CD1d is downregulated at a posttranscriptional level in the presence of HPV E5. Modification of CD1d was interrupted at the level of the ER by interactions between HPV E5 and calnexin. Improper folding and/or ubiquitination of CD1d HC in the presence of HPV then targets CD1d to cellular proteasomal degradation. Others (21) have previously demonstrated that interaction of E5 with calnexin appears to interfere with calnexin-assisted folding of HLA class I molecules. Like the well-described quality control system assuring proper HLA class I HC production and maturation, delayed exit of improperly folded CD1d HC from the ER in the presence of HPV E5 appears to result in movement of CD1d HC to the cytosol and cellular proteasomal degradation of CD1d HC via the unfolded protein response. Finally, we addressed the possible functional significance of CD1d degradation in HPV-infected cells. Decreased cell surface expression of CD1d in the presence of HPV 6E5 and 16E5 completely blocked the secretion of IL-12 in response to CD1d cross-linking. Although several of the assays were not quantitative, the effects of HPV6 and HPV16-derived E5 were similar in all assays and were not statistically different in those that were quantitative. This suggests that a mechanism for immune evasion used in the early phase of HPV infection may be conserved between low-risk and high-risk HPV subtypes.

In planning for these investigations, we chose to use two cell lines. One was a cervical cancer cell line that is unique in being HPV-negative C33A. The other was an endogenous CD1d-bearing keratinocyte cell line established from normal human vaginal epithelial cells. The HPV-negative C33A cell was particularly useful for this study because it allowed us to control for the influence on CD1d of proteins other than E5 that could have been potentially present in an HPV-positive cervical cell line. Via transfection, CD1d could be stably expressed in a cervical cell, and specific HPV proteins could added in isolation to assess their effect on CD1d. Our previous and current immunohistochemical data demonstrated that cells in the basal and parabasal cell layers of a variety of squamous genital epithelia react strongly with anti-CD1d MAbs, in patterns that replicate those seen in normal human skin (9, 25). The distribution of CD1d-bearing epithelial cells within the basal and parabasal cell layers may be required for effective interactions between CD1d and the iNKT cells that reside within submucosal tissues. These interactions may occur primarily through CD1d expressed on the basilar membrane. The immortalized vaginal epithelial cell lines used in the present study have been characterized by Fichorova et al. as being similar to epithelial cells present in basal or parabasal cell layers *in vivo* (17, 18). We have also seen similar patterns of CD1d expression in nondiseased genital tract tissues (25). The data derived from vaginal epithelial cells in the present study allowed us to mimic *in vivo* infection of normal human keratinocytes by HPV and to confirm that the retrovirus vectors used to transduce E5 genes into our cell models did not affect the endogenous CD1d promoter.

CD1d transcription was barely detectable in both C33A cells and HPV-positive cervical cancer cells (HeLa, Caski, and clinical samples). Immunohistochemical data verified that immune reactivity for CD1d was completely abrogated in all cervical cancer lesions. Lack of CD1d expression in cancer-derived cells is unlikely to be associated with HPV E5 protein expression since the E5 gene is deleted when the HPV genome integrates into the host genome. Rather, CD1d may be genomically inactivated during carcinogenesis. Two of eighteen cases with CIN2 and CIN3 showed immune reactivity with the anti-CD1d MAb, although all lesions were positive for high-risk HPV. CD1d expression is known to be induced by inflammatory cytokines such as IFN-γ (9, 25). In some cases, an enhancement in CD1d expression secondary to the immunological microenvironment in the cervix *in vivo* may supercede E5-mediated downregulation. Alternatively, E5-mediated CD1d downregulation in CIN3 lesions may be lessened because most cells may have already integrated the HPV genome

and little E5 remains within the lesion. Statistical analysis, however, reveals a trend toward decreased CD1d expression with progressing CIN.

Previous investigations on HPV-associated immune evasion strategies have highlighted interference with adaptive immune responses against HPV through disruption of HLA molecules (19, 30). Here we focused on CD1d, which serves not only as a sentinel molecule in innate immune response but as a bridge between innate and adaptive immunity. Reports of CD1d expression in epithelial cells lagged behind its detection and functional studies in classic immune cells such as dendritic cells, macrophages, and B cells. In epithelial cells, CD1d encounters a wide array of pathogens and helps to orchestrate innate and adaptive immune responses to these immunologic challenges via interactions with CD1d-restricted iNKT. The interaction of CD1d with CD1d-restricted iNKT cell is lipid antigen dependent; however, this lipid antigen can be derived from invading microbes or from host cellular lipids. In response to some microbes, the rapid effects of CD1d-restricted NKT cells do not require recognition of microbial specific antigens (6, 34, 35). Since HPV has no envelope and therefore no HPV-specific lipid antigens, CD1d may present self lipid antigen for activation of iNKT cells in response to HPV-infected epithelial cells. Recognition of CD1d by iNKT cells can cause rapid release of both IL-4 and IFN-γ from the NKT cell (6). This would be predicted to activate CD1d-restricted iNKT cells and rapidly induce an adaptive immune response to invading microbes. Our previous investigations have also demonstrated that human reproductive tract epithelial cells that express CD1d on their cell surfaces are able to produce cytokines, including IL-12, in response to CD1d ligation (25). IL-12 is a central mediator in both innate and adaptive immunity and is crucial in the prevention of infectious diseases and tumors (40). IL-12 induces IFN-γ-producing NK, NKT, T helper, and cytotoxic T cells and thereby bridges innate and adaptive immune responses. Yue et al. have demonstrated that cross-linking of CD1d rapidly induces phosphorylation of IκB. This, in turn, promotes NF-κB activation and IL-12 production in monocytes and immature dendritic cells (43). As shown here, the induction of IL-12 production in response to CD1d cross-linking is completely abrogated in HPV E5-expressing epithelial cells but never to levels below those produced at baseline. The inhibition of CD1d-mediated cytokine production may be a mechanism by which HPV-infected cells evade (at least temporarily) the bridging of innate and adaptive immune responses that would otherwise occur upon interaction between cell surface CD1d and iNKT cells.

HPV E5 has been reported to play a role in HPV immunoevasion through the downregulation of cell surface HLA class I molecules. Several investigators have demonstrated that the papillomavirus E5 product inhibits the acidification of organelles, including the GA and endosomes (28, 32, 38). Ashrafi et al. have reported that the inhibition of GA acidification mediated by bovine papillomavirus E5 is associated with retention of MHC class I molecules in the GA (4, 41) and that HPV16 E5 retains HLA-A and -B, but not HLA-C and -E, within the GA. These authors hypothesize that the selectivity of HLA class I subtype downregulation may suggest that mechanisms other than GA acidification may be involved (2, 3). Gruener et al. demonstrated that interactions between HPV16 E5 and calnexin interfere with modification of HLA class I HCs and results in heavy-chain retention in the ER (21). Since the synthetic pathways for CD1d and HLA class I HCs are identical, we hypothesized that inhibition of calnexin folding capabilities by HPV E5 was a likely mechanism for decreased cell surface expression of CD1d in HPV-infected cells. Using confocal microscopy, we supported this hypothesis over the acidification mechanism by demonstrating that CD1d HC and calnexin colocalize in the ER rather than the GA. Interestingly, the CD1d HC that was rescued by MG132 treatment in E5-expressing cells was a 48-kDa mature form that was present in a diffuse pattern throughout the intracellular space (Fig. 8 and 9). It appears that CD1d synthesis and trafficking may be fairly robust in the presence of HPV E5 if proteasomal degradation is inhibited. This suggests that HPV E5 does not interfere with the synthesis of CD1d HC but rather delays its exit from the ER and alters its maturation so that CD1d HCs are targeted to proteasomal degradation. Interactions between HPV E5 and calnexin do not appear to interrupt all of the functions of calnexin, but just enough to co-opt the cellular cytosolic proteolytic pathway and effectively degrade CD1d and temporarily inhibits CD1d-mediated innate and adaptive immune pathways early in HPV infection.

CD1d expression and CD1d activation of neighboring iNKT cells may play an important role in the generation of innate and adaptive immune responses to microbial infection of the ectocervix. Our previous and current immunohistochemical data have shown that CD1d immunoreactivity and distribution patterns in ectocervix are similar to those in the penile urethra and vagina, where epithelial cells exhibit CD1d-mediated Th1-type cytokine production (25). It was likely that CD1d-bearing ectocervical epithelial cells were also capable of CD1d-mediated Th1-type cytokine production, and we have here shown that CD1d cross-linking on C33A/CD1d cells promotes the synthesis of IL-12. We therefore suggest a mechanism whereby CD1d downregulation in the presence of low- and high-risk HPV subtypes allows the infecting virus to evade host immune surveillance and establish persistent infection at the primary transmission site. The magnitude of HPV E5 expression and resultant CD1d downregulation may vary among CIN lesions, as shown in our clinical data. If so, variations in CD1d immunoreactivity in biopsy specimens of CIN lesions may be a predictive marker for the fate of early CIN. This topic is currently under investigation.

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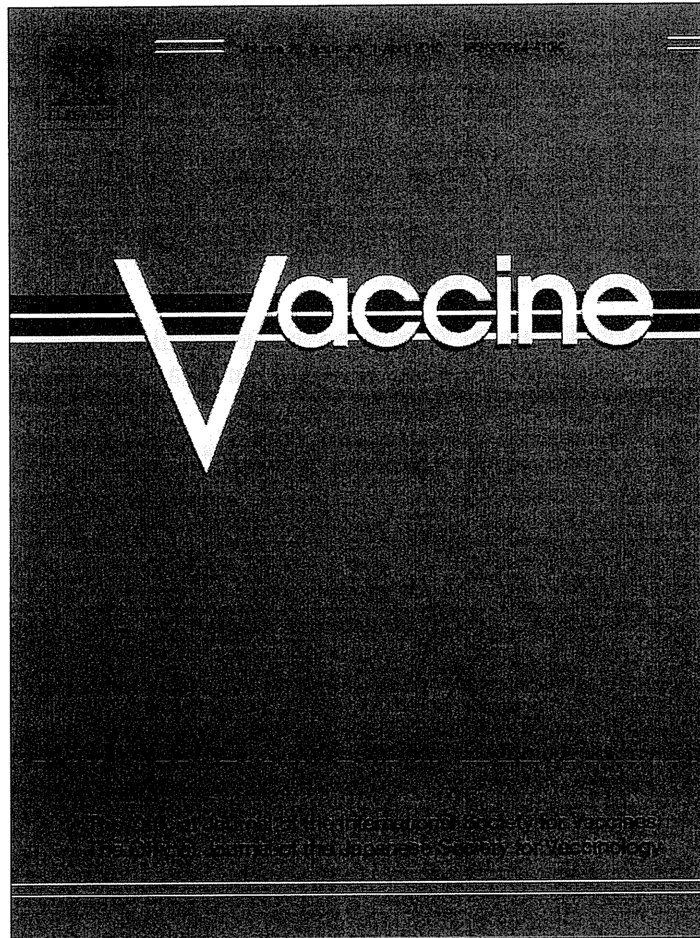

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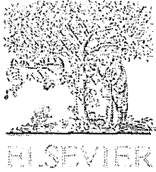

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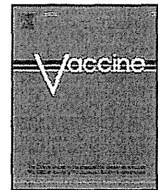

Oral immunization with a *Lactobacillus casei* vaccine expressing human papillomavirus (HPV) type 16 E7 is an effective strategy to induce mucosal cytotoxic lymphocytes against HPV16 E7

Katsuyuki Adachi [a,1], Kei Kawana [a,*], Terufumi Yokoyama [b,2], Tomoyuki Fujii [a,1], Ayako Tomio [a,1], Shiho Miura [a,1], Kensuke Tomio [a,1], Satoko Kojima [a,1], Katsutoshi Oda [a,1], Tomomitsu Sewaki [b,2], Toshiharu Yasugi [a,1], Shiro Kozuma [a,1], Yuji Taketani [a,1]

[a] *Department of Obstetrics and Gynecology, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*
[b] *GENOLAC BL Corp. 503, Okinawa Industry Support Center, 1831-1 Oroku, Naha, Okinawa 901-0152, Japan*

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ABSTRACT

Although many clinical trials on human papillomavirus (HPV) therapeutic vaccines have been performed, clinical responses have not been consistent. We have addressed mucosal cytotoxic cellular immune responses to HPV16 E7 after oral immunization of mice with recombinant *Lactobacillus casei* expressing HPV16 E7 (LacE7). C57BL/6 mice were orally exposed to 0.1–100 mg/head of attenuated LacE7 or vehicle (Lac) vaccines at weeks 1, 2, 4, and 8. Responses to subcutaneous or intramuscular injection of an HPV16 E7 fusion protein using the same timing protocol were used for comparison. Oral immunization with LacE7 elicited E7-specific IFNγ-producing cells (T cells with E7-type1 immune responses) among integrin $\alpha4\beta7^+$ mucosal lymphocytes collected from gut mucosa. An induction of E7-specific granzyme B-producing cells (E7-CTL) exhibiting killer responses toward HPV16 E7-positive cells was also observed. The induction of T cells with specific mucosal E7-type1 immune responses was greater after oral immunization with LacE7 when compared to subcutaneous or intramuscular antigen delivery. Oral immunization with *Lactobacillus*-based vaccines was also able to induce mucosal cytotoxic cellular immune responses. This novel approach at a therapeutic HPV vaccine may achieve more effective clinical responses through its induction of mucosal E7-specific CTL.

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1. Introduction

Human papillomavirus (HPV) is a major risk factor for the development of cervical cancer which is the second most common cancer among women. An estimation of the worldwide cancer incidence and mortality for 2002 showed that persistent HPV infection had caused about 500,000 cases of cervical cancer [1]. Some 99% of cervical cancer cases are associated with genital infection with HPVs. At present, there are about 100 identified genotypes of HPVs and more than 40 subtypes are known to infect human genitalia. HPV type 16 (HPV16) infection is most commonly associated with cervical cancer. It is found in 50% of cervical cancers and cases of high-grade cervical intraepithelial neoplasia (CIN) [2–4] and in 25% of low grade CIN lesions. Since at least 50–80% of women are thought to be exposed to genital HPV in their lifetime and persistent HPV infection is associated with progression to high-grade CIN or cervical cancer [2,5] a vaccine that prevents genital infection with HPV16 should substantially reduce the incidence of cervical cancer. Still, while prophylactic vaccines composed of L1 virus-like particle are available and have been shown to prevent HPV infection with the virus types contained in the vaccine [6], they cannot help the millions of patients who have already been infected [7]. Therapeutic vaccines are also needed.

While some HPV infections may persist and progress to cervical cancer, most infections resolve spontaneously, probably as the result of host cellular immune responses to HPV viral proteins. The HPV E6 and E7 oncogenic proteins are essential to the process of carcinogenesis, and their immunogenicity has given HPV an attractive target for use in immunization strategies to treat CIN or cervical cancer. In fact, several therapeutic vaccines against HPV E6 and/or E7 oncogenic proteins have been developed and clinical vaccination trials using these reagents against CIN and cervical cancer have been completed [8–16]. Using immunohistochemistry, Ressler et al. have demonstrated that high-grade CIN lesions strongly express E7 proteins, suggesting E7 as a target molecule for immunotherapy against high-grade CIN [17].

* Corresponding author. Tel.: +81 3 3815 5411; fax: +81 3 3816 2017.
E-mail address: kkawana-tky@umin.ac.jp (K. Kawana).
[1] Tel.: +81 3 3815 5411; fax: +81 3 3816 2017.
[2] Tel.: +81 98 858 8607; fax: +81 98 858 8607.

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However, no studies to date have demonstrated clinical efficacy of E7-based vaccines. E7-based vaccines have been shown to elicit E7-specific systemic cellular immune responses after subcutaneous or intramuscular delivery, but few have addressed E7-specific cellular mucosal immune responses. Because CIN lesions develop in the cervical mucosa, we believe that cervical mucosal cytotoxic cellular immune responses to E7 are essential for clearance of CIN. Subcutaneous or intramuscular antigen administration may be significantly less effective delivery routes for therapeutic vaccines against HPV-associated CIN.

Poo et al. have shown that oral immunization of C57BL/6 mice with *Lactobacillus casei* (*Lb. casei*) expressing HPV16 E7 reduces tumor formation induced by TC-1 cell administration. TC-1 cells have been immortalized by HPV16 E6/E7 and c-Ha-*ras*. Immunization in these experiments elicited type1 T cell immune responses to E7 in lymphocyte from the spleen and from anogenital regional lymph nodes [9]. Using intranasal mucosal immunization, Bermúdez-Humarán et al. also demonstrated that TC-1 tumors regressed in mice exposed to a *Lactobacillus*-based E7 vaccine and that antigen-specific CTL responses in splenocytes were robust [18]. Although both studies used transmucosal immunization with *Lactobacillus*-based vaccines, they examined E7-specific systemic cellular immune response and regression of subcutaneous TC-1-induced tumors. These investigations provide no insight into mucosal cellular immune responses after immunization and the antigen specificity of mucosal lymphocytes.

Mucosal immunity serves as the first line of defense against microbial infections. These activities include the clearance of microbes from infected lesions and the production of anti-microbial neutralizing antibodies and involve a dynamic immune network that combines innate and acquired mucosal responses. All mucosal sites (lachrymal, nasal, bronchial, gastrointestinal, and genital) share a common immune network that includes induction in mucosa-associated lymphoid tissues (MALT), distinctive effector sites, and specific mucosal lymphocytes (intraepithelial and lamina propria-associated). MALT and mucosal lymphocytes are functionally and anatomically distinct from the systemic lymphoid tissues (e.g. spleen) and lymphocytes (e.g. splenocyte and peripheral white blood cells) of the peripheral immune system. Integrin $\alpha4\beta7$ is a mucosa-associated homing receptor, the expression of which is induced on mucosal lymphocytes only upon interaction with dendritic cells residing in the gut-associated lymphoid tissues (GALT) [19]. Although integrin $\alpha4\beta7^+$ lymphocytes are commonly considered to be gut-derived mucosal lymphocytes, they may be derived in and home to several other distinct mucosal sites. For instance, after binding to natural ligands, such as mucosal addressin cell adhesion molecule (MAdCAM) that is expressed in venular endothelial cells at mucosal sites, integrin $\alpha4\beta7^+$ lymphocytes can also home to nasal, urogenital and other mucosal sites [20–23]. Hawkins et al., have shown that integrin $\alpha4\beta7^+$ lymphocytes home to the chlamydia-infected murine genital tract [24]. In response to these and other findings, oral immunization has become an attractive antigen delivery system that can effectively carry antigen to inductive sites (e.g. GALT such as Peyer's patches) that elicit antigen-specific mucosal immune responses [25]. Further, oral immunization of *Lb. casei* is reported to have an adjuvant effect on type1 T cell immune responses at gut mucosa [26].

Mucosal memory and effector T cells against E7 are essential to the clearance of CIN. Investigations on mucosal immune response to mucosally administered HPV therapeutic vaccines are scarce and no groups have specifically addressed the induction of integrin $\alpha4\beta7^+$ antigen-specific mucosal T cells. In this study, we orally administered *Lb. casei* expressing HPV16 E7 to C57BL/6 mice and assessed mucosal cellular immune responses to HPV16 E7, including the induction of IFNγ-producing type1 T cells and granzyme B-producing $CD8^+$ T cells, and cytotoxic activity against TC-1 cells. Since it was difficult to isolate strictly mucosal T cells from the murine cervix, we used mucosal T cells isolated from gut mucosa as a surrogate for those isolated from the cervix. We demonstrated that oral immunization with *Lb. casei* expressing HPV16 E7 elicited E7-specific mucosal cellular immune responses in gut-derived integrin $\alpha4\beta7^+$ lymphocyte. This may accompany similar homing of primed memory/effector cells from the gut inductive site to effector sites in the cervical mucosa for clearance of high-grade CIN.

2. Materials and methods

2.1. Preparation of attenuated Lb. casei-based vaccines

Lb. casei strain 525 was isolated from sugar apple. *Lb. casei 525* was cultured in MRS medium at 30 °C. The pKV-based expression plasmid was a kind gift from Dr. Sung (Bioleaders Corporation, Korea). The plasmid has a *Lactobacillus*-derived promoter upstream of the integration site for the gene of interest. This plasmid also fuses the gene of interest to pgsA, a peptideglican that traffics the protein of interest to the cytoplasmic membrane as shown previously [9]. In this study, we modified the HPV16 E7 gene by inserting a point mutation into the Rb-binding site (the D, C, and E in the E7 aa21, aa24 and aa26, respectively, were all replaced by a G). Through this mutation, the carcinogenicity of E7 was lost its immunogenicity remained intact [10]. Two plasmid subtypes (pKV/mutated E7 and pKV alone) were created, purified and transformed into *Lb. casei* by electroporation. The recombinant *Lb. casei* expressing HPV16 mutated E7 and the vehicle, *Lb. casei* alone, were named LacE7 and Lac, respectively. LacE7 and Lac were expanded using large scale culture in MRS medium with erythromycin (16 μg/mL) at 30 °C and attenuated using heat. The attenuated *Lb. casei* were purified by washing several times with distilled water, dried to powder and stored at 4 °C until use. The powder weight of the attenuated *Lb. casei* was used to define vaccine inoculum dosage. Semi-quantitative SDS-PAGE and ELISA analyses revealed that 1 g of attenuated LacE7 contained about 7 mg of HPV16 E7 protein (data not shown).

2.2. Immunization of mice with Lactobacillus-based vaccines

Eight-week-old, female SPF C57BL/6 (H-2^b) mice were used for immunization experiments (CLEA Japan. Inc., Japan). Various vaccine doses (0.1, 0.3, 1.0, 3.0, 10, and 100 mg/head) of the attenuated LacE7 or Lac were administered to five mice per dose at weeks 1, 2 and 4 (priming) and at week 8 (booster). LacE7 and Lac powders were insoluble in water-based solvents. All inoculums were suspended in PBS (200 μL/head) and administered via an intra-gastric tube after 3 h of fasting, once per day for 5 days each week. For comparison, 50 μg of purified HPV16 E7-tag fusion protein (kind gift from Dr. Sung, Bioleaders Corporation, Korea) was suspended in complete Freund's adjuvant and administered to mice by intramuscular (i.m.) or subcutaneous (s.c.) injection once per week at weeks 1, 2, 4 and 8.

2.3. Isolation of murine splenocytes and intestinal mucosal lymphocytes

Intestinal mucosal lymphocytes and splenocytes were isolated from immunized mice 1 week after priming or booster inoculations (at week 5 or 9, respectively). After sacrifice, intestines or spleen were obtained from five distinct mice and washed three times in HBSS containing protease inhibitors. The intestines were opened longitudinally and shaken vigorously in RPMI1640 containing 10% FCS, 100 units/mL of penicillin and 100 μg/mL of streptomycin for 30 min at 37 °C. The resulting cell suspensions were passed through a BD Falcon Cell-strainer (BD Bioscience, USA) to remove tissue

debris and were subjected to discontinuous density gradient centrifugation in a 15 mL tube layered from the bottom with 70% and 40% Percoll (Sigma, USA). Approximately 10^{7-8} cells were layered onto the gradients and centrifuged at $600 \times g$ for 20 min at room temperature (LX-130 TOMY SEIKO Co., Ltd., Japan) The interface between the 70% and 40% layers contained lymphocytes with a cell viability of more than 95%. Splenocytes were prepared by gently teasing the spleen in the HBSS. Clumped debris was removed by centrifugation. Approximately $5–10 \times 10^6$ intestinal mucosal lymphocytes and 10^7 splenocytes were obtained from individual mice.

2.4. Flow cytometry

Murine intestinal mucosal lymphocytes were incubated with an anti-CD4 or anti-CD8 mAb conjugated to PE-Cy5 (1 μg/mL, eBioscience, USA or Beckman Coulter, USA), or with an anti-integrin α4β7 (LPAM-1) mAb conjugated to PE (1 μg/mL, Santa Cruz Biotechnology, USA) for 30 min at 4 °C. Controls were exposed to an isotype-matched irrelevant mAb (1 μg/mL, Beckman Coulter). Cells were suspended in 1% paraformaldehyde, and analyzed using a FACSCalibur flow cytometry system (BD Bioscience, USA).

2.5. ELISPOT assay

50 μL of intestinal mucosal lymphocytes or splenocytes (2×10^6 cells/mL) were incubated for 24 h at 37 °C with antigen presenting cells, 50 μL of splenocyte (1×10^6 cells/mL) treated with mitomycin C (75 μg/mL, Nakarai, Japan), and washed four times with PBS. 10 μL of a synthesized peptide (working conc. = 1 μg/mL) corresponding to amino acid 49–57 of HPV16 E7 (a reported CTL epitope for C57BL/6 (H-2^b) mice), mitogen (PMA 40 ng/mL + ionomycine 4 μg/mL), or medium alone (negative control) were added to a 96-well ELIIP plate (Millipore USA) coated to anti-mouse IFNγ or granzyme B monoclonal antibodies (15 μg/mL) according to the manufacturer's protocols for ELISPOT for Mouse Interferon-γ (MABTECH AB, Sweden) and Granzyme B Development Module (R&D systems, USA). Spot numbers of IFNγ and granzyme B-producing lymphocytes were analyzed with a fully automated computer assisted video imaging analysis system, KS ELISPOT (Carl Zeiss Vision, Germany) [10].

2.6. Cytotoxic T lymphocyte (CTL) assays

E7-specific cytotoxicity was measured using a CTL assay system and the Cellular DNA Fragmentation ELISA (Roche, USA). TC-1 cells are derived from a primary lung cell from C57BL/6 mice (allogeneic to the immunized mice) and immortalized by HPV16 E6/E7 plus c-Ha-*ras* and were used in these experiments as target cells (kind gift from Dr. T.C. Wu, Johns Hopkins University, Baltimore, MD USA) [27]. Briefly, target cells were cultured in medium containing BrdU for 12 h at 37 °C. The cells were harvested the following day, washed and plated at a concentration of 2×10^4 cells/well in a 96-well plate. Plates were incubated for 6 h at 37 °C with effecter cells at an effecter cell/target cell (E:T) ratio of 1–20. The release of BrdU from killed cells was measured at OD 450 nm according to the manufacturer's protocol.

2.7. Statistical analysis

ELISPOT data are presented as means ± standard deviations. Experiments were performed independently at least three times. ELISPOT numbers were compared between the immunization groups (5–6 mice/each group) using paired, two-tailed Student's *t*-tests. A *p*-value of <0.05 was considered significant.

3. Results

3.1. Integrin α4β7+ mucosal T cells

Assessment for mucosal cellular immune response requires isolation of mucosal T cells that express homing receptors, including integrins (integrin α4β7 and α4β1). A study in a chlamydia-infected murine model demonstrated that intestinal mucosal lymphocyte also home to the genital tract mucosa. Integrin α4β7 rather than α4β1 is reported to be a central homing receptor directing transit of mucosal lymphocytes to genital tract mucosae [28]. This gut/genital tract similarity allowed us to study mucosal lymphocytes isolated from murine intestinal mucosa in our investigation. These lymphocytes were isolated as described previously [29] and their phenotype was examined using flow cytometry (Fig. 1). About 90% of the lymphocyte were positive for integrin α4β7. The proportions of $CD4^+$ and $CD8^+$ integrin $α4β7^+$ cells were 45% and 56% of the lymphocytes, respectively. A portion of $CD4^+$ or $CD8^+$ integrin $α4β7^+$ cells may be $CD4^+$ and $CD8^+$ (double positive) T cells since the total percentages exceed 100%. The $CD4^+$ and/or $CD8^+$ T cells expressed intermediate levels of integrin α4β7 (bold lines in Fig. 1). A $CD4^-$ and $CD8^-$ subset of integrin $α4β7^+$ cells was presumed to include integrin $α4β7^+$ B, NK, or double negative ($CD4^-$ and $CD8^-$) T cells. The phenotypes of these intestinal mucosal lymphocytes were markedly different from those isolated from peripheral blood and confirmed our isolation of mucosal T cells. 0.3–2% of isolated mucosal lymphocytes were B cells (data not shown).

3.2. Increase in IFNγ-producing type1 T cell after oral immunization with *Lb. casei*

Clearance of cells infected with HPV or immortalized by HPV requires cellular cytotoxic immune responses that are provided

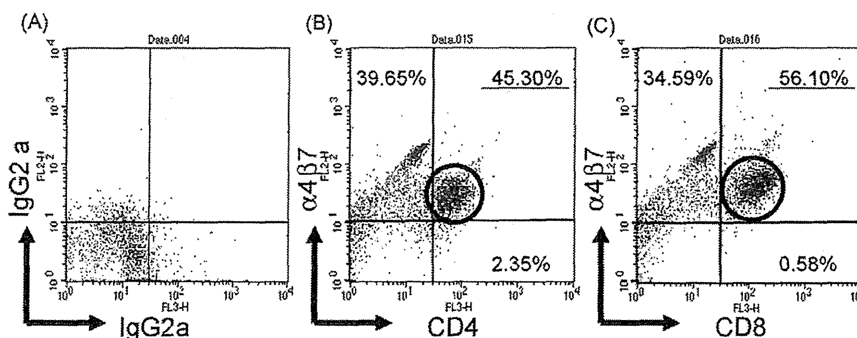

Fig. 1. Flow cytometry analysis of isolated mucosal lymphocytes. Instrument settings for separate samples were identical. The appropriate isotype profile is shown in (A). These profiles depict PE-Cy5 $CD4^+$(B) or $CD8^+$(C) cells on the *x*-axis and PE integrin $α4β7^+$ cells on the *y*-axis. The dual-labeled $CD4^+$ or $CD8^+$/integrin $α4β7^+$ cells cluster in the upper right quadrant (bold line).

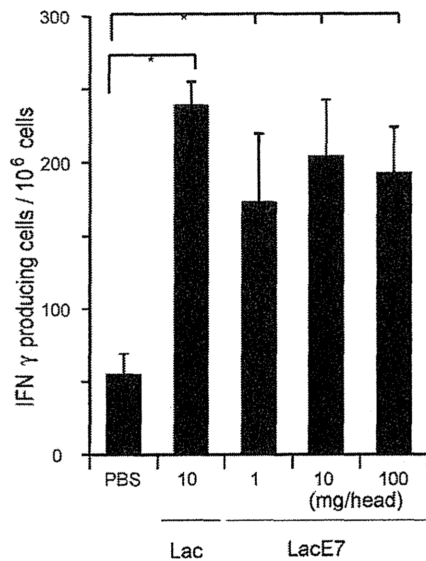

Fig. 2. To examine effect of oral immunization with a recombinant *Lactobacillus casei*-based vaccine on mucosal type1 cell immune responses, the number of non-specific IFNγ-producing cells among the mucosal lymphocytes was assessed using an ELISPOT assay after mitogen stimulation (40 ng/mL of PMA and 4 μg/mL of ionomycin). Groups of five mice received LacE7 (1, 10, and 100 mg/head) or Lac (10 mg/head) orally. Integrin α4β7⁺ intestinal mucosal lymphocytes were collected from each mouse 1 week after final immunization. Asterisks indicate those comparisons (PBS vs. Lac/LacE7) with statistical significance ($p < 0.05$) ($n = 5$).

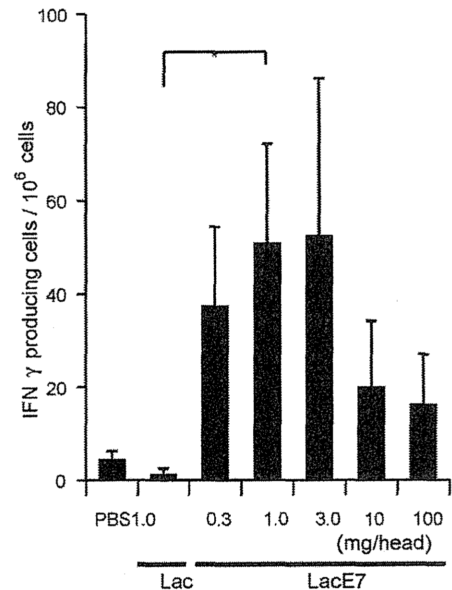

Fig. 3. Various doses of LacE7 (0.3, 1.0, 3.0, 10, and 100 mg/head) or Lac were orally administered to a group of five mice at weeks 1, 2, and 4. The same dose was used for all oral administrations to a given mouse. Integrin α4β7⁺ intestinal mucosal lymphocytes were collected from the immunized mice at week 5. The number of E7-specific IFNγ-producing cells among the mucosal lymphocytes was assessed using an ELISPOT assay after stimulation with 1 μg/mL of a synthetic peptide corresponding to amino HPV16 E7 acids 49–57. Asterisk indicates those comparisons (Lac vs. LacE7) with statistical significance ($p < 0.05$) ($n = 5$).

by IFNγ-producing CD4⁺ helper T cells (type1 immune response-related T cells) and cytotoxic CD8⁺ T (Tc) cells. To examine the effect of oral immunization with a *Lb. casei*-based vaccine on mucosal type1 immune response-related cells, the number of IFNγ-producing cells among all mucosal lymphocytes was assessed by ELISPOT assay (Fig. 2). Each group of five mice was orally exposed to *Lb. casei* bearing HPV16 E7 (LacE7) at 1 mg/head, 10 mg/head, or 100 mg/head or to vehicle (Lac) vaccines at 10 mg/head at weeks 1, 2, and 4. Oral administration occurred once per day for 5 days each week. Intestinal mucosal lymphocytes were collected from each mouse 1 week after the last immunization. For ELISPOT assays, the lymphocytes were stimulated with the mitogens, PMA and ionomycin, and the type1 immune response-related cells were detected as IFNγ positive spots. The number of IFNγ-producing type1 immune response-related cell increased by a similar amount after immunization with equal amounts of Lac and LacE7. Type1 cell increases were also dependent on the dosage of LacE7. This suggested that *Lb. casei* provides a non-specific adjuvant effect on the induction of type1 immune responses at mucosal sites.

3.3. The dose-dependent induction of E7-specific cellular immune response by oral immunization with LacE7

HPV16 E7 amino acids 49–57 are known to represent an E7-CTL epitope that is recognized by the CTL and type1 T cells of C57BL/6 mice [11]. A synthetic peptide with an amino acid sequence corresponding to this CTL epitope was used as a stimulant in E7-specific ELISPOT assays to assess type1 T cell immune responses to HPV16 E7. Various doses of LacE7 (0.3, 1.0, 3.0, 10, and 100 mg/head) or Lac were administered orally to five mice at weeks 1, 2, and 4 as in Fig. 3. Integrin α4β7⁺ intestinal mucosal lymphocytes were collected from the immunized mice at week 5 and examined in an E7-specific ELISPOT assay. The number of type1 immune response-related T cell producing IFNγ after stimulation by the E7 peptide increased significantly in LacE7-immunized mice but not in Lac-immunized mice. This demonstrates that oral immunization of mice with LacE7 elicits type1 mucosal immune response-related T cells that direct E7-specific CTL in the intestinal mucosa. Interestingly, the number of the E7-specific type1 immune response-related T cells peaked at exposure levels of 1.0–3.0 mg/head and decreased with dose escalation over 3.0 mg/head (Fig. 3).

To increase type1 immune response-related T cell cellular immune responses, mice immunized orally with LacE7 or Lac at weeks 1, 2, and 4 received booster oral immunization at week 8. Intestinal mucosal lymphocytes were then isolated and analyzed at week 9. In these experiments, a variety of exposures to 1.0 mg/head or less of LacE7 were used for prime and boost administrations to find the minimum dose that would elicit an optimal type1 immune response-related T cells (Fig. 4). E7-specific, IFNγ-producing type1 cells increased in number with LacE7 dose escalation. 1.0 mg/head of LacE7 oral immunization induced E7-specific type1 cellular immune responses significantly and appeared to be the optimal induction dose while the same dose of Lac alone had no effect on these responses. The booster immunization at week 8 raised E7-specific type1 immune cell numbers 4-fold when compared to the induction at week 5.

3.4. Comparison of mucosal cellular immune responses after oral, intramuscular, and subcutaneous immunizations

Previous studies on HPV therapeutic vaccines have demonstrated E6/E7-specific cellular immune responses in splenocyte or PBMCs in mice [8]. All previous clinical trials in humans have also used peripheral or systemic response read-outs (PBMCs). Prior studies on HPV therapeutic vaccines have also been limited to intramuscular or subcutaneous exposure to E6/E7-fused proteins or plasmid DNA expressing E6/E7 genes [12,13]. Few investigators have addressed specific mucosal cellular immune and few have used mucosal exposure as the route of immunization. Although some studies have analyzed post-exposure lymphocytes collected from minced genital tract tissue or its draining lymph nodes [30,31],

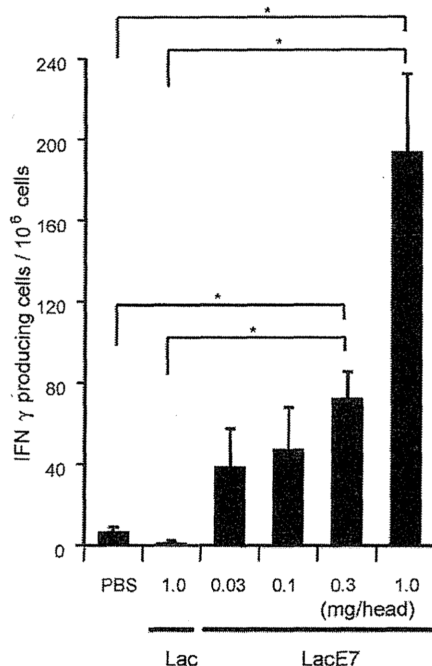

Fig. 4. Various doses of LacE7 (0.03, 0.1, 0.3, and 1.0 mg/head) or Lac were orally administered to a group of five mice at weeks 1, 2, and 4 (priming) and week 8 (boost). The same dose was used for priming and boost exposures in a given mouse. Integrin $\alpha4\beta7^+$ intestinal mucosal lymphocytes were collected from the immunized mice at week 9 and examined using an E7-specific ELISPOT assay as shown in Fig. 3. The boost immunization at week 8 raised E7-specific type1 immune cell numbers 4-fold more than those at week 5. Asterisks indicate those comparisons (PBS/Lac vs. LacE7) with statistical significance ($p < 0.05$) ($n = 5$).

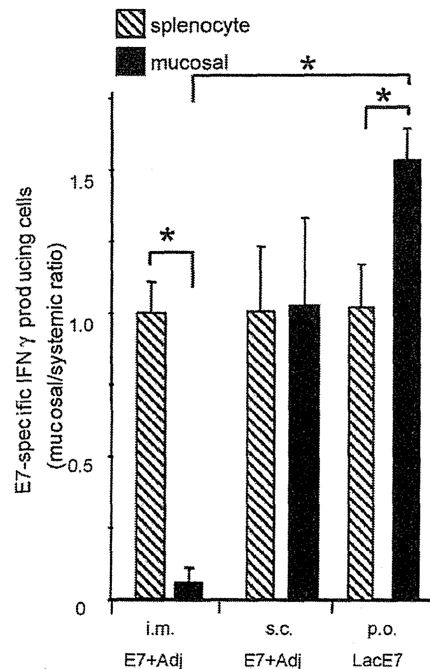

Fig. 5. Oral, intramuscular, or subcutaneous immunizations were compared for the induction of mucosal E7-specific type1 immune responses. Mice were immunized by each route at weeks 1, 2, 4 and 8. Both intestinal mucosal lymphocytes (black) and splenocytes (stripe) were collected from mice immunized by each method and assessed using an E7-specific ELISPOT assay. Each route of immunization elicited similar E7-specific IFNγ-producing type1 immune responses in splenocytes. The number of E7-specific type1 cells among mucosal lymphocytes was normalized to that among splenocytes for each immunization route. This ratio is depicted on the *y*-axis. Asterisks indicate those comparisons (oral vs. other immunization) with statistical significance ($p < 0.05$) ($n = 5$).

these cells are grossly contaminated by peripheral cells in the vessels supplying these tissues and represent local, but not specifically mucosal immune responses.

In this study, intestinal mucosal lymphocytes and splenocytes were collected from immunized mice to represent mucosal and systemic cellular immune responses, respectively. To address the role of route of vaccination on mucosal immunity, mucosal and peripheral responses to oral immunization with LacE7 was compared to intramuscular and subcutaneous immunizations with E7. For intramuscular and subcutaneous injections, a purified E7-fused protein with adjuvant was used since LacE7 was not water soluble and was difficult to use in injections. Each immunization was performed in five mice at weeks 1, 2, 4 and 8 (Fig. 5).

Oral, subcutaneous and intramuscular exposures elicited fairly consistent levels of induction of E7-specific IFNγ-producing type1 immune cells among splenocytes (about hundreds cells/10^6 splenocytes). For comparison, the number of E7-specific type1 immune cells among mucosal lymphocytes was normalized to that among isolated splenocytes for each immunization. Intramuscular immunization induced barely detectable levels of E7-specific type1 mucosal immune cells, significantly less than that found among splenocytes (about one-tenth of that in splenocytes). Subcutaneous immunization induced E7-specific type1 mucosal and peripheral immune responses equally. Oral immunization of LacE7 elicited a predominant mucosal E7-specific type1 immune response, with type1 immune response-related cell levels approximately 1.5–2.0-fold higher than those among splenocytes. The most effective immunization route for the induction of HPV-specific mucosal cellular immune response was oral immunization, followed by subcutaneous immunization. Intramuscular immunization had little effect on mucosal responses.

3.5. E7-specific cytotoxic activity of induced mucosal lymphocytes

E7-specific $CD4^+$ type1 immune response-related cells were detected among the mucosal lymphocytes collected from mice immunized orally with LacE7. To confirm the activity of E7-specific $CD8^+$ CTLs among mucosal lymphocytes, T cells producing granzyme B in response to the E7-CTL epitope (HPV16 E7 amino acids 49–57) were measured using ELISPOT assays and killer activity to HPV16 E7-expressing (TC-1) cells examined. The murine $H\text{-}2^b$ tumor cell line, TC-1, is derived from an allogeneic mouse strain (C57BL/6) and immortalized by HPV16 E6/E7 plus c-Ha-*ras*. TC-1 cells were used as a target cells in killing assays. Mucosal lymphocyte and splenocyte were collected from mice receiving oral immunization of LacE7 at weeks 1, 2, 4, and 8 and used for CTL assays (Fig. 6). ELISPOT assays revealed an induction of E7-specific granzyme B-producing cells in isolated mucosal lymphocyte and splenocyte populations. As shown in Fig. 5, there was a trend toward higher numbers of E7-specific, granzyme B-producing cells among mucosal lymphocytes when compared to splenocytes although this difference was not significant (Fig. 6A). When mucosal lymphocyte were used as effector cells in killer assays with E7-expressing epithelial cell targets derived from C57BL/6 mice (TC-1 cells), the effector cell will recognize target cell MHC class I molecules since both cells are derived from same mouse strain. Mucosal lymphocytes isolated from mice immunized with LacE7 had increased lytic effects against TC-1 cells that were appropriately dependent on the E:T ratio; those isolated from control mice did not. This demonstrates that mucosal lymphocyte killer activity was specifically induced through recognition of the E7-CTL epitope by the mucosal $CD8^+$ T cell.

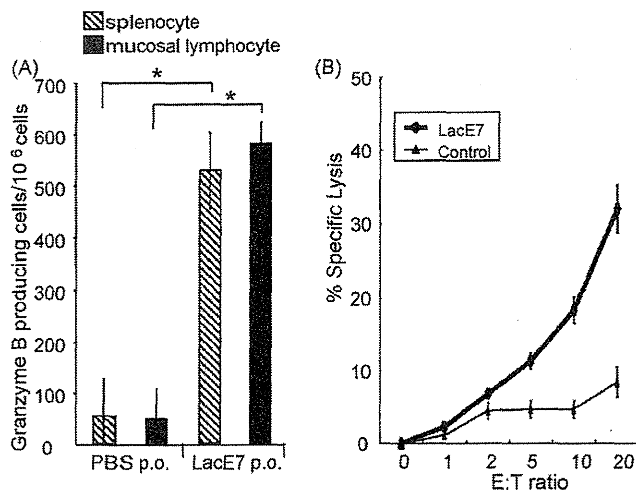

Fig. 6. T cells producing granzyme B in response to E7-CTL epitope exposure were measured using an ELISPOT assay and their killer activity toward TC-1 cells was examined. Mucosal lymphocytes and splenocytes collected from mice receiving oral immunization of LacE7 at weeks 1, 2, 4, and 8 were used for CTL assays. (A) ELISPOT assay revealed an induction of E7-specific granzyme B-producing cells in both mucosal lymphocytes (black) and splenocytes (stripe). The ELISPOT assay was examined as shown in Fig. 3 using an anti-granzyme B antibody. Asterisks indicate those comparisons (PBS vs. LacE7) with statistical significance ($p < 0.05$) ($n = 5$). (B) Mucosal lymphocytes effector cells were mixed with TC-1 target cells at various E:T ratios indicated on the *x*-axis. The cell lytic effect (%) against TC-1 cells is depicted on the *y*-axis.

4. Discussion

HPV E7 expression in cervical neoplasia has been previously verified at transcriptional and translational levels [17,38]. RT-PCR and immunohistochemical assessments have shown that high-grade CIN lesions (CIN 2–3) strongly express the E7 gene and protein but CIN1 lesion exhibit very low expression levels, suggesting that the E7 protein represents an important target molecule for immunotherapy against high-grade CIN (CIN 2–3).

In this study, we demonstrated that mucosal type1 immune response-related T cell activity against HPV16 E7 can be induced by oral immunization with a recombinant *Lactobacillus*-based vaccine (LacE7). Mucosal lymphocytes obtained from the intestinal mucosa were used for assays of cellular immune response. About 90% of the lymphocyte possessed integrin α4β7, a mucosa-associated homing receptor whose expression is induced by dendritic cells (DCs) residing in the GALT via retinoic acid. The proportions of $CD4^+$ and $CD8^+$ cells in the isolated lymphocytes were quite distinct from those in the mucosal lamina propria or among intraepithelial lymphocytes [32]. The mucosal lymphocytes appeared to be a mixture of T cells derived from GALT, mucosal lamina propria and intraepithelial compartments. The number of B cells among isolated mucosal lymphocytes was much less than that in peripheral blood. These flow cytometry data demonstrate that peripheral blood contamination was negligible using the mucosal lymphocyte isolation protocols employed in this study. It is impossible to collect pure mucosal lymphocyte from the murine genital tract mucosa because of its small size. Therefore, previous studies have substituted lymphocytes collected from the lymph nodes draining genital tract (e.g. inguinal and iliac) for the detection of local immune responses [30,31]. Rank et al. have directly demonstrated immune responses in genital tract mucosal cells isolated from guinea pigs [33], but HPV infected target cell lines, like TC-1, are not available in guinea pig models. Although we attempted to use similar methods to isolated genital tract lymphocytes from mice, these cells were massively contaminated by peripheral blood (data not shown) and we could not assure the cells were purely mucosal. In this study, intestinal mucosal lymphocytes were used to address mucosal cellular immune responses to an HPV E7 vaccine in mice. Our data make this manuscript a proof-of-concept paper until the cervix can be assessed directly in humans.

In the mucosal immune system, MALT is the crucial inductive site for adaptive immunity. The cervical mucosa, however, does not possess MALT [34], a characteristic that may help to prevent specific immune attack against sperm deposited repeatedly in the female reproductive tract. Iwasaki's group has demonstrated that DCs in the vaginal mucosa migrate to draining lymph nodes, including inguinal, iliac and sacral lymph nodes, where they present antigen to $CD4^+$ T cells [35–37]. These draining lymph nodes are critical as inductive sites for antigen-presentation after pathogen invasion. Interestingly, these lymph nodes possess endothelial cells that express MAdCAM, a natural ligand for integrin α4β7 [38]. We have shown that integrin $\alpha4\beta7^+$ lymphocytes comprise 50–70% of the cervical lymphocytes isolated from patients with HPV-associated lesions, indicating that GALT-derived cells home to the cervical mucosa in human (Yokoyama et al., unpublished data). Cervical mucosae appear to utilize GALT as alternative inductive sites for antigen-presentation of pathogens that have invaded the genital mucosa. The induced integrin $\alpha4\beta7^+$ memory/effector cells can then traffic to the cervical mucosa and provide cellular immune responses in the cervical mucosa similar to those in the intestinal mucosa. We therefore presumed that mucosal lymphocyte isolated from intestinal mucosa can be used as surrogates for those populating the cervical mucosa and we chose to study oral rather than intranasal immunization. Bermúdez-Humarán et al. demonstrated that intranasal immunization with lactic acid bacteria expressing HPV E7 and IL12 elicits antitumor effects on E7-related murine tumors and assessed CTL responses in splenocytes [18]. The antitumor effects on mucosal neoplastic lesions by mucosal lymphocytes have never been specifically addressed. Oral immunization with lactic acid bacteria should directly stimulate GALT and integrin $\alpha4\beta7^+$ memory/effector cells and this should result in strong mucosal immune responses in the gastrointestinal tract and the cervix. In our investigation, the mucosal type1 immune responses to E7 were quite different depending on the route of immunization. Oral immunization had the predicted advantage of preferential induction of mucosal rather than systemic immunity. Intramuscular immunization, in contrast, was quite suitable for the induction of systemic but not mucosal immunity. This work is the first to compare mucosal cellular immune response to HPV E7 among several routes of vaccine administration.

To induce mucosal immunity to a vaccine antigen, the antigen must be delivered to inductive sites and presented by APC to activated memory cells [39]. The intestinal mucosa possesses many inductive sites, including Peyer's patches, making it an attractive site for the stimulation of protective mucosal immunity. *Lb. casei* has been shown to act as an efficient vaccine carrier that delivers antigen across the gut to GALT as well as a vaccine adjuvant that promotes type1 T cell immune responses [9,26]. Mohamadzadeh et al. demonstrated that *Lactobacillus* species promote type1 immune response polarization through interactions with myeloid dendritic cells (MDC) [40]. *Lactobacillus* activate MDC through TLR-2 and the activated MDC stimulate the proliferation of autologous $CD4^+$ and $CD8^+$ T cells and their secretion of IFNγ [41]. Kajikawa et al. further confirmed that recombinant *Lb. casei* can induce IFNγ production at mucosal sites [26]. Our data demonstrate that oral immunization using *Lb. casei* that lack or express E7 equally elicit non-specific IFNγ-producing type1 immune cells in the intestinal mucosa, indicating that the stimulation of mucosal type1 immune responses is *Lb. casei*- but not E7-specific. The adjuvant effect of *Lb. casei* on type1 immune responses did not change over a range of 1–100 mg/head

exposures, suggesting that *Lb. casei* represents an excellent antigen delivery vehicle when cytotoxic mucosal immune responses to vaccine antigen are desired.

The E7-specific type1 immune response induced by LacE7 was directly dose-dependent over a range of 0.03–1.0 mg/head, but decreased when exposure exceeded a LacE7 level of 1.0 mg/head. These findings could be caused by an aggregation of the attenuated bacteria when levels approach 10 mg/head, resulting in interference with antigen translocation to GALT through M cells. In these investigations we chose LacE7 doses of 1.0 mg/head as optimal for oral immunization to induce E7-specific type1 immune response-related T cell activities and used this level of exposure for CTL detection and killer activity assays. E7-specific type1 immune cell numbers increased after boosting when compared to non-boost protocols, suggesting that mucosal lymphocyte populations include memory T cells that recognize E7.

The lack of an animal model with HPV E7-related mucosal neoplastic lesions hampers assessment of our therapeutic vaccination strategy for preclinical efficacy against CIN. Most previous studies on HPV therapeutic vaccines utilized murine models in which HPV16 E7-transformed TC-1 cells were injected subcutaneously to induce tumor formation [42,43]. This model can assess systemic, but not mucosal, immune responses to HPV-related tumors. In our study, TC-1 cell was used as target cells for mucosal E7-specific CTL in *in vitro* killer activity assays. The HPV-specific killer activity of mucosal lymphocytes was clearly demonstrated by the induction of granzyme B-producing $CD8^+$ T cells. Poo et al. have revealed that oral immunization of mice with *Lb. casei* expressing HPV16 E7 reduces the growth of subcutaneous TC-1 cell tumor and induces E7-specific type1 immune response-related splenic T cells [9]. We have shown that oral immunization with LacE7 preferentially elicits E7-specific type1 T cell responses in mucosal lymphocytes (2-fold higher) when compared to splenocytes. These data strongly suggest that the induced mucosal $CD4^+$ and $CD8^+$ T cells will have antitumor effects on mucosal HPV E7-related neoplastic lesions.

Oral routes of immunization offer many advantages: easy self-administration at home, reduction in hypersensitivity reactions, and decreased costs (no needles, syringes or trained personnel). Further, the production of lactic acid bacteria is also inexpensive. In this study the recombinant *Lb. casei*, LacE7, was heat-attenuated. Attenuation results in the destruction of the expression plasmid and prevention of self-replication. This negates the possibility for transfer of foreign genes to normal bacterial in the gut. The Rb-binding site of HPV16 E7 was mutated in the antigen-producing plasmid, thereby eliminating its oncogenicity but not its immunogenicity [44]. These modifications make the recombinant *Lb. casei* vaccine ensure drug safety. Unfortunately, we must await clinical trials on this promising therapeutic HPV vaccine to assess its actual antitumor effect on mucosal neoplastic lesions. Our data support the development of an initial clinical study on therapeutic vaccination of patients with CIN 2–3 patients using LacE7.

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Comparison Between Conventional Surgery Plus Postoperative Adjuvant Radiotherapy and Concurrent Chemoradiation for FIGO Stage IIB Cervical Carcinoma

A Retrospective Study

AQ:1

Hideomi Yamashita, MD, Kae Okuma, ●●●,* Kei Kawana, ●●●,† Shunsuke Nakagawa, ●●●,† Katsutoshi Oda, ●●●,† Tetsu Yano, ●●●,† Shino Kobayashi, ●●●,* Reiko Wakui, ●●●,* Kuni Ohtomo, ●●●,* and Keiichi Nakagawa, ●●●**

AQ:2

Objective: To compare treatment outcome of conventional surgery followed by adjuvant postoperative radiotherapy (PORT) versus concurrent chemoradiation therapy (cCRT) for stage IIB cervical carcinoma.

Methods: A retrospective analysis was conducted of 59 patients with stage IIB uterine cervical cancer treated with radical surgery plus PORT (N = 34) or cCRT-alone (N = 25) from April 1996 to June 2008. The median follow-up time was 27 months (range, 3–150 months) in the cCRT group and 44 months (range, 4–134 months) in the PORT group. The median age was 59 years (range, 37–85 years) in the cCRT group and 49 years (range, 32–74 years) in the PORT group. All 34 patients in the surgery group underwent hysterectomy with pelvic lymph node dissection and received PORT. Twenty-five patients (42%) were assigned to the cCRT group.

Results: The 3-year overall survival rates for surgery plus PORT and cCRT-alone were 80.0% and 75.1%, respectively. The difference between these 2 treatments was not statistically significant (log-rank $P = 0.5871$). The late complication rate of grade 3–4 was 12% in the cCRT group and 16% in the surgery group.

Conclusion: This retrospective study suggests that survival results with cCRT and with conventional surgery plus PORT for patients with stage IIB cervical carcinoma are comparable.

Key Words: cervical carcinoma, surgery, chemoradiotherapy, high-dose-rate brachytherapy, stage IIB

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Cervical cancer is the most common gynecologic malignancy in Japan, with an estimated 5 new cases per 100,000 females every year. High dose-rate intracavitary brachytherapy (HDR-ICBT) in combination with external beam irradiation (EBRT) has become an acceptable treatment for carcinoma of the cervix.[1] HDR-ICBT has been widely used in treatment of uterine cervical cancer in Asia and Europe. Although some controversy exists in the United States over the use of HDR-ICBT,[2] an increasing frequency of its adoption has been noted.[3,4]

Recently published randomized clinical trials demonstrated a significant improvement in pelvic disease control and survival when concurrent chemotherapy consisting of cisplatin-containing regimens was added to radiotherapy (RT) in patients with locally advanced cervical cancer.[5–7] These results led to significant changes in the standard treatment of cervical cancer.

Radiotherapy has long been recognized as a successful treatment modality for all stages of carcinoma of the uterine cervix. In Japan, however, because patients present first at gynecologic clinics, gynecologists usually determine the treatment modality without additional inputs from radiotherapists. In general, Japanese gynecologists consider surgical treatment to be superior to RT, and, as a result, the majority of patients with stage IIB are subjected to radical hysterectomy plus pelvic, and with or without para-aortic lymphadenectomy followed by preventive postoperative RT (PORT). Consequently, other than this preventive postoperative RT, radiation oncologists in Japan have treated only stage IIB patients who refused surgery or who were not indicated for surgery because of other coexisting disease.

Although RT has been widely used in Western countries, there are only a few reports on definitive RT for early stages (stages I–II) of cervical carcinoma. Some studies indicated that RT for early stage patients was a feasible definitive treatment.[8–11] In Japan, no prior report has compared surgery and RT. We now report the results of a retrospective study in which the survival outcomes of surgery and RT were compared for stage IIB cervical cancer. The hypothesis was to be certified that definitive cCRT is not inferior to survival and less frequency about severe complications than radical hysterectomy plus PORT for stage IIB cervical cancer in this single institution.

PATIENTS AND METHODS

Patients

Between April 1996 and June 2008, a total of 59 consecutive patients were treated for FIGO (International Federation of Gynaecology and Obstetrics classification) stage IIB carcinoma of the cervix with conventional surgery plus adjuvant PORT or concurrent CRT at our institution. All patients with stage IIB treated during the 13-year period (1996–2008) were included in the study. Patients included were those previously untreated and who had a histologic diagnosis of squamous cell carcinoma, or adenocarcinoma in FIGO stage IIB. Patients with adenocarcinoma (n = 13) were also included in this study. Median age was 53 years (range, 32–85 years). Table 1 shows the patients' characteristics. Surgically treated patients comprised 58% (34/59) and cCRT-alone patients 42% (25/59) (Table 1). The patients submitting to definitive CRT were those with comorbidities or who refused surgery in our institution.

T1

Patients were evaluated with a physical and pelvic examination without anesthesia, routine blood counts, blood chemistry profile, chest radiograph, intravenous urogram, and barium enema. Computed tomography (CT) scan and magnetic resonance imaging (MRI) were used only for detecting lymphadenopathy. Pelvic and para-aortic lymph nodes greater than 10 mm in minimum diameter

From the *Departments of Radiology, and †Obstetrics and Gynecology, University of Tokyo Hospital, Tokyo, Japan.
Reprints: Hideomi Yamashita, MD, Department of Radiology, University of Tokyo Hospital, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655 Japan. E-mail: yamashitah-rad@h.u-tokyo.ac.jp.
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TABLE 1. Comparison of Patients' Characteristics

	Surgery Plus PORT	cCRT	*P*
Total no. patients	34	25	
Age			
Median (range) (yr)	49 (32–74)	59 (37–85)	0.0001
Histopathology			
Squamous cell carcinoma	24 (71%)	22 (88%)	0.2026
Adenocarcinoma	10 (29%)	3 (12%)	
Pelvic nodal status			
Positive	17 (50%)	5 (20%)	0.0185
Negative	17 (50%)	20 (80%)	
Paraaortic nodal status			
Positive	3 (8%)	2 (8%)	0.9106
Negative	31 (92%)	23 (92%)	
Maximum tumor diameter (mm)			
>40	19 (56%)	14 (56%)	0.9928
≦40	15 (44%)	11 (44%)	
Median (range)	49.5 (18–100)	45.5 (30–80)	0.6153

detected by CT and MRI were considered to be positive for metastases. Neither lymphangiography nor surgical evaluation of lymph nodes was performed.

Concurrent CRT

EBRT

All patients received EBRT using a linear accelerator with a photon-beam-energy of 10 MV to the whole pelvis with the 4-field box technique for a total dose of 30.6 Gy in 17 fractions (3.4 weeks, 1.8 Gy fractions from Monday to Friday). The irradiated volume was to include the whole uterus, the paracervical, parametrium and uterosacral regions, as well as the external iliac, hypogastric and obturator lymph node. Minimum margins were the upper margin of L-5 (superiorly), the lower margin of the obturator foramen or the lowest extension of the disease (inferiorly), and 2.0 cm beyond the lateral margins of the bony pelvis and its widest plane (laterally). For the lateral fields, the anterior margin was the anterior edge of the symphysis or 3 cm in front of the sacral promontory. The posterior margin was the S2–S3 interspace or the posterior border of the uterine cervix assessed by CT for treatment planning plus a 2 cm margin. After that, a midline block, 4 cm in width at midplane, was inserted with the anteroposterior parallel 2-field technique for a dose of 19.8 Gy in the last 11 fractions (ie, parametrial boost). This block extended to the top of the uterine.

Two patients who had para-aortal lymph node involvement received whole pelvic irradiation plus para-aortal irradiation using the 4-field box or conformal technique. Total dose to para-aortal lymph nodes was 50.4 Gy in 28 fractions.

ICBT

In our department, EBRT preceded ICBT. A midline block was inserted at the same time as the first application of ICBT. Four intracavitary iridium-192 (^{192}Ir) insertions were performed weekly, starting 3.4 weeks after starting EBRT. High-dose-rate intracavitary therapy was used. Brachytherapy was delivered using after-loading applicators placed in the uterine cavity and vagina. A Manchester system applicator (Nucleotron microSelectron HDR source) was used. The dose distribution was calculated for each individual patient and placement. Patients were treated in the dorsal lithotomy position. Point A was defined on radiographs as being 2 cm superior (along the tandem) to the flange abutting the external cervical os and 2 cm lateral from the axis of the tandem. Source loading corresponded to the Manchester System for uterine cervical cancer.[12] HDR-ICBT was performed once a week with a daily dose of 6 Gy at point A. The details of the method for ICBT were shown in our previous report.[13,14]

Total dose to the central area was 54.6 Gy and to the parametrium area was 50.4 Gy. When using biologically effective dose with $\alpha/\beta = 10$ Gy, total dose to the central area was 74.5 Gy and to the parametrium was 59.5 Gy.

Chemotherapy

Concurrent CDDP-based chemotherapy combined with RT for stage IIB has been routinely performed in our department. All patients received platinum series-based chemotherapy combined with RT. All patients received CDDP (75 mg/m^2 in a bolus infusion on days 1, 22, and 43).

Surgery Plus Adjuvant RT

Radical hysterectomy with pelvic lymphadenectomy was performed on 34 patients. Radical hysterectomy at our institution includes resection of the uterus along with its attached parametrial soft tissue and a margin of the upper vagina, as in the world standard. Even when positive nodes were found, radical hysterectomy was continued without stopping. No radical hysterectomy was aborted. Para-aortic lymphadenectomy up to the level of the inferior mesenteric artery was performed for patients with adenocarcinoma or enlarged pelvic lymph nodes assessed by preoperative CT or MRI (N = 13). Because it is well-known that cases with positive pelvic lymph nodes are indicative of a very poor prognosis, lymphadenectomy up to the level of the inferior mesenteric artery was added for these high-risk cases in our institution. The median operation time was 390 minutes (range, 150–550 minutes) and the median quantity of operative blood loss was 1450 mL (range, 400–5600 mL).

All patients in the surgery group received postoperative adjuvant RT because of invasion to the parametrium. Adjuvant RT consisted of external pelvic irradiation (10 MV x-rays) with the 4-portal technique, one fraction of 1.8 Gy daily, with a total dose of 50.4 Gy over 5.6 weeks. The para-aortic region was irradiated with a dose of 50.4 Gy over 5.6 weeks with the conformal technique when metastases were detected in the surgical specimens of para-aortic nodes.

Follow-Up

Both radiation and gynecologic oncologists were involved in the follow-up the treated patients. The patients were seen every month for the first year, every 2 to 3 months for the next 2 years, and at least every 6 months thereafter. No patients were lost to follow-up. Follow-up procedures included pelvic examination, palpation of supraclavicular nodes, cervical Papanicolaou smear, and review of serum squamous cell carcinoma related antigen and cytokeratin 19 fragment antigen values. When central and/or parametrial recurrence was suspected by pelvic examination and/or Papanicolaou smear, a biopsy was taken for confirmation. Intravenously enhanced chest, abdominal, and pelvic CTs were performed annually. Other imaging studies, such as MRI, ultrasound and bone scintigraphy, were not routinely performed. Both acute and late complications were graded in accordance with the National Cancer Institute Common Toxicity Criteria Version 2.0.

Statistical Analysis

Statistical analyses were performed using StatView Dataset File version 5.0 J for Windows computers (Cary, NC). OS, progression-free survival (PFS), and local (ie, within pelvic) recurrence-free survival (LRFS) were calculated from the first date of curative treatment. Survival time was plotted using the Kaplan–Meier

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method. Differences in patients' characteristics were analyzed by the χ^2 test or Fisher exact test for 2 × 2 columns and unpaired t test for a succession of numbers. Differences in survival by treatment were evaluated using the log-rank test.

RESULTS

Patients and Tumor

The age and pelvic nodal status distributions were significantly different between the 2 groups of patients (Student t test or χ^2 test). The median age was 59 years (range, 37–85 years) in the cCRT group and 49 years (range, 32–74 years) in the PORT group (Table 1). The patient FIGO stage, follow-up time, para-aortic nodal status, and histopathology distributions showed no significant differences (Table 1). The Karnofsky Performance Status for all patients was more than 80%. The proportion of patients with tumors less than 4 cm in diameter was 41% (14/34) for the surgery group and 44% (11/25) for the CRT. The median size was 50 mm (range, 18–100 mm) for the surgery group and 45.5 mm (range, 30–80 mm) for the cCRT group. The positive rate of surgical margins for the surgery group was 21% (7 cases). In the surgery group, bilateral parametrial involvement was seen in only one patient, although it was unilateral in the other 33 patients. Of those with unilateral involvement, only one patient had more than half extension of parametrial involvement and the other 32 patients had less than half.

In the surgery group, the positive rate of pelvic nodal metastasis was 32% (11 cases) assessed by clinical method and 50% (17 cases) by histopathological method. The pelvic nodal status showed no significant difference between surgery and definitive CRT groups if assessed by same method (clinically) (χ^2 test, P = 0.2916).

Median follow-up time was 27 months (range, 3–150 months) in the cCRT group and 44 months (range, 4–134 months) in the PORT group. The proportion of the surviving patients was 74% (25/34) for the surgery and 76% (19/25) for the cCRT groups.

The first site of progression was local-alone (ie, within pelvis) in 8% (2 cases) for the definitive CRT group and in 12% (4 cases) for the surgery group. Additionally, it was distant-alone progression in 4% (1 case) for CRT group and in 18% (6 cases) for surgery group, and both local and distant in 8% (2 cases) for CRT group and 0% for the surgery group. In other words, the recurrent rate within the pelvis was 14% (8/59 cases).

Survival

When this analysis was closed, 44 of 59 patients were alive. Deaths resulted in all 15 patients with cervical cancer (6 patients for definitive CRT group and 9 patients for surgery group). No treatment-related deaths were encountered. Figure 1 shows a comparison of the OS curves for the definitive CRT and surgery groups. The 3-year OS rates were 75% for the cCRT group and 80% for the surgery, respectively; the difference between these 2 rates was not significant (log-rank P = 0.5871). The 3-year PFS rates were 79% for the cCRT group and 70% for the surgery, respectively (P = 0.7247, HR = 0.825, 95% CI = 0.281–2.422). The 3-year LRFS rate was 83% for the cCRT group and 86% for the surgery, respectively (P = 0.4908, HR = 1.622, 95% CI = 0.404–6.513). Clearly, none of these analyses was of any significance.

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Complications

In the cCRT group, nonhematological acute toxicity and all late toxicity (complications that persisted or occurred for more than 60 days after treatment) of grade 3+ were noted in 3 patients (12%). Small bowel perforation without tumor recurrence (grade 4) and melena from radiation proctitis in 2 cases (both grade 3) were seen at 5, 8, and 11 months after the completion of CRT. Moreover, grades 1 or 2 melena and lymphoedema of the lower limbs were seen in each of 4 patients (16%).

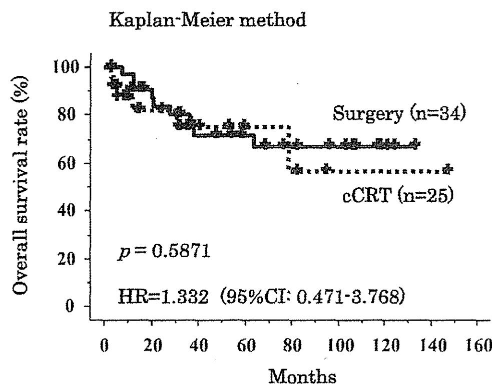

FIGURE 1. Overall survival curves in stage IIB patients comparing surgery plus postoperative radiotherapy (surgery) and concurrent chemoradiation (cCRT).

In the surgery group, the bladder damage was seen in one example for a complication during operation. Nonhematological acute toxicity of grade 3+ was seen in 9 patients (16%). Postoperative ileus (grade 4 in 1 patient and grade 3 in 3 patients), grade 3 pelvic lymphocyst in one patient, grade 3 pulmonary infarction in 2 patients, grade 3 bilateral hydronephrosis in one patient, and lymphangitis of lower extremities (grade 3) in one patient were seen. Moreover, grade 2 hypertension, urinary tract infection, unilateral functionless kidney, and pelvic lymphocyst were seen in each of a single patient (total was 16%). No ureteral stricture was seen in the neither PORT or surgery groups.

DISCUSSION

This is a retrospective analysis of 59 patients with FIGO stage IIB cervical cancer treated with surgery plus PORT (n = 34) or concomitant CRT-alone (n = 25). Some centers especially in Japan still consider both treatment modalities as a standard for FIGO stage IIB cervical cancer. In this study, the 3-year OS, PFS, and LRFS were the same for both groups, although the surgery cases had at least a debulking of lymph nodes when positive as compared with the definitive CRT group. Also, CRT patients received a slight low dose for tumor control: only 50.4 Gy with EBRT plus 4 × 6 Gy with HDR-ICRT. These techniques are quite different from those used to treat the same category of patients in the United States and Europe. Additionally, surgery plus PORT presented 16% of grade 3+ complications. Patients who received definitive CRT were at higher risk, when age and probable comorbidities were considered. This suggests that there is no treatment of choice with respect to local control of disease. Conceivably, the patient population might be too small to draw any conclusions about the superiority of either one of the 2 treatment modalities.

The type of radical hysterectomy performed was III and ureteral resection was performed, although we had 21% of positive margins. The median and mean number of excised nodes was 61 and 63.4 (range, 25–133).

Many previously published results[8–11,15,16] suggest that radical hysterectomy or definitive RT is standard treatments for IB–IIA. In the United States and Europe, definitive RT has been selected in many cases. Radical hysterectomy is not a world standard for stage IIB patients. In contrast, surgery is preferentially used over RT in Japan even for stage IIB cervical cancer, although those patients with stage IIB ideally should have been treated with concomitant

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CRT. RT is usually selected only for the elderly or inoperable cases because of coexisting disease in Japan.

The age of the patients and pelvic nodal status were significantly different between those patients going to surgery and cCRT patients (Table 1). The mean age of the cCRT patients was significantly greater than that of patients in the surgery group (P = 0.0001). There was a tendency for cCRT to be performed for elderly patients who were fearful of surgery or general anesthesia. Regardless of these fears, the cCRT patients had survival rates comparable with the surgery patients.

For the surgery group, pelvic nodal status was pathologically assessed in the surgical specimen, whereas clinically assessed by CT or MRI in the cCRT group. The positive rate of pelvic nodal status for the surgery group was significantly higher than in the cCRT group (50% vs. 20%, $P = 0.0185$). In the surgery group, the positive rate of pelvic nodal metastasis was 32% (11 cases) assessed clinically. The pelvic nodal status showed no significant difference between surgery and definitive CRT groups if assessed by the same method (clinically) (χ^2 test, $P = 0.2916$).

Horn et al[17] concluded that tumor size, when bulky disease was defined as tumors larger than 4 cm, was also of prognostic importance in FIGO stage II cervical carcinomas. In this study, there was no significant difference in the maximum tumor diameter between the 2 groups ($P = 0.6153$), although stage IIB varied from minimal to medial and even to lateral parametrial invasions, just short of pelvic wall fixation. To determine the size of the tumor, pathologic evaluation was used in the PORT group and pretreatment MRI in the CRT group.

Rotman et al[18] concluded that pelvic RT after radical surgery significantly reduced the risk of recurrence and prolonged PFS in women with stage IB cervical cancer whereas PORT appeared to be particularly beneficial for patients with tumors comprised of adenocarcinoma or adenosquamous histologies. In this study, there was no significant difference in the number of adenocarcinomas between the 2 groups ($P = 0.2026$).

It was shown in a previous publication[19] that for the stage IIB patients with lateral parametrial involvement had significantly higher rates of pelvic failure and of survival in comparison with those patients with medial parametrial involvement.

In our experience, the low rate of major complications after cCRT suggests that this approach is well tolerated in most patients. Treatment-related toxicity of grade 3+ developed in 16% of the surgery patients and in 12% of the definitive CRT group. This difference was not significant, probably because of the small numbers of patients in both groups.

Our results confirm earlier findings that have suggested that cCRT is not inferior to surgery plus PORT for FIGO stage IIB cervical carcinoma regardless of age bias, although the 2 treatment groups were not similar (ie, the cCRT group was older and had more comorbidities) and the pelvic node status was different as well. The 3-year outcomes in both groups were also been shown to be compatible with previous reports.[2–8] Because the t test based on a sample of 59 is underpowered, clinical trails with more patients should be needed to further confirm the efficacy of cCRT or surgery plus PORT on FIGO stage IIB cervical carcinoma. By the power analysis, to detect the difference between 2 independent groups, when the input is assumed that tail = one, effect size $r = 0.5$ (large), 0.3 (medium), or 0.1 (small), α err probability = 0.05, power (1-β err probability) = 0.8, and allocation ratio N2/N1 = 1, total sample size is calculated as 102, 278, or 2476, respectively. This study is too underpowered to conclude whether such both techniques as radical hysterectomy plus PORT and definitive CRT are feasible.

In this study, it was to be certified that definitive cCRT is not inferior with regards to survival and less frequency about severe complications than radical hysterectomy plus PORT for stage IIB cervical cancer in this single institution. The limitations of our study included the retrospective nature of the study, heterogeneity of the patient population in the 2 treatment arms, shorter follow-up and physician's bias in the selection of the patients. A matched-pair analysis would be better to compare the 2 groups but could not be done in our study because of the small number of patients. Nevertheless, we hope that our experience will initiate further prospective studies especially in Japan.

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Advanced age is a significant determinant of poor prognosis in patients treated with surgery plus postoperative radiotherapy for endometrial cancer

Kae Okuma[1], Hideomi Yamashita[1], Kei Kawana[2], Shunsuke Nakagawa[2], Katsutoshi Oda[2] and Keiichi Nakagawa[1]

Departments of [1]Radiology and [2]Obstetrics and Gynecology, University of Tokyo Hospital, Hongo, Tokyo, Japan

Abstract

Aim: A review was conducted in which the effect of age on survival was assessed in a population of endometrial cancer patients treated with surgery and adjuvant radiation therapy in our institution.

Methods: From 1988 to 2008, 111 endometrial cancer patients underwent total abdominal hysterectomy and adjuvant whole pelvic radiation therapy (RT). After surgery, for patients with low or intermediate risk without lymph node metastasis, no postoperative adjuvant therapy was performed. For patients with high risk or positive cytology from the abdominal cavity, postoperative radiation therapy was performed. A total dose of 50–50.4 Gy of RT was delivered sequentially. Forty-four patients (44%) were given chemotherapy consisting of epirubicin/cisplatin/carboplatin or paclitaxel/carboplatin. Univariate and multivariate analyses were performed to identify significant prognostic clinicopathological factors.

Results: With a median follow-up time of 59.2 months, the 5-year overall survival was 74% for those 60 years or older versus 90% for those younger than 60 years ($P = 0.044$). For disease-free survival, it was 65% for those 60 years or older, versus 85% for those younger than 60 years ($P = 0.013$). On multivariate analysis, poor disease-free survival was associated with age $\geq$ 60 years ($P = 0.035$).

Conclusions: Older patients (age $\geq$ 60 years) with endometrial cancer had significantly lower overall survival and disease-free survival following postoperative RT independent of other prognostic factors and/or treatment technique.

Key words: age, endometrial carcinoma, prognostic factors, radiation therapy, treatment.

Introduction

The preponderance of data in the literature indicates that advanced age is a predictor of poor outcome in patients with endometrial carcinoma.[1–4] Whether the poor outcome among elderly patients can be accounted for entirely by a more advanced stage at the time of diagnosis, staging, treatment or that endometrial carcinoma among the elderly is intrinsically more aggressive than in younger patients remains to be determined.[5,6] In general, older patients with endometrial carcinoma tend to have deep myometrial invasion, poorly differentiated histology, or extra-uterine spread.[7,8] Consequently, the perception of a negative influence of advanced age on outcome was prevalent even in patients who underwent full surgical staging or those with well- to moderately differentiated tumors.[2,9]

Poor outcome in some of the published reports may be attributed to the less aggressive adjuvant therapy (i.e. radiation therapy), offered to elderly patients.[6] This

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Reprint requests to: Dr Hideomi Yamashita, Department of Radiology, University of Tokyo Hospital, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: yamashitah-rad@h.u-tokyo.ac.jp
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is conceivably a valid argument, especially when dealing with elderly patients who are less likely than younger patients to consent to and tolerate recommended adjuvant therapy in general.[10,11] According to Alekitar *et al.*,[12] even when treated in a similar fashion, endometrial carcinoma patients aged ≥ 70 years appeared to fare worse than younger patients independent of other prognostic factors, thus mandating further improvement in their treatment strategies. Therefore, to determine whether advanced age is an intrinsically poor prognostic factor or whether it is due to less aggressive adjuvant therapy, a comparison was made of the outcomes according to age in a group of patients who all received adjuvant radiation therapy.

Methods

Patients

A total of 111 consecutive endometrial cancer patients were treated with postoperative radiation therapy in our institution between October 1988 and January 2008. All patients were followed in detail and evaluated. This was a retrospective study in a single institution.

In this study, several categories of risk were defined as follows:

1 Intermediate–low risk ($n = 2$, 2%): Stage IA + histological International Federation of Gynecology and Obstetrics (FIGO) grade 3, Stage IB + grade 2, and Stage IIA + grade 1–2 + <50% myometrial invasion (MI)
2 Intermediate–high risk ($n = 30$, 28%): Stage IB + grade 3, Stage IIA + grade 3 + <50% MI, Stage IC + grade 1–2, Stage IIA + grade 1–2 + ≥50% MI, or lymph vascular space invasion or 1/3 above + age ≥70, 2/3 above + age <50–69, or 3/3 above + age <50
3 High risk ($n = 76$, 70%): Stage IC + grade 3, Stage IIA + grade 3 + ≤50% MI, Stage IIB + any grade, or uterine papillary serous carcinoma or clear cell carcinoma, or Stage III–IV.

Basically, simple total abdominal hysterectomy (TAH), bilateral salpingo-oophorectomy (BSO), pelvic lymphadenectomy (PLA), para-aortic lymphadenectomy (PALA), and peritoneal washing cytology (PWC) were applied for the endometrial cancer patients with stage I or II where the disease was confined to the uterine body. Abdominal radical hysterectomy (ARH) or modified radical hysterectomy (mRH) was carried out in place of TAH for the patients with clinically obvious interstitial infiltration. TAH was performed in 12 cases (11%) and RH in the other cases (89%) including mRH for eight cases (7%). PLA was performed in 98 cases (88%), PALA in 92 cases (83%), and bilateral and ipsilateral SO in 60 (54%) and six cases (5%) in this study.

After surgery, for patients with low or intermediate risk without lymph node metastasis, no postoperative adjuvant therapy was performed. For patients with high risk or positive cytology from the abdominal cavity, postoperative radiation therapy (PORT) was performed.

Postoperative chemotherapy

For patients with lymph node metastasis, those with only one lymph node metastasis were given PORT alone and those with two or more lymph node metastases were given chemotherapy followed by external beam radiation therapy. From 2003, the cyclophosphamide, doxorubicin, and cisplatin (CAP) regimen was administered to patients with histological FIGO grade 1 and without vascular invasion and the paclitaxel and carboplatin (TC) regimen was administered for patients with histological FIGO grade 2 or 3 and/or with vascular invasion. Before 2003, only the CAP regimen had been used for postoperative chemotherapy. The CAP regimen consisted of three cycles of 70 mg/m^2 of cisplatin, 500 mg/m^2 of cyclophosphamide, and 50 mg/m^2 of doxorubicin. The TC regimen consisted of three cycles of paclitaxel at 175 mg/m^2 and carboplatin with an area under the curve (AUC) of 6, tri-weekly or monthly.

PORT

The whole pelvis was irradiated in all cases. For para-aortic lymph node metastasis, the para-aortic area and the whole pelvis were irradiated. Two parallel ports, the anterior–posterior and posterior–anterior, were used for whole pelvis irradiation until the year 2000. Thereafter, four ports (box field) were used. The upper edge included the bifurcation of the common iliac artery (around L4–5). The lower edge was between the obturator foramen and the ischial tuberosity, and the lateral edge was 1.5–2 cm outside the small pelvic cavity. The energy was basically 10 MV. The prescribed irradiation dose was 50–50.4 Gy/25–28 fractions. On the irradiation to the para-aortic area, the upper edge was between the 11th and 12th thoracic vertebrae, and the lateral edge included the transverse process.

Evaluation and follow up

Response to radiotherapy was evaluated using pelvic examination, computed tomography, and cytology. Follow up after PORT was usually conducted every

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month for the first 2 years and every 3 months thereafter. Follow-up computed tomography was performed every 6 months and cytology every month. Swab samples were obtained from the vaginal stump. For patients suspected of recurrent disease, the follow up was conducted at more frequent intervals in consideration of alternative salvage treatment. Follow-up examination included physical and pelvic examinations and cytology.

In addition, toxicity was scored using the Common Terminology Criteria for Adverse Events (CTCAE) v3.0.

Statistical analysis

Associations between variables were assessed using the χ^2-test, Fisher's exact test, and linear-by-linear exact tests. The Kaplan–Meier product–limit method was used to estimate the probability of overall survival (OS) and disease-free survival (DFS); the log–rank test was used to estimate any differences. Multivariate analyses were performed using the Cox proportional hazards regression model. OS was calculated in months from the date of surgery to the date of death from any cause or to January 2009. Patients who were still alive in June 2008 were treated as censored. P-values < 0.05 were regarded as statistically significant. Statistical analyses were carried out using StatView Dataset File version 5.0 J for Windows.

Results

Patients

The patient characteristics are shown in Table 1. Of the 111 patients receiving PORT, the median age was 57 years (range, 28–78). One of these 111 patients was not followed up after PORT, and was therefore excluded from the analysis. Thirty-six patients had positive pelvic lymph nodes, 71 patients were negative, and others

Table 1 Univariate analysis of OS and DFS

Factor	*n*	%	5-y OS	*P*-value	5-y DFS	*P*-value
Age						
<60 y	67	61	90%	0.044	85%	0.013
≥60 y	43	39	74%		65%	
PLN						
(+)	36	34	81%	0.36	71%	0.150
(–)	71	66	87%		81%	
PALN						
(+)	24	22	72%	0.12	52%	0.001
(–)	86	78	88%		85%	
FIGO stage						
I	36	34	93%	0.11	87%	0.040
II–III	69	66	79%		71%	
Histological type						
EA	84	78	83%	0.51	76%	0.32
Not EA	24	22	87%		80%	
FIGO grade						
1	41	48	97%	0.35	81%	0.440
2	25	29	82%		76%	
3	18	23	77%		70%	
Risk group						
High	76	70	82%	0.61	72%	0.180
Intermediate–high	30	28	88%		86%	
Intermediate–low	2	2	100%		100%	
Chemotherapy						
With	46	44	82%	0.75	66%	0.13
Without	58	56	84%		84%	
Depth						
a–b†	18	18	100%	0.19	75%	0.67
c–d‡	80	82	83%		77%	

†<50% myometrial invasion. ‡>50% myometrial invasion. DFS, disease-free survival; EA, endometrioid adenocarcinoma; FIGO, International Federation of Gynecology and Obstetrics; OS, overall survival; PALN, para-aortic lymph node; PLN, pelvic lymph node.

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