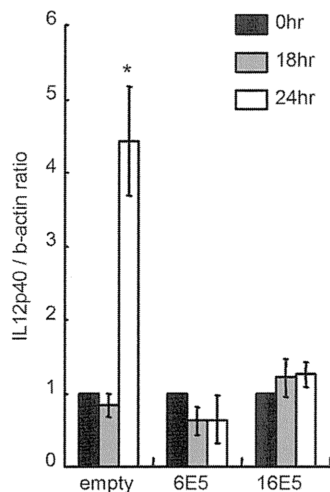

FIG. 10. Autocrine cytokine production upon CD1d cross-linking in C33A/CD1d-empty, C33A/CD1d-6E5, and C33A/CD1d-16E5 cells. An anti-CD1d 51.1 MAb was added at a dosage of 10 μg/ml to cultured epithelial cell monolayers, followed by incubation for 1 h. After being washed with PBS, 10 μg of a goat anti-mouse 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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REFERENCES

1. **Araibi, E. H., B. Marchetti, G. H. Ashrafi, and M. S. Campo.** 2004. Downregulation of major histocompatibility complex class I in bovine papillomas. J. Gen. Virol. **85:**2809–2814.

2. **Ashrafi, G. H., M. Haghshenas, B. Marchetti, and M. S. Campo.** 2006. E5 protein of human papillomavirus 16 downregulates HLA class I and interacts with the heavy chain via its first hydrophobic domain. Int. J. Cancer **119:** 2105–2112.
3. **Ashrafi, G. H., M. R. Haghshenas, B. Marchetti, P. M. O'Brien, and M. S. Campo.** 2005. E5 protein of human papillomavirus type 16 selectively downregulates surface HLA class I. Int. J. Cancer **113:**276–283.
4. **Ashrafi, G. H., E. Tsirimonaki, B. Marchetti, P. M. O'Brien, G. J. Sibbet, L. Andrew, and M. S. Campo.** 2002. Downregulation of MHC class I by bovine papillomavirus E5 oncoproteins. Oncogene **21:**248–259.
5. **Balk, S. P., S. Burke, J. E. Polischuk, M. E. Frantz, L. Yang, S. Porcelli, S. P. Colgan, and R. S. Blumberg.** 1994. Beta 2-microglobulin-independent MHC class Ib molecule expressed by human intestinal epithelium. Science **265:** 259–262.
6. **Behar, S. M., and S. A. Porcelli.** 2007. CD1-restricted T cells in host defense to infectious diseases. Curr. Top. Microbiol. Immunol. **314:**215–250.
7. **Bilenki, L., S. Wang, J. Yang, Y. Fan, A. G. Joyee, and X. Yang.** 2005. NK T-cell activation promotes *Chlamydia trachomatis* infection in vivo. J. Immunol. **175:**3197–3206.
8. **Blumberg, R. S., C. Terhorst, P. Bleicher, F. V. McDermott, C. H. Allan, S. B. Landau, J. S. Trier, and S. P. Balk.** 1991. Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. J. Immunol. **147:**2518–2524.
9. **Bonish, B., D. Jullien, Y. Dutronc, B. B. Huang, R. Modlin, F. M. Spada, S. A. Porcelli, and B. J. Nickoloff.** 2000. Overexpression of CD1d by keratinocytes in psoriasis and CD1d-dependent IFN-γ production by NK-T cells. J. Immunol. **165:**4076–4085.
10. **Brigl, M., and M. B. Brenner.** 2004. CD1: antigen presentation and T-cell function. Annu. Rev. **22:**817–890.
11. **Brigl, M., L. Bry, S. C. Kent, J. E. Gumperz, and M. B. Brenner.** 2003. Mechanism of CD1d-restricted natural killer T-cell activation during microbial infection. Nat. Immunol. **4:**1230–1237.
12. **Canchis, P. W., A. K. Bhan, S. B. Landau, L. Yang, S. P. Balk, and R. S. Blumberg.** 1993. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. Immunology **80:**561–565.
13. **Cho, S., K. S. Knox, L. M. Kohli, J. J. He, M. A. Exley, S. B. Wilson, and R. R. Brutkiewicz.** 2005. Impaired cell surface expression of human CD1d by the formation of an HIV-1 Nef/CD1d complex. Virology **337:**242–252.
14. **Colgan, S. P., R. M. Hershberg, G. T. Furuta, and R. S. Blumberg.** 1999. Ligation of intestinal epithelial CD1d induces bioactive IL-10: critical role of the cytoplasmic tail in autocrine signaling. Proc. Natl. Acad. Sci. U. S. A. **96:**13938–13943.
15. **Conrad, M., V. J. Bubb, and R. Schlegel.** 1993. The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-kilodalton pore-forming protein. J. Virol. **67:**6170–6178.
16. **Fehrmann, F., and L. A. Laimins.** 2003. Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation. Oncogene **22:** 5201–5207.
17. **Fichorova, R. N., A. O. Cronin, E. Lien, D. J. Anderson, and R. R. Ingalls.** 2002. Response to *Neisseria gonorrhoeae* by cervicovaginal epithelial cells occurs in the absence of Toll-like receptor 4-mediated signaling. J. Immunol. **168:**2424–2432.
18. **Fichorova, R. N., and D. J. Anderson.** 1999. Differential expression of immunobiological mediators by immortalized human cervical and vaginal epithelial cells. Biol. Reprod. **60:**508–514.
19. **Frazer, I. H.** 2009. Interaction of human papillomaviruses with the host immune system: a well evolved relationship. Virology **384:**410–414.
20. **Gravitt, P. E., C. L. Peyton, T. Q. Alessi, C. M. Wheeler, F. Coutlee, A. Hildesheim, M. H. Schiffman, D. R. Scott, and R. J. Apple.** 2000. Improved amplification of genital human papillomaviruses. J. Clin. Microbiol. **38:**357–361.
21. **Gruener, M., I. G. Bravo, F. Momburg, A. Alonso, and P. Tomakidi.** 2007. The E5 protein of the human papillomavirus type 16 downregulates HLA-I surface expression in calnexin-expressing but not in calnexin-deficient cells. Virol. J. **4:**116.
22. **Gumperz, J. E., C. Roy, A. Makowska, D. Lum, M. Sugita, T. Podrebarac, Y. Koezuka, S. A. Porcelli, S. Cardell, M. B. Brenner, and S. M. Behar.** 2000. Murine CD1d-restricted T-cell recognition of cellular lipids. Immunity **12:** 211–221.
23. **Ho, G. Y. F., R. Bierman, L. Beardsley, C. J. Chang, and R. D. Burk.** 1998. Natural history of cervicovaginal papillomavirus infection in young women. N. Engl. J. Med. **338:**423–428.
24. **Kang, S. J., and P. Cresswell.** 2002. Calnexin, calreticulin, and ERp57 cooperate in disulfide bond formation in human CD1d heavy chain. J. Biol. Chem. **277:**44838–44844.
25. **Kawana, K., J. Matsumoto, S. Miura, L. Shen, Y. Kawana, T. Nagamatsu, T. Yasugi, T. Fujii, H. Yang, A. J. Quayle, Y. Taketani, and D. J. Schust.** 2008. Expression of CD1d and ligand-induced cytokine production are tissue specific in mucosal epithelia of the human lower reproductive tract. Infect. Immun. **76:**3011–3018.
26. **Kawana, K., A. J. Quayle, M. Ficarra, J. A. Ibana, L. Shen, Y. Kawana, H. Yang, L. Marrero, S. Yavagal, S. J. Greene, Y. X. Zhang, R. B. Pyles, R. S. Blumberg, and D. J. Schust.** 2007. CD1d degradation in *Chlamydia trachomatis*-infected epithelial cells is the result of both cellular and chlamydial proteasomal activity. J. Biol. Chem. **282:**7368–7375.
27. **Kim, H. S., J. Garcia, M. Exley, K. W. Johnson, S. P. Balk, and R. S. Blumberg.** 1999. Biochemical characterization of CD1d expression in the absence of β2-microglobulin. J. Biol. Chem. **274:**9289–9295.
28. **Marchetti, B., G. H. Ashrafi, E. Tsirimonaki, P. M. O'Brien, and M. S. Campo.** 2002. The bovine papillomavirus oncoprotein E5 retains MHC class I molecules in the Golgi apparatus and prevents their transport to the cell surface. Oncogene **21:**7808–7816.
29. **Mattner, J., K. L. Debord, N. Ismail, R. D. Goff, C. Cantu III, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, K. Hoebe, O. Schneewind, D. Walker, B. Beutler, L. Teyton, P. B. Savage, and A. Bendelac.** 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature **434:**525–529.
30. **O'Brien, P. M., and M. Saveria Campo.** 2002. Evasion of host immunity directed by papillomavirus-encoded proteins. Virus Res. **88:**103–117.
31. **Sanchez, D. J., J. E. Gumperz, and D. Ganem.** 2005. Regulation of CD1d expression and function by a herpesvirus infection. J. Clin. Invest. **115:**1369–1378.
32. **Schapiro, F., J. Sparkowski, A. Adduci, F. Suprynowicz, R. Schlegel, and S. Grinstein.** 2000. Golgi alkalinization by the papillomavirus E5 oncoprotein. J. Cell Biol. **148:**305–315.
33. **Shiraki, Y., Y. Ishibashi, M. Hiruma, A. Nishikawa, and S. Ikeda.** 2006. Cytokine secretion profiles of human keratinocytes during *Trichophyton tonsurans* and *Arthroderma benhamiae* infections. J. Med. Microbiol. **55:**1175–1185.
34. **Skold, M., X. Xiong, P. A. Illarionov, G. S. Besra, and S. M. Behar.** 2005. Interplay of cytokines and microbial signals in regulation of CD1d expression and NKT cell activation. J. Immunol. **175:**3584–3593.
35. **Skold, M., and S. M. Behar.** 2003. Role of CD1d-restricted NKT cells in microbial immunity. Infect. Immun. **71:**5447–5455.
36. **Stanic, A. K., J. S. Bezbradica, J. J. Park, L. Van Kaer, M. R. Boothby, and S. Joyce.** 2004. Cutting edge: the ontogeny and function of Va14Ja18 natural T lymphocytes require signal processing by protein kinase Cθ and NF-κB. J. Immunol. **172:**4667–4671.
37. **Stoler, M. H., C. R. Rhodes, A. Whitbeck, S. M. Wolinsky, L. T. Chow, and T. R. Broker.** 1992. Human papillomavirus type 16 and 18 gene expression in cervical neoplasias. Hum. Pathol. **23:**117–128.
38. **Straight, S. W., B. Herman, and D. J. McCance.** 1995. The E5 oncoprotein of human papillomavirus type 16 inhibits the acidification of endosomes in human keratinocytes. J. Virol. **69:**3185–3192.
39. **Taniguchi, M., and T. Nakayama.** 2000. Recognition and function of V[alpha]14 NKT cells. Semin. Immunol. **12:**543–550.
40. **Trinchieri, G.** 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat. Rev. Immunol. **3:**133–146.
41. **Tsirimonaki, E., R. Ullah, B. Marchetti, G. H. Ashrafi, L. McGarry, B. Ozanne, and M. S. Campo.** 2006. Similarities and differences between the E5 oncoproteins of bovine papillomaviruses type 1 and type 4: cytoskeleton, motility and invasiveness in E5-transformed bovine and mouse cells. Virus Res. **115:**158–168.
42. **Yuan, W., A. Dasgupta, and P. Cresswell.** 2006. Herpes simplex virus evades natural killer T-cell recognition by suppressing CD1d recycling. Nat. Immunol. **7:**835–842.
43. **Yue, S. C., A. Shaulov, R. Wang, S. P. Balk, and M. A. Exley.** 2005. CD1d ligation on human monocytes directly signals rapid NF-κB activation and production of bioactive IL-12. Proc. Natl. Acad. Sci. U. S. A. **102:**11811–11816.
44. **zur Hausen, H.** 2002. Papillomaviruses and cancer: from basic studies to clinical application. Nat. Rev. Cancer **2:**342–350.

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

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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 ≥ 60 years ($P = 0.035$).

Conclusions: Older patients (age ≥ 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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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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($n = 3$) could not be clearly determined. For the para-aortic lymph nodes, 24 patients were positive and 86 patients were negative. There were 36 patients (32%) in stage I, 15 patients (14%) in stage II, and 54 patients (50%) in stage III according to the FIGO staging. Five patients (4%) could not be staged because of insufficient FIGO staging information. Endometrioid adenocarcinoma, with 84 patients (76%), was the most frequent histological type encountered. Six other types were detected with considerably fewer frequencies. The histological types for two patients were not described in their medical records.

Among the cases not receiving postoperative chemotherapy, just the whole pelvis was irradiated in 86 cases (78%) and the para-aortic lymph node area plus the whole pelvis were irradiated in 24 cases (22%). As for the cases with postoperative chemotherapy, the CAP regimen was used in 32 cases (29%) and the TC regimen in 14 cases (13%).

To account for the high risk of local recurrence after surgery, a strong primary possibility was deep invasion in over 50% in 80 patients (82%) (Table 1). Secondarily, there were positive lymph node metastases in 36 patients (34%) (Table 1). Other reasons were ovarian invasion in five patients and cervical invasion in one patient. PORT was performed even for four patients with FIGO stage IA or IB with high risk of local recurrence. The reasons were histological FIGO grade 3 in three patients, and adenosquamous carcinoma in one patient. Although the intermediate low-risk group was not a target of PORT, two patients with intermediate low risk were given PORT because the residual lesions were strongly doubted by the surgeon in spite of negative histopathology.

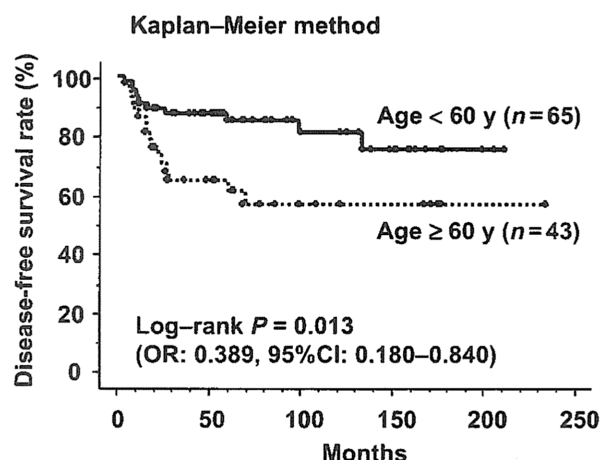

Figure 1 Disease-free survival curves by age (less than 60 years old vs 60 years or older). CI, confidence interval; OR, odds ratio.

Survival

The median follow-up time for all patients was 59.2 months (range; 6.5–235.2 months). The number of survivors at the end of the observation period was 93 (84%), and the number of disease-free survivors was 81 (73%). The OS rate at 5 years was 84%, and the DFS rate at 5 years was 77% for all patients. Both OS and DFS reached a plateau at approximately 3 years. Patients younger than 60 years had significantly better OS (log–rank $P = 0.044$, odds ratio [OR] = 0.383, and 95% confidence interval [CI] = 0.145–1.008) and DFS (log–rank $P = 0.013$, OR = 0.389, and 95%CI = 0.180–0.840) than patients 60 years or older (Fig. 1). Moreover, when limited to the histological type of endometrioid adenocarcinoma ($n = 84$), there was a significant difference in DFS between patients 60 years or older, and those under 60 years old (log–rank $P = 0.004$, OR = 0.289, and 95%CI = 0.119–0.703). However, when the age was raised to 65 years, the earlier significant difference in DFS shown for those younger than 60 years vanished ($P = 0.067$). When the age was raised to 70 years, there was a significant difference in DFS ($P = 0.0030$, OR = 0.274, and 95%CI = 0.109–0.685). The numbers of patients aged ≥65 and 70 years old were 25 (23%) and 10 (9%), respectively.

Certain prognostic factors (as determined by univariate analysis, Table 1) for both OS and DFS were examined. Pathological stage, with or without lymph node metastasis, and with or without chemotherapy, and age were studied. The 5-year OS and DFS rates were 93% and 87% in FIGO pathological stage I cases, 85% and 79% in stage II cases, and 83% and 74% in stage III cases, respectively. A significantly higher DFS rate in stage I ($n = 36$) emerged when compared with stages II–III ($n = 69$) (log–rank $P = 0.040$). Significant differences for DFS were not shown for those with and without lymph node metastasis (log–rank $P = 0.15$) and with and without postoperative chemotherapy (log–rank $P = 0.13$). However, for those with and without para-aortic lymph node metastasis the difference was significant (log–rank $P = 0.0006$, OR = 0.285, 95%CI = 0.133–0.612) (Table 1).

On multivariate analysis (Table 2), poor DFS correlated only with age ≥ 60 years ($P = 0.035$).

The distribution of variables according to age (age <60 vs age ≥60) is summarized in Table 3. There were significantly more cases of endometrioid

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Table 2 Multivariate analysis of disease-free survival

Factor	P-value	OR	95%CI
Age			
<60 y	0.035	0.427	0.193–0.944
≥60 y			
PALN			
(–)	0.067	0.451	0.192–1.058
(+)			
FIGO stage			
I	0.18	0.484	0.167–1.405
II–III			

CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; OR, odds ratio; PALN, para-aortic lymph node.

adenocarcinoma on histological type ($P = 0.019$) and fewer high-risk cases ($P = 0.017$) in patients ≥60 years old than in patients <60 years old.

Complications

According to the CTCAE v3.0, lower limb edema, intestinal obstruction, and diarrhea were adverse events of grade 3 or more. Regarding complications of grade 3 or more, lower limb edema was seen in 16 patients (14%), intestinal obstruction was seen in nine patients (8%), and diarrhea was seen in three patients (3%). In this study, lower limb edema was recorded in 15 patients with grade 1 complications and 13 patients with grade 2 complications. There was no grade 3 or 4 myelosuppression in any of the cases.

Discussion

In this retrospective study of endometrial cancer in our institution, prognostic factors were evaluated in endometrial cancer patients and were focused particularly on the effect of advanced age on the outcome of surgery and PORT. The limitations of our study included the retrospective nature of the study and the heterogeneity of the patient population in the two arms (<60 years vs ≥60 years).

In a prospective randomized trial of postoperative radiation therapy in endometrial carcinoma (PORTEC) for stage I disease, Creutzberg *et al.*[13] reported that patient age ≥60 years was an independent predictor of death from endometrial carcinoma (hazard ratio of 3.1 and 95%CI , 1.2–8; $P = 0.02$). The data in the literature also suggest that there is an incremental increase in the risk of dying from endometrial carcinoma with increasing age. In a review of 819 patients with stage I–II endometrial carcinoma from the Gynecologic Oncology Group database, Zaino *et al.* demonstrated that the relative risk (RR) increased from 1.0 for patients who were aged ≤45 years (reference) at the time of diagnosis to 2.0 for patients aged 55 years, to 3.4 for patients aged 65 years, and to 4.7 for patients aged ≥75 years.[2] According to Alektiar *et al.*,[12] patient age ≥70 years was found to be an independent predictor of poor locoregional control (RR: 3 and 95%CI , 1–10; $P = 0.019$), DFS (RR: 2 and 95%CI , 1–13; $P = 0.03$), and OS (RR: 4 and 95%CI , 2–7; $P = 0.001$). Jolly *et al.*[14] concluded from a retrospective study that older endometrial cancer (age >63 years) patients had a significantly decreased OS, cause-specific survival, and greater risk of recurrence following PORT that were independent of other prognostic factors and/or treatment technique. According to Lee *et al.*,[15] their study ($n = 51\,471$) of a large population of uterine cancer patients demonstrated that those 40 years or younger have an OS advantage compared with women older than 40 years, independent of other clinicopathological prognosticators. Farley *et al.*[16] concluded that age (older than 50) is a specific and significant predictor of outcome in endometrioid adenocarcinoma of the uterus ($n = 328$). The frequent association between older age in endometrial carcinoma patients on the one hand and deep myometrial invasion and aggressive histologies always raises the possibility that the poor outcome in older patients is entirely the result of such an association. Why older patients with early-stage endometrial carcinoma tend to fare worse independent of other factors is not clear. Nevertheless, clinical efforts should be directed toward maximizing the therapeutic ratio in those patients. The notion of limited life expectancy should not hinder that effort because survival to the age of 80 years and beyond has been reported to have increased in many developed countries.[17] The remaining life expectancy of a white US woman aged 75 years is estimated to be 11.7 years.[18]

The treatment methods were changed for postoperative adjuvant therapy in our institution due to a pathological result after operation. Seeing the treatment outcome, the 5-year DFS rate of each FIGO stage was 82% in stage I, 79% in stage II, and 74% in stage III. These outcomes are comparable to other institutions. In the gynecology tumor committee report of 1993 in Japan,[19] the OS rate for five years was 84.0% in stage I, 73.5% in stage II, and 54.8% in stage III. As for our outcome results, only those cases receiving PORT in our department were evaluated, and it is likely that the results would show further improvement for stage I if the patients in the low-risk group were included. There

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Table 3 Distribution of variables according to age

Variables	<60 years	(*n* = 67)	≥60 years	(*n* = 43)	*P*-value (×2)
PLN					
(+)	21	(41%)	15	(27%)	0.96
(−)	30	(59%)	41	(73%)	
PALN					
(+)	15	(23%)	9	(20%)	0.67
(−)	50	(77%)	36	(80%)	
FIGO stage					
I	20	(33%)	16	(36%)	0.65
II–III	41	(67%)	28	(64%)	
Histological type					
EA	43	(69%)	41	(89%)	0.019
not EA	19	(31%)	5	(11%)	
FIGO grade					
1	25	(50%)	16	(47%)	0.81
2	14	(28%)	11	(32%)	
3	11	(22%)	7	(21%)	
Risk group					
High	51	(80%)	25	(57%)	0.017
Intermediate–high	12	(19%)	18	(41%)	
Intermediate–low	1	(1%)	1	(2%)	
Chemotherapy					
With	30	(49%)	16	(37%)	0.21
Without	31	(51%)	27	(63%)	
Depth					
a–b†	10	(19%)	8	(18%)	0.27
c–d‡	44	(81%)	36	(82%)	

†<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.

were only 15 examples for stage II, and the number of cases might not be sufficient to analyze treatment results. On the other hand, there were 55 examples of stage III, which constitutes an excellent OS rate. A phase III randomized trial showed improved survival with the use of chemotherapy for stage III and IV endometrial cancer.[20,21] However, pelvic and abdominal failure rates were alarmingly high, which appears to be persuasive for the integration of radiation and chemotherapy as performed in our institution.

According to our multivariate analysis of DFS, being a senior citizen is in itself an independent risk factor. More intensive treatment may be necessary for senior citizens than for young people. A total dose of approximately 50 Gy in PORT has already been prescribed, and because any further dose increase is difficult, the inclusion of postoperative chemotherapy can be expected. According to a recent Japanese Gynecologic Oncology Group study,[22] adjuvant CAP chemotherapy may be a useful alternative to PORT for intermediate-risk endometrial cancer. Moreover, adjuvant vaginal high-dose-rate brachytherapy alone may be a safe and effective alternative to pelvic external beam PORT for surgical early stage endometrial cancer.[23,24]

References

1. Kosary CL. FIGO stage, histology, histologic grade, age and race as prognostic factors in determining survival for cancers of the female gynecological system: An analysis of 1973–1987 SEER cases of cancers of the endometrium, cervix, ovary, vulva, and vagina. *Semin Surg Oncol* 1994; **10**: 31–46.
2. Zaino RJ, Kurman RJ, Diana KL, Morrow CP. Pathologic models to predict outcome for women with endometrioid adenocarcinoma: The importance of the distinction between surgical stage and clinical stage – a Gynecologic Oncology Group study. *Cancer* 1996; **77**: 1115–1121.
3. Abeler VM, Kjørstad KE. Endometrial adenocarcinoma in Norway. A study of a total population. *Cancer* 1991; **67**: 3093–3103.
4. Irwin C, Levin W, Fyles A, Pintilie M, Manchul L, Kirkbride P. The role of adjuvant radiotherapy in carcinoma of the endometrium: results in 550 patients with pathologic stage I disease. *Gynecol Oncol* 1998; **70**: 247–254.

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5. Poulsen MG, Roberts SJ. Prognostic variables in endometrial carcinoma. *Int J Radiat Oncol Biol Phys* 1987; **13**: 1043–1052.
6. Mundt AJ, Waggoner S, Yamada D, Rotmensch J, Connell PP. Age as a prognostic factor for recurrence in patients with endometrial carcinoma. *Gynecol Oncol* 2000; **79**: 79–85.
7. Grigsby PW, Perez CA, Kuten A *et al*. Clinical stage I endometrial cancer: Prognostic factors for local control and distant metastasis and implications of the new FIGO surgical staging system. *Int J Radiat Oncol Biol Phys* 1992; **22**: 905–911.
8. Lindahl B, Ranstam J, Willén R. Five year survival rate in endometrial carcinoma stages I-II: Influence of degree of tumour differentiation, age, myometrial invasion and DNA content. *Br J Obstet Gynaecol* 1994; **101**: 621–625.
9. Alektiar KM, McKee A, Venkatraman E *et al*. Intravaginal high-dose-rate brachytherapy for Stage IB (FIGO Grade 1, 2) endometrial cancer. *Int J Radiat Oncol Biol Phys* 2002; **53**: 707–713.
10. Samet J, Hunt WC, Key C, Humble CG, Goodwin JS. Choice of cancer therapy varies with age of patient. *JAMA* 1986; **255**: 3385–3390.
11. Schrag D, Cramer LD, Bach PB, Begg CB. Age and adjuvant chemotherapy use after surgery for stage III colon cancer. *J Natl Cancer Inst* 2001; **93**: 850–857.
12. Alektiar KM, Venkatraman E, Abu-Rustum N, Barakat RR. Is endometrial carcinoma intrinsically more aggressive in elderly patients? *Cancer* 2003; **98**: 2368–2377.
13. Creutzberg CL, Van Putten WL, Koper PC *et al*. Surgery and postoperative radiotherapy versus surgery alone for patients with stage-1 endometrial carcinoma: Multicentre randomized trial. PORTEC Study Group. Post Operative Radiation Therapy in Endometrial Carcinoma. *Lancet* 2000; **355**: 1404–1411.
14. Jolly S, Vargas CE, Kumar T *et al*. The impact of age on long-term outcome in patients with endometrial cancer treated with postoperative radiation. *Gynecol Oncol* 2006; **103**: 87–93.
15. Lee NK, Cheung MK, Shin JY *et al*. Prognostic factors for uterine cancer in reproductive-aged women. *Obstet Gynecol* 2007; **109**: 655–662.
16. Farley JH, Nycum LR, Birrer MJ, Park RC, Taylor RR. Age-specific survival of women with endometrioid adenocarcinoma of the uterus. *Gynecol Oncol* 2000; **79**: 86–89.
17. Manton KG, Vaupel JW. Survival after the age of 80 in the United States, Sweden, France, England, and Japan. *N Engl J Med* 1995; **333**: 1232–1235.
18. Taeuber CM. Sixty-five plus in America. Current Population Reports. Special Studies. Washington, DC: US Department of Commerce, Economics and Statistics Administration, Bureau of the Census, 1996; publication no. P23–173RV.
19. The Cancer Committee of the Japan Society of Gynecology. [The 41st treatment annual report. 5-year result of treatment started in 1993 for cervical and endometrial carcinoma.] *J Jpn Soc Obstet Gynecol* 2008; **60**: 1876–1901. (In Japanese.)
20. Randall ME, Brunetto VL, Muss H. Whole abdominal radiotherapy versus combination doxorubicin-cisplatin chemotherapy in advanced endometrial carcinoma: A randomized phase III trial of the Gynecologic Oncology Group. *Proc Am Soc Clin Oncol* 2003; **22**: 2.
21. Randall ME, Spirtos NM, Dvoretsky P. Whole abdominal radiotherapy versus combination chemotherapy with doxorubicin and cisplatin in advanced endometrial carcinoma (phase III): Gynecologic Oncology Group Study no. 122. *J Natl Cancer Inst Monogr* 1995; **19**: 13–15.
22. Susumu N, Sagae S, Udagawa Y *et al*. Japanese Gynecologic Oncology Group.Randomized phase III trial of pelvic radiotherapy versus cisplatin-based combined chemotherapy in patients with intermediate- and high-risk endometrial cancer: A Japanese Gynecologic Oncology Group study. *Gynecol Oncol* 2008; **108**: 226–233.
23. Jolly S, Vargas C, Kumar T *et al*. Vaginal brachytherapy alone: An alternative to adjuvant whole pelvis radiation for early stage endometrial cancer. *Gynecol Oncol* 2005; **97**: 887–892.
24. Solhjem MC, Petersen IA, Haddock MG. Vaginal brachytherapy alone is sufficient adjuvant treatment of surgical stage I endometrial cancer. *Int J Radiat Oncol Biol Phys* 2005; **62**: 1379–1384.

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ORIGINAL PAPER

Aromatase inhibitor anastrozole as a second-line hormonal treatment to a recurrent low-grade endometrial stromal sarcoma: a case report

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Abstract Low-grade endometrial stromal sarcoma (ESS) is a rare neoplasm and is generally an indolent tumor with estrogen and progesterone receptors. Objective responses by hormonal treatment with progestin or aromatase inhibitor have been reported, however, long-term management of this disease could be difficult if it becomes refractory to one of these hormonal therapies. A 34-year-old woman was diagnosed with stage I low-grade ESS at the time of hysterectomy for presumed uterine fibroma. Five years later, she recurred with multiple tumors in the lower abdomen. After an optimal surgery, she was free from progression for 6 years with progestin treatment (medroxyprogesterone acetate: MPA, 200–600 mg daily). Thereafter, she recurred twice during the MPA treatment and received debulking surgery each time. MPA was discontinued at age of 53, because another recurrent tumor grew up to 13 cm in diameter. Aromatase inhibitor anastrozole was then given at a daily dose of 1 mg with partial response (the tumor size decreased to 7 cm in diameter) for a duration of 9 months. After complete resection of the recurrent tumor, she remains progression-free for 16 months. Anastrozole was effective to recurrent low-grade ESS even after being refractory to progestin therapy. Aromatase inhibitor treatment may be a useful option as a second-line hormonal treatment to low-grade ESS.

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Keywords Low-grade endometrial stromal sarcoma · Uterine corpus · Recurrence · Aromatase inhibitor · Progestin therapy · Hormonal treatment

Introduction

Endometrial stromal sarcoma (ESS) is a rare neoplasm, accounting for 0.2% or less of gynecologic malignancies [1]. Low-grade ESS usually expresses estrogen receptors (ER) and progesterone receptors (PR), and estrogen acts as a growth stimulus [2, 3]. Objective responses have been obtained with progestin therapy, such as megestrol acetate and medroxyprogesterone acetate (MPA) [4, 5]. More recently, the efficacy of a non-steroid aromatase inhibitor has been also reported [6, 7], as it inhibits estrogen synthesis. Although either type of hormonal therapy might be useful as a first-line therapy, it is still uncertain whether a second-line hormonal treatment is effective to repetitively recurrent ESS with resistance to a first-line therapy.

We report a case of recurrent low-grade ESS with long-term survival, treated with MPA for 13 years as a first line and aromatase inhibitor anastrozole for 9 months as a second-line hormonal therapy.

Case report

A 34-year-old woman (gravida 4, para 2) underwent a total abdominal hysterectomy for presumed uterine fibroma at her local hospital in 1988. The histopathological result revealed stage I low-grade ESS of the corpus uteri. In December 1993, she was referred to our hospital, and a computed tomography (CT) scan revealed a 9-cm pelvic mass, bilateral ovarian masses (4 cm on the left and 7 cm

on the right), and para-aortic lymph node enlargement. She underwent secondary debulking surgery, including bilateral salpingo-oophorectomy, omentectomy, bowel resection, and biopsy of para-aortic lymph nodes. All the residual tumors were less than 1 cm in diameter. The final pathology revealed recurrence of the low-grade ESS (Fig. 1a), involving the bilateral adnexae, ileum, appendix, colon, omentum, and para-aortic lymph nodes. Immunohistochemical analysis showed a strong nuclear staining for both ER and PR (Fig. 1b, c), as well as CD10 (Fig. 1d) and vimentin, and a negative staining for HHF35, 1A4, Desmin, and CD34. Postoperatively, she was started on MPA at a daily dose of 600 mg. Three years after the MPA therapy, complete response was pathologically confirmed by second look laparoscopy. MPA was continued at a daily dose of 200–400 mg without any appreciable adverse effects.

In April 2000, surgical biopsy of a 2-cm mass around the liver confirmed the recurrence of the disease on peritoneum. Two years later, she received another debulking procedure with partial liver resection for a 5-cm tumor and resection of another 5-cm pelvic tumor. After the surgery, she was hospitalized four times within 2 years due to grade 2 ileus. In June 2006, a CT scan showed a 5-cm solid mass in the left upper quadrant. The patient did not choose a debulking surgery and was kept treated with MPA at a daily dose of 200–400 mg. Eight months later, she was found to have progression of disease, represented by enlargement of the mass up to 13 cm in diameter and appearance of 4 cm mesenteric mass in the pelvis (Fig. 2a). Then, MPA treatment was discontinued, and anastrozole at a daily dose of 1 mg was started with an informed consent. After 9 months of the treatment, the tumor in the left upper quadrant was decreased to 7 cm in diameter and the mesenteric tumor was undetected (Fig. 2b). Anastrozole was discontinued because of arthritis with grade 2 joint-function disorder. Then, she underwent complete resection of the recurrent tumor. Pathological findings also revealed the significant effect of anastrozole. As shown in Fig. 3, the majority of the tumor cells was necrotic and replaced by numerous foamy histiocytes. The viable cells remained partly in the marginal lesion with expression of ER and PR. She recovered from the joint-function disorder shortly after the surgery and remains asymptomatic and progression-free for 16 months.

Discussion

ESS is subdivided histopathologically into low-grade and undifferentiated (or high-grade) forms depending on the morphology, number of mitoses, cellularity, and necrosis. The primary treatment for low-grade ESS is mainly surgery, including an abdominal hysterectomy with bilateral salpingo-oophorectomy. Adjuvant treatment, such as radiotherapy or chemotherapy, is not routinely recommended

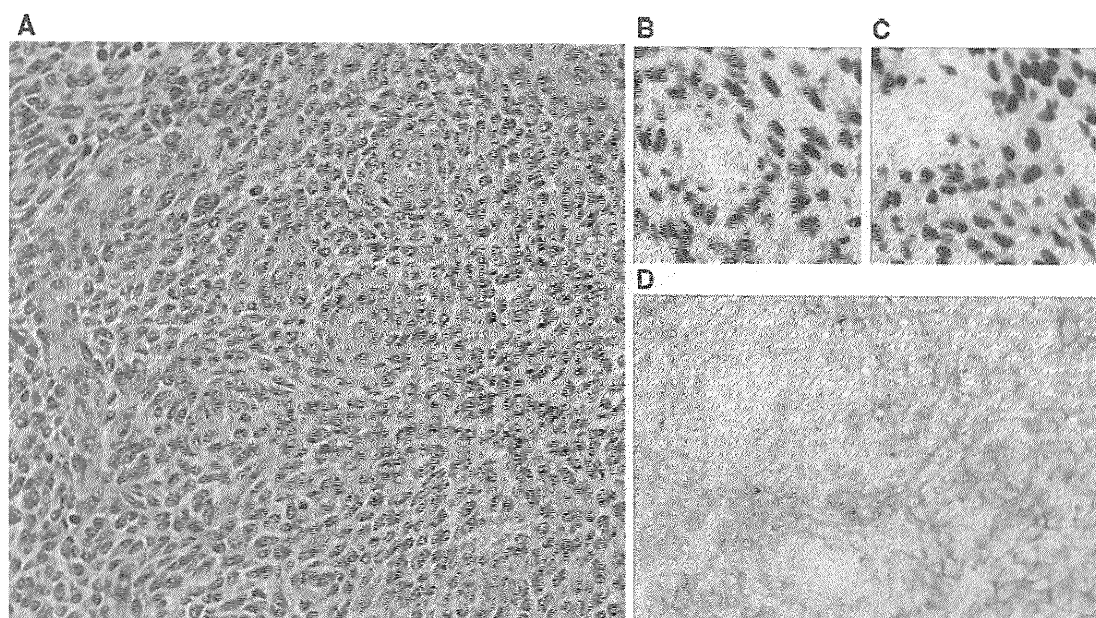

Fig. 1 Histological findings of the tumor, excised before MPA treatment. **a** High Power: Tumor cells in the pelvis, showing proliferation of endometrial stromal cells without significant atypia or pleomorphism, diagnosed as low-grade ESS. **b–d** High Power: Tumor cells are strongly positive for estrogen receptor (**b**) and progesterone receptor (**c**) and are diffusely positive for CD10 (**d**) by immunohistochemistry

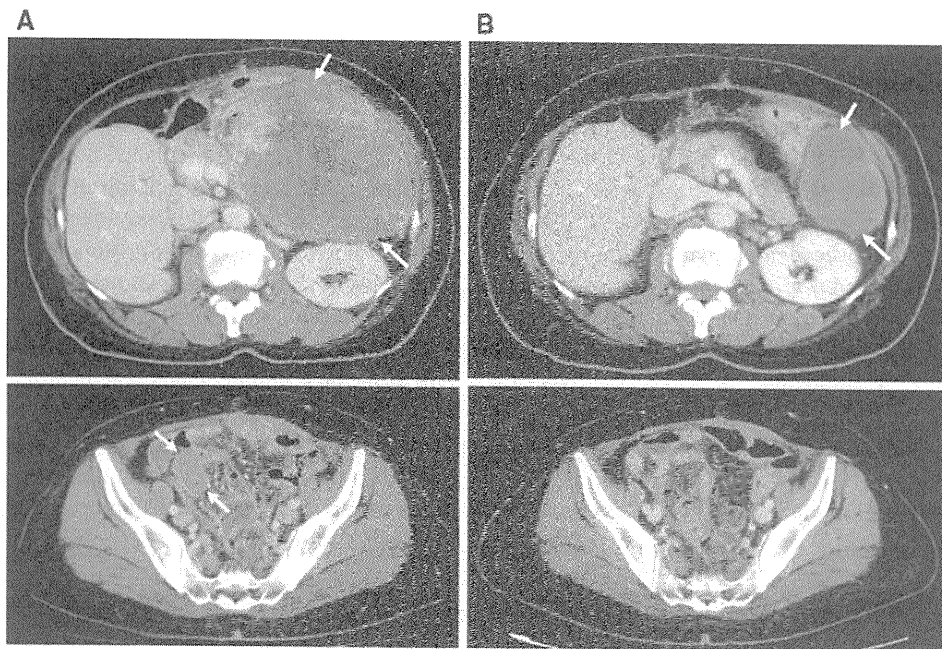

Fig. 2 Images of CT scan before and after anastrozole treatment. **a** Recurrent tumors with 13 cm in diameter in the left upper quadrant (*Upper*) and 4 cm in diameter in the pelvis (*Lower*). **b** The recurrent tumors were diminished to 7 cm in diameter (*Upper*) or became undetectable (*Lower*)

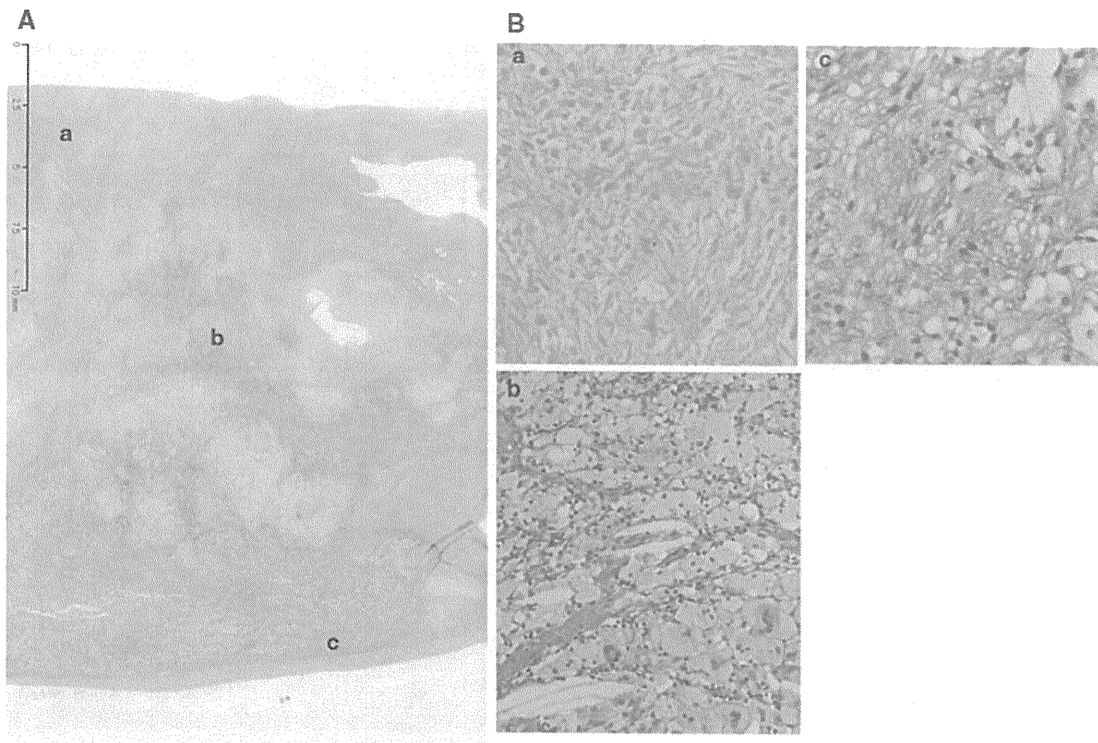

Fig. 3 Histological findings of the tumor, excised after anastrozole treatment. **a**. Low Power: Tumor cells with massive necrosis. **b** High Power: (*a*) Lesion with coagulative tumor cell necrosis, which occupies the majority of the tumor. (*b*) Center lesion with numerous foamy histiocytes. (*c*) Marginal lesion of the tumor with viable cells partly remaining

[8]. Although the prognosis of low-grade ESS is generally favorable with more than 90% of 5-year overall survival, the recurrence-free survival rate is reported to be about 50% [9, 10]. In addition to surgical resection, treatment option to recurrent low-grade ESS is hormonal therapy with progesterone derivative or aromatase inhibitor. MPA and megestrole acetate are synthetic derivatives of progesterone that exert an anti-estrogenic effect after binding to PR. The sensitivity to these progestin therapies is associated with the presence of ER and PR [11]. Aromatase inhibitors reduce estrogen levels by inhibiting its synthesis in peripheral sites. The distinct function suggests that suppressing aromatase might be still effective to recurrent ESS with resistance to progestin therapy.

The patient reported here suffered from repeated recurrences after becoming refractory to MPA treatment. Positive PR expression of the recurrent tumors suggests that the resistance to MPA therapy is caused by PR-independent manner. As a second-line hormonal therapy, anastrozole showed significant response to these recurrent tumors, suggesting that aromatase inhibitor might be useful for progestin-resistant low-grade ESS tumors. It is to be elucidated whether aromatase inhibitor is also effective to recurrent ESS tumors with negative PR expression.

Acknowledgments Conflict of interest statement All the authors declare no conflict of interest.

References

1. Koss LG, Spiro RH, Brunschwig A. Endometrial stromal sarcoma. Surg Gynecol Obstet. 1965;121:531–7.
2. Sabini G, Chumas JC, Mann WJ. Steroid hormone receptors in endometrial stromal sarcomas. A biochemical and immunohistochemical study. Am J Clin Pathol. 1992;97:381–6.
3. Wade K, Quinn MA, Hammond I, Williams K, Cauchi M. Uterine sarcoma: steroid receptors and response to hormonal therapy. Gynecol Oncol. 1990;39:364–7.
4. Gloor E, Schnyder P, Cikes M, Hofstetter J, Cordey R, Burnier F, et al. Endolymphatic stromal myosis. Surgical and hormonal treatment of extensive abdominal recurrence 20 years after hysterectomy. Cancer. 1982;50:1888–93.
5. Tsukamoto N, Kamura T, Matsukuma K, Imachi M, Uchino H, Saito T, et al. Endolymphatic stromal myosis: a case with positive estrogen and progesterone receptors and good response to progestins. Gynecol Oncol. 1985;20:120–8.
6. Maluf FC, Sabbatini P, Schwartz L, Xia J, Aghajanian C. Endometrial stromal sarcoma: objective response to letrozole. Gynecol Oncol. 2001;82:384–8.
7. Leunen M, Breugelmans M, De Sutter P, Bourgain C, Amy JJ. Low-grade endometrial stromal sarcoma treated with the aromatase inhibitor letrozole. Gynecol Oncol. 2004;95:769–71.
8. Reich O, Regauer S. Hormonal therapy of endometrial stromal sarcoma. Curr Opin Oncol. 2007;19:347–52.
9. Pink D, Lindner T, Mrozek A, Kretzschmar A, Thuss Patience PC, Dorken B, et al. Harm or benefit of hormonal treatment in metastatic low-grade endometrial stromal sarcoma: single center experience with 10 cases and review of the literature. Gynecol Oncol. 2006;101:464–9.
10. Kim WY, Lee JW, Choi CH, Kang H, Kim TJ, Kim BG, et al. Low-grade endometrial stromal sarcoma: a single center's experience with 22 cases. Int J Gynecol Cancer. 2008;18:1084–9.
11. Katz L, Merino MJ, Sakamoto H, Schwartz PE. Endometrial stromal sarcoma: a clinicopathologic study of 11 cases with determination of estrogen and progestin receptor levels in three tumors. Gynecol Oncol. 1987;26:87–97.

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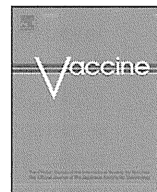

Oral immunization with a *Lactobacillus casei* vaccine expressing human papillomavirus (HPV) type 16 E7 is an effective strategy to induce mucosal cytotoxic lymphocytes against HPV16 E7

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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 $\alpha 4\beta 7^{+}$ 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].

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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

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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 × 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 × 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 $\alpha4\beta7^+$ 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 $\alpha4\beta7^+$ cells were 45% and 56% of the lymphocytes, respectively. A portion of $CD4^+$ or $CD8^+$ integrin $\alpha4\beta7^+$ 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 $\alpha4\beta7^+$ cells was presumed to include integrin $\alpha4\beta7^+$ 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

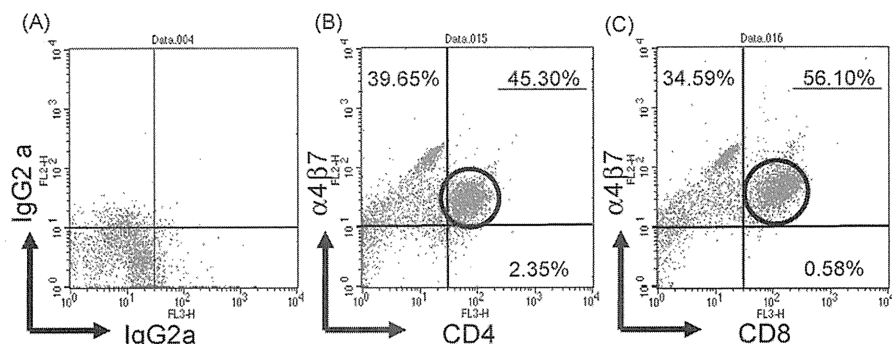

Fig. 1. Flow cytometry analysis of isolated mucosal lymphocytes. Instrument settings for separate samples were identical. The appropriate isotype profile is shown in (A). These profiles depict PE-Cy5 $CD4^+$(B) or $CD8^+$(C) cells on the *x*-axis and PE integrin $\alpha4\beta7^+$ cells on the *y*-axis. The dual-labeled $CD4^+$ or $CD8^+$/integrin $\alpha4\beta7^+$ cells cluster in the upper right quadrant (bold line).

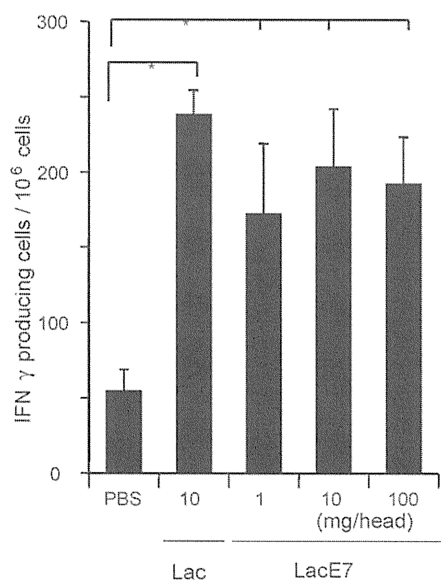

Fig. 2. To examine effect of oral immunization with a recombinant *Lactobacillus casei*-based vaccine on mucosal type1 cell immune responses, the number of non-specific IFNγ-producing cells among the mucosal lymphocytes was assessed using an ELISPOT assay after mitogen stimulation (40 ng/mL of PMA and 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 ($p < 0.05$) ($n = 5$).

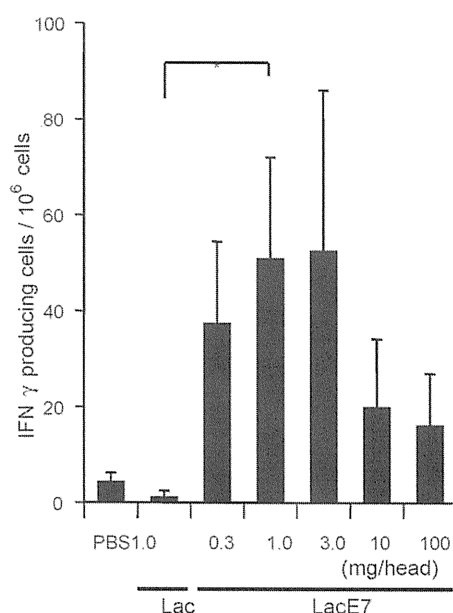

Fig. 3. Various doses of LacE7 (0.3, 1.0, 3.0, 10, and 100 mg/head) or Lac were orally administered to a group of five mice at weeks 1, 2, and 4. The same dose was used for all oral administrations to a given mouse. Integrin α4β7$^+$ intestinal mucosal lymphocytes were collected from the immunized mice 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],

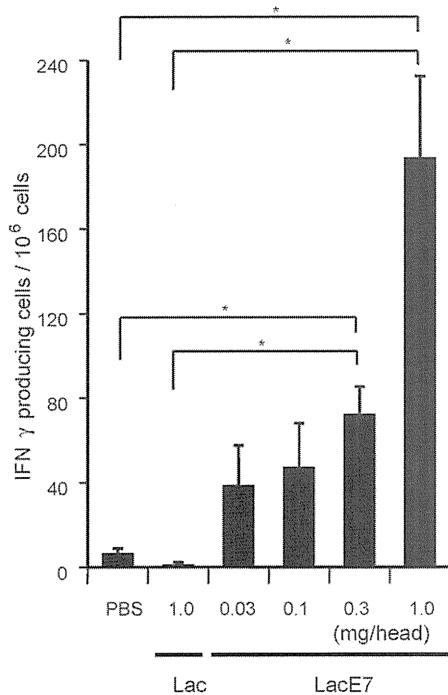

Fig. 4. Various doses of LacE7 (0.03, 0.1, 0.3, and 1.0 mg/head) or Lac were orally administered to a group of five mice at weeks 1, 2, and 4 (priming) and week 8 (boost). The same dose was used for priming and boost exposures in a given mouse. Integrin $\alpha 4\beta 7^+$ intestinal mucosal lymphocytes 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 ($p < 0.05$) ($n = 5$).

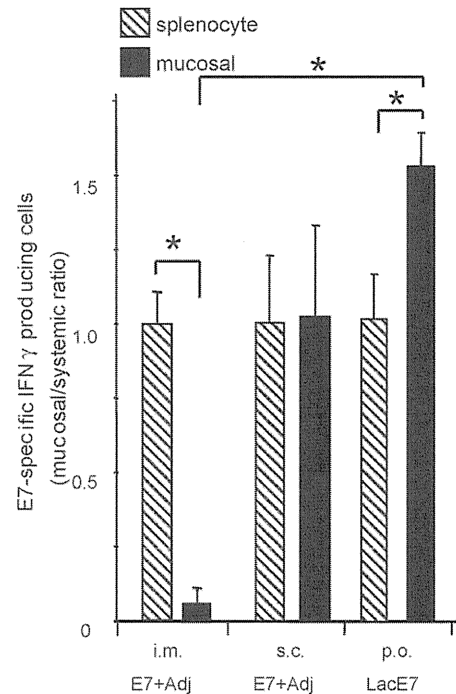

Fig. 5. Oral, intramuscular, or subcutaneous immunizations were compared for the induction of mucosal E7-specific type1 immune responses. Mice were immunized by each route at weeks 1, 2, 4 and 8. Both intestinal mucosal lymphocytes (black) and splenocytes (stripe) were collected from mice immunized by 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-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.

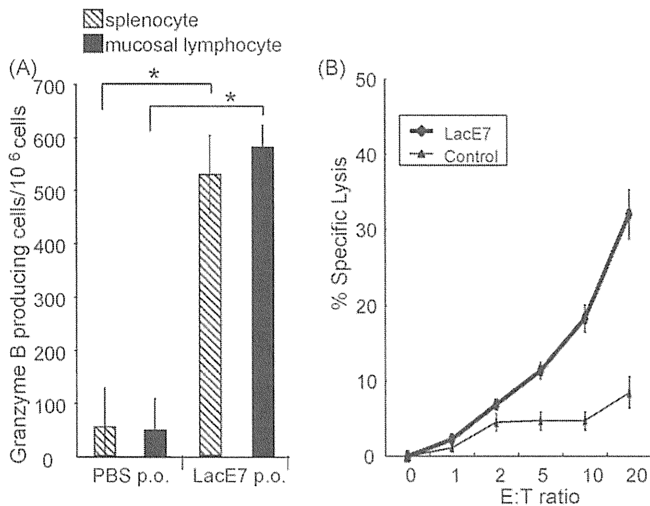

Fig. 6. T cells producing granzyme B in response to E7-CTL epitope exposure were measured using an ELISPOT assay and their killer activity toward TC-1 cells was examined. Mucosal lymphocytes and splenocytes collected from mice receiving oral immunization of LacE7 at weeks 1, 2, 4, and 8 were used for 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 $\alpha 4\beta 7$, 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 $\alpha 4\beta 7$ [38]. We have shown that integrin $\alpha 4\beta 7^+$ 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 $\alpha 4\beta 7^+$ 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 $\alpha 4\beta 7^+$ 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