Increased Response of Human T-Lymphocytes by Dendritic Cells Pulsed with HPV16E7 and Pleurotus sajor-caju-β-glucan (PBG)

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ABSTRACT

Background: Infection with human papillomavirus type 16 (HPV-16) is known to cause cervical cancer, hence the several HPV therapeutic vaccines are developed in E7 oncoproteins and targeted on cell-mediated immunity. Human dendritic cells (HuDCs) are extensively employed in HPV therapeutic vaccines as the carrier or platform for inducing adaptive immune responses. However, the immunomodulators need to be further investigated for vaccine effects. Gray oyster mushroom (Pleurotus sajor-caju) containing β-glucans is a potent immunomodulator with potential to be used in vaccines. Objective: To study the effect of Pleurotus sajor-caju-β-glucan Polysaccharides (PBG) on human T-lymphocytes by use of the HuDCs’ antigen presentation platform for HPV16 vaccine. Methods: The HPV16-E7 recombinant proteins were constructed in E. Coli. HuDCs pulsed with E7 peptide were cocultured with the T-lymphocytes treated with and without PBG. The number of T-lymphocytes(CD4; CD8) was detected by flowcytometry, and the viral response of T-lymphocytes was measured via IFN-γ release. Results: The PBG treated group of T-lymphocytes cocultured with the HuDCs pulsed by the HPV16-E7 proteins showed significantly higher numbers of T-lymphocytes and IFN-γ release compared with T-lymphocytes without PBG in vitro. Moreover, a significant improvement in the level of specific IgG neutralizing antibodies to HPV was found in a murine model. Further observed was an increase in the expansion of helper and cytotoxic T-cells and IFN-γ releases in human system. Conclusion: PBG treatment of T-lymphocytes could be a useful option for prophylactic and therapeutic vaccines in cervical cancer.
INTRODUCTION

Cervical cancer is mostly caused by chronic infections of types 16 and 18 high-risk human papillomaviruses (HPV) (1-3). The HPV gene is integrated into the genome of the host cell and the overexpression of the E6 and E7 proteins causes the deregulation of the retinoblastoma protein (pRB), a tumor suppressor, and p53 (4). Therefore, HPV vaccines targeting the HPV16-E6 and E7 proteins, have been recently generated (1,5) as a safe vaccine for human testing and/or clinical studies (1). Currently, HPV therapeutic vaccines are more powerful than prophylactic vaccines due to the fact that commercial HPV vaccines can induce only prophylactic antibodies to prevent the viral infection of cervical epithelial cells; furthermore, its high efficacy has merely been demonstrated in young women. However, women with HPV need to continuously use therapeutic vaccines to eliminate cancer (2,6,7). Consequently, therapeutic vaccines for HPV have been intensively investigated and combined with a new immunomodulator so as to improve efficacy (6). Moreover, previous studies have shown that using L1 and L2 capsid proteins can be a strategy for HPV therapeutic vaccines. Nevertheless, cytotoxic T-lymphocytes (CTL) responses in L1 and L2 (viral capsid protein) have been found to induce weak responses (8); E7 oncoprotein, on the other hand, has proved to be potent and developed for peptide-based antitumor vaccines (9).

Recently, therapeutic vaccine strategies have focused on specific T-helper type 1 (Th1) and CTL responses to HPV16-E7 (9, 10). In the researcher’s previous study, the immunotherapeutic strategy was also focused on the E7 protein due to the ability to be continuously expressed by the target epithelial cell upon viral integration and cellular transformation since the E7 protein amino acid sequences were highly conserved among the HPV genotypes (11). The E7 protein-pulsed DCs vaccine is considered as the “gold standard” for cervical cancer immune therapy and an effective strategy for targeting a specific CTL response with E7 protein pulsed-DCs (9, 12). However, the peptide vaccine has a poor immunogenicity and inefficient antigen presentation in some of them (13). Therefore, the potent immunomodulator needs intensive investigation if the efficacy of the vaccine is to be enhanced (14).

Several studies have reported that certain species of mushrooms contain immunomodulatory activities (15). An example is the grey oyster mushroom (Pleurotus sajor-caju), regularly found in tropical regions. This oyster mushroom has a high amount of fungal compounds as polysaccharides (mostly β- and some α-glucans) and has been reported as a potent immunomodulator (16) for stimulating the immune system. The immunomodulation of a mushroom polysaccharide is to enhance the expansion of the CD4+/CD8+ T-cells (15).

In this study, the researcher demonstrated that Pleurotus sajor-caju-β-glucan polysaccharides (PBG) were able to highly enhance the expansion of human T-lymphocytes in a human HPV16 vaccine model in vitro. With that in mind, the HPV16-E7 protein pulsed human dendritic cells (HuDCs) in vitro were performed as the professional antigen presentation system to induce human T-lymphocytes. This novel type of immunomodulator is conducive to develop a prophylactic, therapeutic HPV vaccine and dendritic cells vaccine formulations for human use.
MATERIALS AND METHODS

Mice. Female BALB/c mice (8 weeks of age) were delivered from the National Laboratory Animal Center, Mahidol University, Thailand. The mice were cultured under pathogen-free conditions and handled following the Animal Care and Handling guidelines of the Faculty of Medical Technology, Western University, Kanchanaburi, Thailand.

Antigens. The recombinant E7 protein of HPV-16 (Accession: EU430687.1, GI: 167996746) was inserted in the Escherichia coli (E.coli) bacterial strain BL21γDE3 (Novagen, Darmstadt, Germany), as previously described [20] and was induced to express the recombinant protein (His-Tag) by isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, MO, USA). The bacteria were lysed and purified on a Ni-NTA agarose column (QIAGEN, Hilden, Germany) and washed with imidazole (30 mM) to remove the lipopolysaccharide; they were further extracted for the recombinant protein by urea (8 M in the PBS). The purified proteins were eluted with imidazole (100 mM in 8 M of urea) and dialyzed against the PBS. The purified protein was then lyophilized for the enzyme-linked immunosorbent assay (ELISA) technique.

Pleurotus sajor-caju-β-glucan polysaccharides. The mushrooms (Pleurotus sajor-caju) were ground prior to being extracted with hot water using percolation and Soxhlet techniques in accordance with Kim et al.’s study (15). The organic solvent was evaporated, and the extraction was then freeze-dried into lyophilized powder. Subsequently, the powder extract was diluted into the PBS for concentration as 1 g/ml. The β-glucan level in the mushroom extract was quantified by a β-glucan kit (specific for mushroom and yeast, Megazyme, USA). The beta-glucan of the mushroom was quantified in triplicate to measure with the standard levels of beta-glucan as 0.25, 0.50, 0.75, and 1.0 mg by spectrophotometry (UV/visible, 510 nm).

Culture of human dendritic cells. Human dendritic cells (HuDCs) were obtained from peripheral blood mononuclear cells (PBMC) as explained by Delirezh et al. (17). Briefly, 10 ml of a heparinized peripheral female blood sample without HPV infection was prepared for mononuclear cells separation by Ficoll–Hypaque density gradient centrifugation (Sigma–Aldrich, MO, USA). The monocytes were isolated adhering to the plastic surface at the bottom of the culture plates, and non-adherent cells were removed after 48 hours of culture, whereupon the fresh culture medium was replaced. Adherent immature monocyte-derived HuDCs were cultured with the completed culture medium (RPMI-1640 (Gibco/BRL, Carlsbad, CA, USA) supplemented with 10% of fetal calf serum (FCS), 25mM of HEPES, 2mM of L-glutamine, 1mM of sodium pyruvate, and 100µg/ml of penicillin/streptomycin). On days 2 and 4 of the culture, the supernatant was discarded and a fresh medium containing 20 ng/ml of human granulocyte-macrophage-colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) was added into the cultures. The cells were incubated at 37oC in 5% of CO2. HuDCs were divided into four sets of three. The first set was pulsed with the PBS (HuDCs 1), and the second (HuDCs 2), third (HuDCs 3) and fourth sets (HuDCs 4) were pulsed with E7 at a concentration of 0.1 mg/ml. All sets of the HuDCs were further co-cultured with lymphocytes (non-adherent cells).

Maturation of the HuDCs. After seven days of culture, the HuDCs specific markers were determined by the flow cytometry (FACSortTM flow cytometer, Becton Dickinson, NJ, USA). The adherent cells (1 x 10^5 ) cells/ml were labeled using mouse FITC-anti-human CD80 (B7-1) and phycoerythrin (PE)-anti-human CD86 (B7-1) antibody (e-Bioscience, CA, USA). The cells were incubated with the specific
monoclonal antibody for 30 minutes on ice with anti-human CD80/CD86 in the PBS/1% BSA (w/v). After washing with the PBS/1% BSA (w/v), the labeled cells were analyzed with the FACSortTM flow cytometer (Becton Dickinson, NJ, USA).

**Analysis of the T-Lymphocytes prior to co-culturing with the HuDCs.** The non-adherent cells (2 x 10^6 cells/ml) in the PBMC were regarded as lymphocytes and suspended in a RPMI 1640 culture medium (Gibco/BRL) supplemented with 10% of FCS, 25mM of HEPES, 2mM of L-glutamine, 1mM of sodium pyruvate, and 100 µg/ml of penicillin/streptomycin. The lymphocytes (2 x 10^6 cells/ml) were analyzed for CD4+, CD8+ T-cells before co-culturing with the HuDCs pulsed by PBS, PBG, E7 and the E7PBG protein. The CD4+ T-cells were identified by a PE-anti-human CD4-antibody, and the CD8+ T-cells were detected through the use of a phycoerythrin-cyanine 5 (PE-Cy5)-anti-human CD8-antibody (e-Bioscience, CA, USA). The available number of the CD4+ and CD8+ T-cells were counted by the FACSortTM flow cytometer (Becton Dickinson, NJ, USA).

**IFN-γ ELISA assay.** The interferon gamma (IFN-γ) was mainly obtained from the activated antigen-specific CTL, HuDCs and other lymphocytes that also secreted a low level of IFN-γ and were harvested from the culture supernatant of lymphocytes co-cultured with the HuDCs for 72 hours. The culture supernatants were lyophilized and re-suspended in 300 µl of the PBS. The levels of IFN-γ in the culture supernatant of lymphocytes co-cultured with the HuDCs were pulsed by the E7 protein at a concentration of 0.1 mg/ml (LympE7); lymphocytes co-cultured with HuDCs were pulsed by the E7 protein and PBG at concentrations of 0.1 mg/ml and 0.2 mg/ml, respectively (LympE7PBG); lymphocytes co-cultured with HuDCs were pulsed by PBG (LympPBG) at a concentration of 0.2 mg/ml, and the lymphocytes co-cultured with the HuDCs pulsed by the PBS (LympPBS) were evaluated using the IFN-γ ELISA kit (Dakewe Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. Human IFN-γ standard proteins at 0, 5, 10, 20, and 40 pg, were measured as the standard point of OD at 450 nm. The data were representative of the three independent assays.

**Mice antibody response.** The mice were divided into four groups of three for four subcutaneous immunizations (50 µl per injection) with complete Freund’s adjuvant on the first day and incomplete Freund’s adjuvant on day 14. The first group of mice was administered PBS as a saline control. The second group of mice was administered 4 mg/ml of the PBG. The third and fourth groups of mice were administered E7 peptide and E7PBG (4 mg/ml), respectively. After day 28, a sample of 200 µl of blood was taken from each mouse’s tail. The antibodies against E7 were evaluated from mouse serum by indirect ELISA. E7 protein in PBS was coated on microtiter plates overnight at 4°C with 25 µg/well of antigen. Afterwards, the plates were blocked with skim milk in the PBS (200 µl) for 2 hours at 37°C and washed three times with PBS. The mouse serum was diluted to 1:20 with skim milk in PBS. The plates were incubated for 2 hours with E7 antigen on the plates. After incubation, the plates were washed three times with PBS. Mice antibodies to E7 protein bound to E7 antigen on the plates were detected by goat anti-mouse IgG-horseradish peroxidase (e-Bioscience, CA, USA) in PBS. After incubating for 2 hours at 37°C, following washes, the reaction was detected by the TMB substrate (Bio-rad, CA, USA). The plates were added for acid stop solution and measured for OD at 450 nm by the ELISA reader. The mice HPV16-E7 antibody was quantified through comparison
with the standard monoclonal HPV16-E7 antibody (ab30731, Abcam, UK) in a serial concentration (0, 0.5, 1.0, 2.0 and 2.5 µg/µl) as the standard point of OD at 450 nm.

**Human CD4+, CD8+ T-cell analysis.** The T-cell numbers in the percentages of LympE7, LympE7PBG, LympPBG, and LympPBS were evaluated for the cytotoxic T-cell surface marker (CD8+ T-cell) and helper T-cell marker (CD4+ T-cell) measurement. The cells were incubated with specific monoclonal antibody for 30 minutes in PBS/1% BSA (w/v) and then unspecific binding was washed with PBS/1% BSA (w/v). The CD4+ T-cells were identified by PE-anti-human CD4-antibody (e-Bioscience, CA, USA). The CD8+ T-cells were labeled via phycoerythrin-cyanine 5 (PE-Cy5)-anti-human CD8-antibody (e-Bioscience, CA, USA). The percentage of the human CD4+ and CD8+ T-cells was analyzed by the FACSort™ flow cytometer (Becton Dickinson, NJ, USA).

**Statistical analysis.** The significant differences between the means were specified by a two-tailed Student's t-test. The P-values <0.05 were considered to be statistically significant.

**RESULTS**

**E7PBG induced the highest level of humoral immune response.** The antibody responses to PBS, PBG, E7, and E7PBG were evaluated in mice serum, since mice carry E7 antibody. The mice antibody response to PBS, PBG, E7 and E7PBG are shown in OD450 nm as 0.292 ± 0.02, 0.470 ± 0.03, 0.672 ± 0.05, and 0.945 ± 0.05, respectively (Figure 1A). The mice antibody concentration was quantified through HPV16-E7 standard antibody as 0.5, 1.0, 1.5, 2.0, and 2.5 µg/µl, respectively (Figure 1B). It was found that the mice into which E7 (1.5 µg/µl) and E7PBG (2.0 µg/µl) were administered, produced significantly higher levels of antibody (n=3, p<0.001) compared with mice administered PBS (1.0 µg/µl) and PBG (0.5 µg/µl). These data indicate that E7 and E7PBG administered mice were able to induce adaptive immunity and humoral immune response by producing a specific IgG to E7 protein. The highest-level antibody was observed in the E7PBG-treated mice (Figure 1).

**Figure 1.** Mice Antibody. Certain groups of mice (n=3) were subcutaneously vaccinated twice on days 0 and 14 with PBS, PBG, E7 and E7PBG. Mice antibody levels were determined by indirect ELISA. Bars represent the arithmetic mean value (n=3) ± SD.*, P<0.005 between PBS, PBG, E7 (A). The antibody concentration. Groups of mice (n=3) were treated with PBS, PBG, E7 and E7PBG. Mice antibody concentration was quantified with HPV16-E7 standard antibody as 0.5, 1.0, 1.5, 2.0, 2.5 µg/µl and E7PBG (B).
**Maturation of HuDCs.** The maturation of HuDCs was associated with an expression of HuDCs surface markers CD80, and CD86. The surface markers were detected by gating the monocytes population in the flow cytometry analysis. The monocyte-derived HuDCs with the surface markers of CD80 and CD86, on day 7, were counted in the four groups of cultures (HuDCs 1, HuDCs 2, HuDCs 3 and HuDCs 4) as 77.62 ± 3.83 (776 ± 38 cells), 81.03 ± 7.10 (810 ± 71 cells), 74.15 ± 3.86 (741 ± 39 cells), and 76.21 ± 4.31% (762 ±43 cells) (Figure 2A). There was no significant difference concerning the number of mature HuDCs among the groups.

**T-lymphocytes numbers were analyzed prior to co-culturing with the HuDCs.** The human lymphocytes were analyzed for their number of CD4+T-cells and CD8+ T-cells in the percentage before co-culturing with HuDCs. These results are shown in Figure 2B, where CD4+ T-cells for PBG, E7 protein and E7PBG treated group are 11.33 ± 0.58 (2,226 ± 116 cells), 12.33 ± 1.53 (2,466 ± 306 cells), 12.57 ± 0.91 (2,514 ± 182 cells), and 12.87 ± 1.37% (2,574 ± 274 cells), respectively. The CD8+ T-cells for PBS, PBG, E7 protein and E7PBG treated group was 8.01 ± 1.38 (1,602 ± 276 cells), 9.27 ± 0.64 (1,854 ± 128 cells), 9.91 ± 0.65 (1,982 ± 130 cells), and 9.53 ± 0.93 (1,906 ± 186 cells) % respectively. There was no difference in CD4+ and CD8+ T-cell numbers before co-culturing with HuDCs (Figure 2B).

**Figure 2.** The number of HuDCs and lymphocytes in the culture. Sets of HuDCs were measured on CD80 and CD86 for maturation before co-culture to lymphocytes treated with PBS, PBG, E7 and E7PBG. Bars represent the arithmetic mean value (n=3) ± SD (A). Sets of lymphocytes (Lymp) were measured on CD4 and CD8 for the T-lymphocytes in the culture system before pulsing with PBS, PBG, E7, and E7PBG. Bars represent the arithmetic mean value (n=3) ± SD (B).

**E7PBG augmented IFN-γ production.** The IFN-γ levels in the culture supernatant of the LympPBS, LympPBG, LympE7, and LympE7PBG groups were compared as is demonstrated in Figure 3. IFN-γ levels in LympPBS, LympPBG, LympE7, and LympE7PBG were 5 pg, 10 pg, 20 pg and 40 pg, respectively (Figure 3B). The LympE7PBG group showed the highest levels of IFN-γ secretion (n=3, p<0.001, Figure 3A). The LympPBS and LympPBG groups were also determined as the baseline OD.
point of the IFN-γ (0.61±0.08) and (0.82±0.02), respectively. Further observed was a significantly higher level of IFN-γ from LympE7PBG compared with LympE7 (n=3, p<0.001; Figure 3A).

Figure 3. IFN-γ levels. The average levels of IFN-γ in each group of LympPBS, LympPBG, LympE7 and LympE7PBG are shown at OD 450 nm. Bars represent the arithmetic average (n=3) (A). IFN-γ concentration. The concentrations of IFN-γ in each group of LympPBS, LympPBG, LympE7 and LympE7PBG were quantified with IFN-γ standard at 0,5,10,20,40 pg. ± SD. *, P < 0.05 between LympPBS, LympPBG, LympE7 and LympE7PBG (B).

An expansion of the helper and cytotoxic T-cells was enhanced by E7PBG. The expansions of T-lymphocytes induced by PBS, PBG, E7 and E7PBG were evaluated in vitro for helper (CD4) and cytotoxic (CD8) T-cells (Figure 4). The significant expansion of human CD4+ and CD8+ T-cells was shown by the flow cytometry analysis data between the two groups of the culture T-lymphocytes with E7 and E7PBG (Figure 4). The percentages of the CD4+ T-cells from PBS, PBG, E7 and E7PBG were 12.33 ± 1.53 % (2,466 ± 306 cells), 17.33 ± 1.53 % (3,466 ± 306 cells), 28.24 ± 1.79 % (5,648 ± 358 cells), and 34.87 ± 2.37% (6,974 ± 474 cells), respectively. The percentages of the CD8+ T-cells from the PBS, PBG, E7 and E7PBG were 12.00 ± 1.50 % (2,400 ± 300 cells), 15.67 ± 1.56 % (3,134 ± 312 cells), 20.57 ± 0.84 % (4,114 ± 168 cells), and 20.87 ± 2.37% (4,174 ± 474 cells), respectively. There was a significant increase in the human CD4+ and CD8+ T-cells population from E7PBG when compared to E7-treated group, suggesting that E7PBG was able to more significantly enhance the expansion of helper and cytotoxic T-cells compared with E7 peptide alone (n=3, p<0.05) (Figure 4).
Enhancing Human T-Lymphocytes by Dendritic Cells and Pleurotussajor-caju-β-glucan

Figure 4. Analysis of T-lymphocytes’ surface markers. The lymphocytes treated with PBS, PBG, E7 and E7PBG (LympPBS, LympPBG, LympE7, LympE7PBG) in the culture were monitored for CD4+ and CD8+ T-cells through the use of flow cytometry analysis. Percentages of CD4+ and CD8+ T-cells are shown as bars which correspond to the arithmetic mean value (n=3) ± SD. *, P<0.05 between PBS, PBG, E7, and E7PBG.

DISCUSSION

An uptake of viral protein antigen by antigen presenting cells (APCs), such as dendritic cells facilitates the antigens for intracellular processes; moreover, antigen presentation by MHC class I and II pathways induce both CD4+ and CD8+ T-cell responses. In this study, the researcher applied HuDCs pulsed with E7 that served as the carrier or platform for inducing a higher human cell-mediated immune response. HuDCs with E7PBG enhanced the efficacy of the prophylactic and therapeutic vaccine. As per the previous study, the use of DCs is a good strategy for effective immunotherapy for cervical cancer (9).

For viral vaccine, the focus is intensively on human lymphocytes which represent the whole adaptive immune system response against the virus and probably consequent cancer. The mice antibody response evaluated the antigenicity of E7 for studying the humoral immune responses. The prophylactic antibody to E7 was studied in E7-treated mice, revealing that E7 could increase the specific antibody response, hence able to enhance the humoral immune response and could be used as a vaccine to prevent HPV16.

In addition, the fungal cell wall of the mushroom is well known for containing polysaccharides. The β-glucans of polysaccharides have been considered as potent immunomodulators for T-cells and antigen presenting cells (16). Macrophages,
dendritic cells (DCs) and B lymphocytes are professional antigen presenting cells as far as presenting exogenous antigens to helper T lymphocytes is concerned (18). There is a report on β-glucan’s ability to induce innate immunity as a potent immunomodulator (16). In this study, E7PBG was capable of enhancing the humoral immune response as it has induced significantly higher antibody levels compared with E7 peptide alone; it is, therefore, recommended as a vaccine regimen for preventing HPV16. Regarding therapeutic vaccines, HuDCs pulsed with E7PBG served as the carrier or platform for inducing a higher human cell-mediated immune response. The researcher applied HuDCs pulsed with E7 to improve the efficacy of therapeutic vaccine. As in the researcher's previous study, the use of DCs is a good strategy for effective immunotherapy for cervical cancer. (9)

In this study, the group of lymphocytes in both CD4 and CD8 T-cells with E7PBG, produced a significantly higher expansion and level of IFN-γ compared with the group only treated with E7 peptide. IFN-γ is a crucial cytokine in the host immune response against viral infections secreted by effector CD8+ T-cells, Th1 CD4+ T-cells, as well as NK and NK T-cells (19). The effect of IFN-γ was to enhance the cytotoxic T-cells activity (20) and activate the NK cells in order to help differentiation of the CD4+ T-cells toward a Th1 response to promote the differentiation (21) and it is assumed that the activity of the cytotoxic T-cells could be enhanced by increasing IFN-γ.

Conclusion: In PBGE-treated group of T-lymphocytes, adaptive immunity to HPV was enhanced by a) triggering the humoral immune response comprised of viral antigen-specific IgG virus-neutralizing antibodies in a murine model, and b) increasing the human cellular immune response characterized by the expansion of helper and cytotoxic T-cells and IFN-γ release. This regimen is conducive to developing prophylactic and therapeutic vaccines for cervical cancer.

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REFERENCES


