Phenotypic Analysis of Monocyte-derived Dendritic Cells Loaded with Tumor Antigen with Heat-shock Cognate Protein-70

SHINICHIRO ITO1, YASUHIRO NAGATA2, SEIYA SUSUMU1, AKIRA YONEDA1, MITSUTOSHI MATSUO3, KATSUYUKI YUI4, HEIICHIRO UDONO5, SUSUMU EGUCHI1 and TAKASHI KANEMATSU1,6

Departments of 1Surgery and 4Molecular Medicine, Division of Immunology, Nagasaki University Graduate School of Biomedical Science, Nagasaki, Japan; 2Department of Surgery, National Hospital Organization, Nagasaki Medical Center, Nagasaki, Japan; 3Department of Surgery, Nagasaki Memorial Hospital, Nagasaki, Japan; 5Department of Immunology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; 6Nagasaki Municipal Hospital, Nagasaki, Japan

Abstract. Background/Aim: The cross-presentation system of tumor antigen by monocyte-derived dendritic cells (mo-DCs) has been observed under appropriate conditions. Both CD14-negative and CD1a-positive phenotypes were critical in our previous study. This study compared the phenotype of mo-DCs and identified the conditions that favored T helper-1 (Th1) cytokine production after stimulation with the hsc70 and NY-ESO-1 p157-165 epitope fusion protein (hsc70/ESO p157-165). Materials and Methods: The mo-DCs were induced from healthy donors. Their surface markers and cytokine production were examined after stimulation with hsc70/ESO p157-165. Results: CD1a+ and CD1a – mo-DCs were generated in half of the healthy donors. The concentration of fetal calf serum in the culture medium was critical for the induction of CD1a+ DCs, which were able to produce interleukin-12 (IL-12), but not IL-10. Neutralizing IL-6 and IL-6R antibodies affected the expression of CD1a. Conclusion: Anti IL-6 analogs may be effective adjuvants for the development of mo-DC-based cancer vaccine.

NY-ESO-1 is a promising target antigen for specific immune recognition of cancer because it has restricted expression in normal tissue but frequently occurs on human tumors (1-4). Clinical trials with this antigen have been conducted using the NY-ESO-1 peptide, full-length protein, and DNA (5, 6). These cancer vaccines are designed to enhance effector T-cell responses to tumor antigens. An appropriate antigen-presenting cell is required to induce favorable T-cell responses (7).

Dendritic cells (DCs) are the most potent antigen-presenting cells and they have been shown to play a critical role in the generation of immune responses. The unique features of antigen presentation by DCs have generated considerable interest in their use as therapeutic vehicles, especially for vaccination (8, 9). DCs-alone or in complexes with tumor antigens are expected to be a powerful tool in the development of cancer vaccines (10, 11). However, no consensus has yet been reached on the most appropriate DC population to be employed for immunization.

A fusion protein containing the human heat-shock cognate protein-70 (hsc70) and ESO p157-165, epitope of NY-ESO-1 was constructed, as part of the development of a new strategy to vaccinate cancer patients with tumor antigens (12-14). A previous study demonstrated that monocyte-derived (mo)-DCs capture and endogenously process the hsc70/ESO p157-165 fusion protein to major histocompatibility complex (MHC) class I molecules through the cross-presentation pathway (15, 16). However, this cross-presentation system could not always work. This study was conducted to define the appropriate conditions in order to use mo-DCs for vaccination after loading with the hsc70/ESO p157-165 fusion protein.

Materials and Methods

Expression and purification of the hsc70 and NY-ESO-1 p157-165 epitope fusion protein. The hsc70/ESO p157-165 fusion protein was manufactured as previously described (15). Briefly, human cDNA of
hsc70 was generated by reverse transcription-polymerase chain reaction (RT-PCR) from the mRNA obtained from the peripheral blood mononuclear cells (PBMCs) of a healthy volunteer. The total mRNA was extracted from the PBMCs with an Isogen kit (Wako, Osaka, Japan). The mRNA was transcribed to cDNA with oligo (dT) 16 primer using AMV reverse transcriptase (Promega, Tokyo, Japan). The cDNA encoding hsc70 was amplified by LA Taq polymerase (Takara, Tokyo, Japan) using the primers AT GGATCC C ATG TCC AAG GGA CCT G (forward) and AT GGATCC TTA ATC AAC CTC TTC AAT G (reverse). The amplified cDNA was cloned into a pQE31 expression vector (Qiagen, Tokyo, Japan) at 5' BamHI and 3' KpnI restriction sites. The hsc70/ESO p157-165 fusion protein was generated by incorporating a mini-gene encoding NY-ESO-1 p157-165 in either the forward or reverse primers containing the 5' BamHI and 3' KpnI restriction sites. Escherichia coli strain M15 was transformed by the constructed plasmids and grown in an LB medium, containing ampicillin (50 μg/ml) and Kanamycin (20 μg/ml). Protein expression was induced by 0.1 M IPTG in LB medium, containing ampicillin (50 μg/ml) and kanamycin (20 μg/ml). The lysate was centrifuged at 10,000 × g, and the supernatant was treated with Kurimover I and II (Kurita Incorporation, Tokyo, Japan) and then concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore, Bedford, MA, USA). The fusion proteins were dialyzed against phosphate buffered saline (PBS) (pH 7.4) to remove imidazole containing 200 mM imidazole. The eluate was extensively dialyzed and then concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore, Bedford, MA, USA). The fusion proteins were treated with Kurimover I and II (Kurita Incorporation, Tokyo, Japan) to remove the contaminating lipopolysaccharide (LPS). The level of His-tagged protein was re-folded by washing with 15 column volumes of urea-free Tris buffer (pH 7.5) and then concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore, Bedford, MA, USA). The fusion proteins were treated with Kurimover I and II (Kurita Incorporation, Tokyo, Japan) to remove the contaminating lipopolysaccharide (LPS). The level of LPS was determined by the Limulus ES-II test (Wako, Osaka, Japan).

**Peptide.** The human leukocyte antigen (HLA)-A201 restricted NY-ESO-1 peptide p157-165 (SLLMWTQGC) was identified by reactivity with activity of differentiation 8 (CD8) T-cell from patients with spontaneous NY-ESO-1 immunity. This epitope (ESO p157-165) was selected to analyze the CD8 T-cell response (17, 18). The peptide was synthesized by using the Multiple Peptide Systems, with a purity of >86%, as determined by reversed-phase high-performance liquid chromatography (HPLC).

**Generation of dendritic cells from PBMCs.** Mononuclear cells were isolated from the peripheral blood of healthy individuals by using Ficoll-Paque density gradient centrifugation after obtaining informed consent (19, 20). The CD14+ monocytes were enriched by negative isolation using magnetic beads (Dynal, Oslo, Norway). Monocytes were seeded at a density of 1.0×10⁶ cells/well in 24-well plates in 2 ml of RPMI 1640 medium with 2.5% or 10% fetal calf serum (FCS), 100 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) (Leukine; Immunix, Seattle, WA, USA) and 50 ng/ml IL-4 (R&D Systems, Minneapolis, MN, USA). The culture was incubated at 37°C in a humidified atmosphere with 5% CO₂ for 5 days. The harvested cells were characterized by flow cytometry and were then stimulated with hsc70/ESO p157-165 fusion protein or p157-165 peptide.

**Flow cytometry.** The cells were processed for double-staining using fluorescein isothiocyanate (FITC)-CD14, FITC-CD1a, phycoerythrin (PE)-CD83, PE-HLA-DR, PE-CD86 (B7.2) monoclonal antibodies (mAbs) (BD Pharmingen, San Diego, CA, USA). Fluorescence acquisition was carried out on a FACSCan (BD Biosciences, San Diego, CA, USA), and data analysis was carried out using the CellQuest software package (BD Biosciences).

**Stimulation of DCs and measurement of cytokines.** The harvested cells were incubated with GM-CSF and IL-4 for 5 days and exposed to p157-165 (5 μg/ml) or hsc70/ESO p157-165 fusion protein (350 μg/ml) in RPMI 1640 for 12 h. The cell culture supernatants were collected and then particulates were removed by centrifugation. The concentration of the cytokines (IL-10 and IL-12) in the supernatants was measured by an enzyme-linked immunosorbent assay (ELISA; Quantikine, R&D Systems, Minneapolis, MN, USA).

**Neutralization of IL-6 for differentiation of DCs from monocytes.** mo-DCs were generated as described above. Anti-human IL-6 and/or IL-6R neutralizing antibodies, at a concentration of 2.5 μg/ml (R&D Systems), were added to the cultures at day 0 and 3 (21).

**Results**

**Phenotype of mo-DCs from healthy donors.** The population of monocytes isolated from PBMCs exhibited a unique phenotype (Figure 1a). However, the mo-DCs from the healthy donor were differentiated into two distinct phenotypes (Figure 1b). After 5 days, culture in RPMI, containing 10% FCS with GM-CSF and IL-4, the expression of CD14 was down-regulated in cells from half of the donors. On the other hand, CD1a was expressed in those cases. The expression of CD83 was negative in cells from all donors.

**IL-10 production of mo-DCs from each donor.** IL-10 was measured in the supernatants of DCs stimulated for 12 h by hsc70/ESO p157-165 fusion protein or p157-165 (Figure 2). CD14⁺CD1a⁻ DCs secreted significant amounts of IL-10 in response to hsc70/ESO p157-165 fusion protein. However, there was less IL-10 secretion stimulated by hsc70/ESO p157-165 fusion protein in CD14⁺CD1a⁺ DCs from donors 1 and 3.

**The expression of CD1a and production of cytokines from mo-DCs.** CD14⁺CD1a⁻ DCs (donor 5) produced IL-10, but IL-12, which plays a prominent role in the induction of the T-helper 1 (Th1) immune response against cancer, was barely secreted. Meanwhile, CD14⁺CD1a⁺ DCs from donor 6 exhibited a reversed pattern of cytokine production for IL-10 and IL-12 (Figure 3). The expression of surface markers CD14 and CD1a of mo-DCs had an effect on the balance of Th1 and Th2 response.

**Mo-DCs from the same donor exhibited different surface marker phenotype after induction under different conditions.** CD14⁺CD1a⁺ DCs were generated from donor 7 following
culture in RPMI with 10% FCS. However, CD14+CD1a– DCs were generated under culture conditions of RPMI with 2.5% FCS, using cells from the same donor. This phenotypic conversion changed the function of DCs, which exhibited IL-10 production rather than IL-12, in response to stimulation with hsc70/ESO p157-165 fusion protein (Figure 4).

The expression of CD1a and IL-6 antagonists. IL-6 affects the differentiation of monocytes into DCs and macrophages. The addition of IL-6 and/or IL-6R antibodies during the generation of mo-DCs up-regulated the expression of CD1a. The expression of CD1a was remarkable following blocking of both IL-6 and IL-6R (Figure 5). Although the DCs generated in medium containing 2.5% FCS were almost all CD1a– cells, the expression of CD1a was positive following the addition of neutralizing IL-6 and IL-6R antibodies on day 0 and 3. The function of the DCs differed according to their phenotypic features and they produced IL-12 in response to the activation by hsc70/ESO p157-165 fusion protein (Figure 6).

Figure 1. a: Phenotype of monocytes isolated by magnetic beads from peripheral blood mononuclear cells. Cells were enriched and harvested immediately. b: Phenotype of monocyte-derived dendritic cells. Peripheral blood mononuclear cells were isolated from the peripheral blood of healthy individuals. The CD14+ monocytes enriched by negative isolation were incubated in RPMI plus 10% fetal calf serum, granulocyte macrophage colony-stimulating factor and interleukin-4, for 6 days. The phenotypes (CD14, CD1a, CD83, HLA-DR, CD86) of the harvested cells were analyzed by flow cytometry.

Figure 2. Interleukin-10 (IL-10) production of dendritic cells (DCs). CD14–CD1a+ DCs and CD14+CD1a– DCs were stimulated by hsc70/ESO p157-165 fusion protein or p157-165 in RPMI medium for 12 h. The IL-10 in the supernatants of pulsed DCs was measured by an enzyme-linked immunosorbent assay.
Figure 3. Interleukin-10 (IL-10) and interleukin-12 (IL-12) production of dendritic cells (DCs). CD14+CD1a+ DCs and CD14+CD1a- DCs were activated, as described in Materials and Methods by the hsc70/ESO p157-165 fusion protein. IL-10 and IL-12 in the supernatants of cells were measured by an enzyme-linked immunoassorbent assay.

Figure 4. Cytokine production of dendritic cells (DCs) in different culture medium. The percentage of fetal calf serum (FCS) in RPMI medium for generation of monocyte-derived DCs was changed from 10% to 2.5%.
Discussion

NY-ESO-1 is a prototype cancer/testis antigen that is expressed in a variety of human malignancies, but not in normal tissues, except for the testis. Spontaneous immune responses involving an antibody, as well as CD4+ and CD8+ T-cells directed against a broad range of MHC class I- and class II-restricted NY-ESO-1 peptides are observed in patients with advanced NY-ESO-1-expressing tumors (22). Therefore, NY-ESO-1 is thought to be a favorable target for use as a cancer vaccine. The initial trials of peptide vaccination against NY-ESO-1 were particularly effective in generating CD8+ T-cell responses. However, the clinical outcome remains unsatisfactory.

The major role of heat shock proteins (HSPs) is to act as a molecular chaperone, binding immature peptides during their synthesis and assisting in their folding (23-25). The peptides are thought to be degraded in the cytoplasm and are then transferred to the endoplasmic reticulum by binding to HSP70 or HSP90, but not by natural diffusion (26). In addition, HSPs are thought to bind to a diverse array of antigenic peptides in tumor cells, and that the tumor-derived HSP-antigenic peptide complexes can be purified for vaccination against cancer (27).

A mini-gene encompassing the NY-ESO-1 cytotoxic T-lymphocyte (CTL) epitope p157-165 (ESO p157-165) was genetically fused to the human heat shock cognate protein-70 (hsc70), and the resulting fusion proteins were expressed in E. coli. mo-DCs captured and endogenously processed the hsc70/ESO p157-165 fusion protein to MHC class I molecules through the cross-presentation pathway. Finally, NY-ESO-1-specific CTL were generated by in vitro stimulation with hsc70/ESO p157-165 fusion protein on mo-DCs.

DCs play a crucial role in the initiation of antigen-specific immune responses, exhibiting a variety of specializations that contribute to their efficiency as antigen-presenting cells (9, 28). One major population of DCs is myeloid DCs which include specific subtypes, including Langerhans cells, interstitial DCs and mo-DCs that have unique phenotypic features. The CD14+ monocytes are the most common source of DCs and can be enriched by negative isolation from PBMCs and incubated in RPMI with 10% FCS, GM-CSF and IL-4 to generate DCs (29, 30). However, not all DCs generated or cultured under the same conditions are equivalent (31). They appear to be derived from multiple lineages and, depending on their origin, site of residence, or the type of maturation stimulus received, they program different T-cell outcomes (32). The generated mo-DCs exhibit various expression levels of CD1a, CD14, CD83, human leucocyte antigen-DR (HLA-DR) and CD86 according to the culture conditions and individuals from which they are sourced. mo-DC subsets are defined by their phenotypic features and have a functional diversity of cytokine production that regulates the polarization of naïve T-cells to Th1 or Th2 (33-36). However, this diversity creates difficulties in their clinical application in cancer immunotherapy using DCs. The optimal phenotypic features of DCs and appropriate conditions for clinical applications must be determined.

CD1a is one of the common DC subset markers (37, 38). The proportion of CD1a+ and CD1a- DCs varies in individuals.

Figure 5. The population of CD1a+ dendritic cells (DCs) after neutralizing Interleukin-6 (IL-6) and/or IL-6R. Antibodies to human IL-6 and IL-6R were added to the culture on day 0 and 3 of the generation of DCs from monocytes, in a medium with 2.5% fetal calf serum (FCS). The CD1a expression of harvested DCs was analyzed by flow cytometry on day 6.
CD1a+ DCs are able to secrete more IL-12 in response to stimulation with LPS than do CD1a− DCs. On the other hand, the IL-10 production of CD1a+ DCs is less, or similar to that of CD1a− DCs (39). The present study generated two types of DCs, CD1a+ and CD1a−, under the same conditions. Interestingly, there were different patterns of CD1a expression under the different conditions during induction, even when using cells from the same donor. This strongly suggests that the conditions of DC culture were critical for induction of the appropriate antigen-presenting cells in vivo.

Humoral factors in the serum also affect the differentiation of immature DCs (40, 41). Culture medium with 2.5% FCS converted the phenotype of DCs from CD1a+ to CD1a−. This conversion also changed the cytokine production from IL-12 to IL-10. This is a critical conversion associated with the polarization of Th1 and Th2 cells. A major question was whether this conversion was reversible. IL-6 inhibits the differentiation of monocytes to DCs by promoting their differentiation toward macrophages (42, 43). On the other hand, an antagonist of IL-6 can drive monocytes to form immature DCs. Therefore, the present study compared the effect of anti-IL-6 agents on CD1a expression. Cells treated with both antibodies to IL-6 and IL-6R, recovered CD1a expression and secreted IL-12. These results suggest that anti-IL-6 analogs may be used as an effective adjuvant for the development of a mo-DC-based cancer vaccine with the hsc70/ESO p157-165 fusion protein.

Acknowledgements

This work was supported by a Grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (no. 16591263).

References

Ito et al: Phenotypic Analysis of (mo)-DCs in Complex with Tumor Antigen with hse70


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