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**Pluripotent stem cell-derived dendritic cells for immunotherapy**

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## 1. ABSTRACT

Dendritic cell (DC) is regarded as a powerful means for anti-cancer immunotherapy. Clinical trials of cancer therapy with DC loaded with cancer antigens, such as tumor cell-lysates or HLA class I-binding antigenic peptides, have been conducted. Antigen-specific negative manipulation of the immune response by DC is a potential treatment for autoimmune diseases and also for control of allo-reactive immune responses in transplantation medicine. Currently, DC for clinical use are generated from peripheral blood monocytes of the patients. However, the number of monocytes obtained from the patients is limited and the potential of monocytes to differentiate into DC varies depending on the blood donor. Thus, the issue of limited cells is a serious obstacle for DC therapy. ES cells and iPS cells have pluripotency and unlimited propagation capacity and may be an ideal cell source for DC-therapy. Several groups, including us, have developed methods to generate DC from ES cells or iPS cells. This review introduces the studies on generation, characterization, and genetic modification of DC derived from ES cells or iPS cells.

## 2. PHYSIOLOGICAL FUNCTIONS AND MEDICAL APPLICATION OF DENDRITIC CELLS

Dendritic cells (DC) are the most potent antigen presenting cells and they are known to play a pivotal role in the development of immune responses. DC, a type of hematopoietic cell, exist in various tissues in the body. Upon invasion of exogenous antigens to the tissues, tissue-localized DC capture the antigen through phagocytosis, receptor-mediated endocytosis, or pinocytosis. DC digest the ingested protein antigen to produce oligo peptides. In the steady state, DC are efficient in capturing antigens and similar to macrophages. However, after exposure to the constituents of exogenous micro-organisms, such as bacteria and fungi, that transduce danger signals, DC become potent T cell-stimulators. Accompanying this functional change termed "maturation", their morphology changes and DC begin to form dendritic protrusions. At the molecular level, they up-regulate the cell surface expression of MHC class I and class II, CD80, CD86, and CD40, molecules involved in the T-cell stimulation or DC-T cell interaction, and they produce cytokines such as TNF-α and IL-12.

After antigen capture, DC migrate to the draining lymphoid tissues such as the lymph nodes. In the lymphoid tissues, DC present the antigenic peptides in the context of MHC class I and MHC class II. Circulating T cells migrate from the blood stream into the T-cell area of the lymph nodes and recognize the complex of MHC plus peptide on the cell surface of DC. Importantly, mature DC are very potent in stimulating T cells, and can prime naive T cells that have not yet experienced antigenic stimulation. Antigen-specific T cells stimulated by DC proliferate vigorously and produce various kinds of cytokines that stimulate immune cells, consequently resulting in the development of an anitgen-specific immune response. Therefore, the stimulation of naive antigen-specific T cells by antigen-presenting DC is a crucial step for the initiation of immune responses.

In addition, several studies have indicated that DC are involved also in the induction and maintenance of immunological self-tolerance (1). The mechanisms of negative regulation of immune response by DC include expansion of $CD4^+$ $CD25^+$ regulatory T cells (2) (3) and catabolism of tryptophan (4). Collectively, DC physiologically regulate immunity both positively and negatively.

Because DC are immune-regulating cells, the application of DC for clinical immunotherapy is an attractive option. Studies in mice have demonstrated that cellular vaccination with antigen-bearing DC is efficient in stimulating antigen-sepcific T cell responses (5). DC are now regarded as a powerful tool for anti-cancer immunotherapy. Genetically engineered DC with enhanced T cell-stimulating capacity should yield an even more powerful anti-cancer therapy. To treat patients who suffer from autoimmune or inflammatory diseases, it is desirable to down-modulate immune responses in an antigen-specific manner without causing systemic immune suppression. GVHD and graft rejection are the most serious problems in transplantation medicine, and control of allo-reactive immune response is the key to overcome them. Antigen-specific negative regulation by DC is considered to be a promising means also in the field of tranplantation medicine (6).

Within the past decade, various clinical anti-cancer therapy trials using DC have been conducted. Cancer antigens such as tumor cell-lysates or HLA class I-binding antigenic peptides are loaded onto DC and transferred to the patients, where a cancer antigen-specific T cell response is expected, in particular a cytotoxic T lymphocyte (CTL) response. DC used for clinical anti-cancer therapy are generated from peripheral blood monocytes of cancer patients. In order to obtain sufficient number of monocytes, apheresis procedure is used to isolate white blood cells from a large volume of patient blood, and subsequently monocytes are isolated from the white blood cell population. However, the number of monocytes obtained from the peripheral blood is limited even with apheresis. In addition, the DC-differentiation potential of monocytes varies depending on the blood donor. Therefore, this continuing cell source limitation remains one of the major obstacles for DC therapy.

## 3. GENERATION OF DENDRITIC CELLS FROM MOUSE ES CELLS

ES cells are characterized by pluripotency and infinite propagation capacity. Therefore, if we can use ES cells as a source of DC, the cell source limitation for DC therapy can be overcome. We and others developed methods to generate DC from mouse ES cells, and the functions of ES cell-derived DC (esDC or ES-DC), including the stimulation of allogeneic T cells, processing and presentation of antigenic proteins, and migration upon *in vivo* transfer, were comparable to those of DC generated *in vitro* from bone marrow cells (7) (8).

For hematopoietic differentiation of ES cells *in vitro*, Embryoid Body (EB)–mediated methods and the feeder-coculture method have been established. OP9 is a bone marrow stromal cell line that originated from a Macrophage Colony Stimulating Factor (M-CSF)-defective op/op mouse, and generation of various hematopoietic cells from ES cells using OP9 cells as feeder cells has been reported, with ES cells yielding granulocytes, erythrocytes, and B lymphocytes (9). The method has been applied to a number of molecular and cellular analyses for investigations of hematopoiesis.

In 2000, Fairchild and colleagues reported the first study on the generation of functional dendritic cells from mouse ES cells (7). Their method is based on the formation of EB. At the initiation of the differentiation, ES cells were grown in suspension to allow EB formation. After culturing for 14 days, EB were cultured in medium supplemented with Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF) and Interleukin (IL)-3. The combination of these 2 cytokines uniquely supported the development of cells with an appearance suggestive of DC within 4 days of culture. These cells expanded rapidly over the following 7–10 days to form lightly adherent clusters, reminiscent of those observed in cultures of immature DC derived from bone marrow cells. The cells released from these clusters seeded uncolonised areas of the dish and displayed typical dendritic morphology, and they were designated as esDC.

A flowcytometry analysis showed that esDC expressed CD11c, MHC class II, CD40, CD54, CD80, and CD86. On the other hand, expression of CD8 and Dec-205 (CD205) was not observed, thus suggesting that esDC were derived from a myeloid lineage. RT-PCR analysis revealed that esDC expressed mRNAs for IL-20, IL-18, and IL-1β converting enzyme (ICE). They possessed a potent capacity to process a protein antigen, Hen Egg Lysozyme (HEL), and present the resultant epitope in the context of $H\text{-}2E^k$, as demonstrated by the response of co-cultured antigen-specific T cell hybridoma. Upon co-culture with allogeneic T cells, esDC stimulated the T cells to vigorously proliferate, indicating that esDC had a strong T cell-stimulating capacity. Collectively, the ES cell-derived cells with DC-like morphology were fully functional in antigen-presentation and T cell-stimulation.

Hematopoietic differentiation of mouse ES cells by co-culture with M-CSF-deficient mouse stromal

cells, OP9, was developed by Nakano and colleagues in 1994 (9). Similarly, we established a method to generate dendritic cells from mouse ES cells by aid of OP9 stromal cells (8).

In our method, mouse ES cells were transferred onto the OP9 cell layers and cultured in αMEM medium containing fetal calf serum without exogenous cytokines. ES cell colonies changed their morphology from undifferentiated dome-like structure to a differentiated flattened morphology in 4 to 5 days. To induce DC differentiation, the mesodermally differentiated ES cell–derived cells harvested from a 5 or 6 -day culture on OP9 feeder layers were cultured on fresh OP9 cell layers in the presence of exogenous GM-CSF. In comparison to the culture without exogenous GM-CSF, addition of this cytokine resulted in appearance of a larger number of floating cells in a few days, indicating that cells grew in response to GM-CSF.

At day 3 following transfer, we observed many round and relatively homogenous floating cells, most of which expressed CD11b, thus suggesting their commitment to the myeloid cell lineage. On day 10 or 11, we recovered the cells floating or loosely adherent to feeder cells and transferred them to bacteriologic Petri dishes without feeder cells. After this passage, approximately half of the transferred cells adhered to the dish surface and resembled macrophages. In addition, clusters of floating cells were observed after days 17 to 19, and the cell clusters gradually increased. The cells were of irregular shape and possessed protrusions. We designated them ES-DC. Addition of maturation stimuli, IL-4, TNF-α, plus anti-CD40 antibody or LPS, to the cells resulted in typical morphology of mature DC with long protrusions.

ES-DC were positive for MHC class I, MHC class II, CD80, CD86, CD40, DEC205, and CD11c. They had the capacity to process and present protein antigens to T cells. After exposure to maturation stimuli, the expression of MHC class I, MHC class II, CD80, CD86 and CD40 were increased. Mature ES-DC were highly competent in stimulating T cells, based on the proliferative response of the allogeneic T cells co-cultured with ES-DC. ES-DC were positive for F4/80 and CD11b and negative for CD8, thereby suggesting that they were of myeloid lineage.

## 4. MOUSE STUDIES ON THE INDUCTION OF ANTI-CANCER IMMUNITY BY GENETICALLY ENGINEERED ES-DC

Non–virus-mediated methods for gene transfer, including targeted gene integration and procedures for isolation of appropriate transfectant cell clones, have been established for ES cells. We developed a strategy for the genetic modification of mouse ES-DC. Expression vectors were introduced into ES cells by electroporation and subsequently the transfected ES cell clones were induced to differentiate into ES-DC. The capacity of ES-DC introduced with an ovalbumin (OVA)-expression vector to prime OVA-specific T cells *in vivo* was demonstrated (8).

In anti-cancer immunotherapy with administration of DC, the efficiency of T cell-priming *in vivo* by transferred DC is critical. T cell-priming *in vivo* by injected DC should depend on their encounter with T cells. When exogenous antigen was injected intracutaneously, about 25% of the DC capturing the antigen migrated to the T cell area of draining lymph nodes (10), where they presented the antigen to prime naive T cells specific to the antigen. On the contrary, when BM-DC or splenic DC were transferred exogenously by s.c. or i.p. injection, the absolute number of the DC found within the draining lymph nodes represented only a small proportion (0.1-1%) (11) (12). It has been also reported that almost all the transferred DC remained at the s.c. immunization site up to 24 hours after transfer in the patients who received DC therapy (13). The inefficient migration of the exogenous DC to lymphoid organs may lower the frequency of their encounter with T cells. It may be possible to improve the efficacy of exogenously transferred DC to prime immune responses by augmenting their encounter with T cells. We attempted to improve the capacity of DC to prime T cells *in vivo* by genetic modification to express a chemokine with T cell-attracting properties. We generated double-transfectant DC expressing a chemokine along with OVA by sequential transfection of ES cells with 2 kinds of expression vectors and then induction of DC differentiation (14).

Several kinds of chemokines with the capacity to attract T cells are produced by different cell types. CCL21 (Secondary lymphoid tissue chemokine, SLC) is produced in T cell regions of lymph nodes and spleen and also by high endothelial venules in lymph nodes. SLC chemoattracts T cells, NK cells, B cells, and DC. CXCL9 (Monokine induced by IFN-γ, Mig) is produced by macrophages and binds to the chemokine receptor CXCR3, which mediates the recruitment of predominantly Th1 cells and activated NK cells. XCL1 (Lymphotactin, Lptn), produced by activated T cells, has chemoattractive properties on $CD4^+$ and $CD8^+$ T cells and on NK cells. We comparatively evaluated the effect of these 3 kinds of chemokines.

All three types of double-transfected ES-DC primed OVA-specific CTL *in vivo* more efficiently than did ES-DC expressing only OVA, and co-expression of SLC or Lptn was more effective than that of Mig. Immunization with DC expressing OVA plus SLC or Mig provided protection from OVA-expressing tumor cells more potently than that with OVA only, and SLC was more effective than Mig. Conversely, the co-expression of Lptn gave no additive effect on protection from the tumor. Collectively, among the three chemokines, expression of SLC was the most effective in enhancing anti-tumor immunity by transferred ES-DC *in vivo*.

NKT cells are a group of T cells expressing invariant T cell receptors and recognize lipid ligands, for example alpha-galactosylceramide (αGalCer), in the context of CD1d, a nonclassical MHC class I molecule. On stimulation with αGalCer, NKT cells rapidly produce large amount of cytokines, resulting in activation of conventional T cells and NK cells. ES-DC loaded with αGalCer potently

stimulated NKT cells, and αGalCer-loaded ES-DC expressing OVA elicited anti-tumor activity against peritoneally injected tumor cells expressing the OVA antigen (15). Expression of SLC with OVA further enhanced the anti-tumor activity of αGalCer-loaded ES-DC expressing OVA. These results suggest that αGalCer-loaded ES-DC expressing tumor-associated antigen along with SLC can stimulate multiple subsets of effector cells to induce a potent therapeutic effect against peritoneally disseminated tumor cells.

The anti-tumor immunity obtained by ES-DC directed to natural tumor antigens was examined. Glypican-3 is a tumor antigen expressed in hepatocellular caricinoma and melanoma (16) (17). ES-DC expressing Glypican-3 could induce anti-tumor activity against Glypican3-transfected tumor cells and also against the mouse melanoma B16-F10, which endogenously expresses Glupican3 (18). Immunization of mice with a mixture of ES-DC expressing GP100, SPARC, and Tyrosinase-related protein-2 induced potent immunity against B16-BL6 melanoma in the peritoneal dissemination model and also lung metastasis models (19). Collectively, we have demonstrated that genetically modified ES-DC are highly potent in induction of anti-tumor immunity in mouse models.

## 5. STUDIES ON THE TREATMENT OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS BY ES-DC

We investigated whether we could negatively modulate immune responses with genetically modified ES-DC expressing specific antigen along with immune-suppressive molecules. Experimental Autoimmune Encephalomyelitis (EAE), characterized by neurological impairment resulting from de-myelination in the central nervous system caused by activated myelin antigen-reactive $CD4^+$ T cells, is induced by immunization with myelin antigens such as Myelin Oligodendrocyte Glycoprotein (MOG) or Myelin Basic Protein (MBP). We attempted to prevent MOG-induced EAE by pre-treating mice with genetically modified DC presenting a MOG peptide in the context of MHC class II molecules and simultaneously expressing molecules with T cell-suppressive property (20).

For efficient presentation of the MOG peptide in the context of MHC class II molecules, we used a previously devised expression vector in which cDNA for human MHC class II-associated invariant chain (Ii) was mutated to contain antigenic peptide in the CLIP (class II-associated invariant chain peptide) region. An epitope inserted in this vector is efficiently presented in the context of co-expressed MHC class II molecules (21).

The T cell-suppressive molecules we tested were TNF-Related Apoptosis-Inducing Ligand (TRAIL) and Programmed Death-1 Ligand (PD-L1). TRAIL is a member of the TNF superfamily and is constitutively expressed in a variety of cell types including lymphocytes, NK cells, and neural cells. $TRAIL^{-/-}$ mice are hypersensitive to collagen-induced arthritis and streptozotocin-induced diabetes. PD-L1, a ligand for PD-1 and member of the CD28/CTLA-4 family, is expressed on DC, IFN-γ-treated monocytes, activated T cells, placental trophoblasts, myocardial endothelium, and cortical thymic epithelial cells. $PD\text{-}1^{-/-}$ mice spontaneously develop a lymphoproliferative / autoimmune disease, a lupus-like disease, arthritis, and cardiomyopathy (22). Thus, abrogation of either of these two molecules make mice autoimmune-prone, suggesting that these molecules play significant roles in maintaining immunological self-tolerance in physiological situations.

ES cells were sequentially transfected with an expression vector for TRAIL or PD-L1 and an MHC class II-associated invariant chain-based MOG epitope-presenting vector. Subsequently, double-transfectant ES cell clones were induced to differentiate into ES-DC expressing the introduced genes, ES-DC-TRAIL/MOG and PDL1/MOG.

The severity of MOG peptide-induced EAE was significantly reduced in mice pre- or post-treated with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG. The severity of myelin basic protein-induced EAE was also reduced by ES-DC-TRAIL/MOG but not by PD-L1/MOG. In addition, adoptive transfer of $CD4^+$ T cells from ES-DC-TRAIL/MOG-treated mice protected the recipient mice from subsequent induction of EAE, thus suggesting the involvement of $CD4^+$ T cells with immune-regulatory function in the disease-preventative effect of ES-DC-TRAIL-MOG (23). These results demonstrate the prevention of autoimmune disease by treatment with ES-DC expressing target antigen along with immune-suppressive molecules, and also suggest the potential benefits of future clinical application of this technology.

Currently, corticosteroids and other immune suppressants are commonly used for treatment of subjects with autoimmune diseases. The medication with these drugs often leads to systemic immune suppression and consequent opportunistic infections. Thus, it is desirable to develop a therapeutic means to down-modulate immune responses in an antigen-specific manner without causing systemic immune suppression. Our results demonstrated that genetically modified ES-DC expressing antigenic protein along with immune-regulatory molecules control autoimmunity without causing general immune suppression.

In the clinical settings, a lager number of immune cells may be necessary to control autoimmunity by administration of immune regulatory cells. Although *in vitro* expanded antigen-specific T cells with regulatory function (Treg cells) may be useful for such purpose, it is currently difficult to expand human Treg cells specific to a certain antigen. On the other hand, unlimited number of DC with immune regulatory functions can be generated by using pluripotent stem cells as cell source.

## 6. GENERATION OF DC FROM HUMAN ES CELLS

In 2004, Zhan and colleagues reported the generation of functional antigen presenting cells from

human ES cells (24). Similar to the method of generating dendritic cells from mouse ES cells as described by Fairchild and colleagues, Zhan and colleagues induced the formation of EB by suspension cultures of human ES cells, and then cultured the EB in the presence of hematopoietic cytokines to generate leukocytes with antigen presenting functions.

In their method, human ES cells were cultured at a high density on Matrigel before induction of differentiation. After reaching confluency, undifferentiated ES cell colonies were harvested with dispase. Cell colonies were cultured in medium for induction of differentiation with fetal calf serum and without basic Fibroblast Growth Factor (bFGF). To inhibit adherence to the plastic surface of the culture plates, ES cell colonies were plated in low-attachment plates. Cystic embryonic bodies emerged after 5-20 days in the suspension culture. When harvested at days 10-20, 50-80% of EB were cystic, each EB containing apporximately 10,000 cells.

Whole EB formed in suspension were transferred onto tissue culture plates and allowed to differentiate into hematopoietic and other cell types. To stimulate production of hematopoietic progenitor cells and also dendritic cells, they added Stem Cell Factor (SCF), FLT3-ligand, and Thrombopoietin (TPO), cytokines widely used to maintain human postnatal hematopoietic stem cells. To expand dendritic cell-committed progenitor cells, IL-3, GM-CSF, and IL-4 were added. A part of the resulting leukocytes expressed MHC class II, CD80, CD86, CD40, and CD83. The MHC class $II^+$ leucocytes resembled dendritic cells and macrophages. They functioned as stimulator cells capable of eliciting allogeneic T-cell responses in culture.

After the above study was published, Su and colleagues and Tseng and colleagues reported similar methods of EB-mediated generation of DC from human ES cells (25) (26).

We generated ES-DC from human ES cells by using OP9 feeder layers (27). The protocol for the differentiation culture to generate ES-DC from human ES cells developed in our study is composed of 3 steps. At the beginning of the differentiation culture, undifferentiated ES cells were plated on OP9 feeder cell layers. Thereafter, the ES cells grew and formed clusters composed mainly of epithelial cell-like large flat cells. Clusters of round, cobble stone-like cells also appeared at about day 8. The size and number of round cell clusters gradually increased, and, by around day 15, covered 20-30 % of the surface area.

On days 15-18 of step 1, cells were recovered from the dishes using trypsin/EDTA and non-adherent cells were isolated, and then the cells were seeded onto freshly prepared OP9 cell layers, beginning step 2. The culture medium containing GM-CSF and M-CSF was used in this step. Thereafter, small round cells, floating or loosely adhering to the feeder layer, appeared and gradually increased in number. The growth of the round cells depended primarily upon GM-CSF, thus suggesting that they grew in response to that factor. These cells expressed CD31, CD43, and CD11b, thus collectively indicating a commitment to a myeloid cell lineage.

On days 7-10 of step 2, the floating or loosely adherent cells were harvested by pipetting and they were transferred to Petri dishes without feeder cells and cultured in the presence of GM-CSF and IL-4 (step 3). Following this passage, the cells changed their morphology from round to irregular shape, and some cells displayed protrusions. Cells with protrusions gradually increased and more than 50% of the cells exhibited a DC-like irregular shape after 2-3 days. The floating cells expressed CD86 and CD40, but scarcely expressed CD80 and CD83.

The simultaneous addition of TNF-α, LPS, soluble CD40-ligand, and IL-4 to the cells induced maturation. Flow cytometric analysis revealed increased expression of CD86 and the expression of CD80, CD83, and HLA-DR. Collectively, the cells exhibited the characteristics of DC in morphology and expression of surface molecules, and thus they were designated as human ES-DC. OK-432 is a Streptococcus-derived immunotherapeutic agent and known to be very potent in stimulating dendritic cells to produce IL-12. The production of TNF-α was profoundly induced by either LPS or OK432. OK432 but not LPS induced the production of IL-12, consistent with reports that OK432 is an efficient inducer of IL-12 (28) (29). Generation of ES-DC from the non-human primate, cynomolgus monkey, ES cells was also achieved by this method (27).

Slukvin and colleagues preceded us in reporting a method for generation of human ES cell-derived DC using OP9 feeder layers (30). Slukvin's method is also composed of 3 steps and is different from ours in that OP9 feeders were used only in the first step.

The capacity of the human ES-DC to stimulate T cells was examined based on the proliferative response of allogeneic T cells co-cultured with ES-DC. ES cell-derived floating cells recovered from the 2nd step (pre-ES-DC) showed little capacity to induce a response of T cells. In contrast, ES-DC following the 3rd step before the addition of maturation stimuli (immature ES-DC) showed a weak but definite stimulation, and following exposure to maturation stimuli (mature ES-DC) showed a strong capacity to stimulate allogeneic T cells to proliferate. The antigen presenting capacity of ES-DC was confirmed by their ability to present Glutamic Acid Decarboxylase 65 (GAD65) antigen to GAD65-specific HLA-DR53-restricted human T cell clone, SA32.5 (31).

Su and colleagues introduced mRNA for green fluorescence protein (GFP) or prostate-specific antigen (PSA) into DC generated from human ES cells (25). They showed induction of CTL specific to the antigens by the *in vitro* stimulation of semi-allogeneic HLA-A*0201$^+$ donor-derived T cells with genetically modified DC.

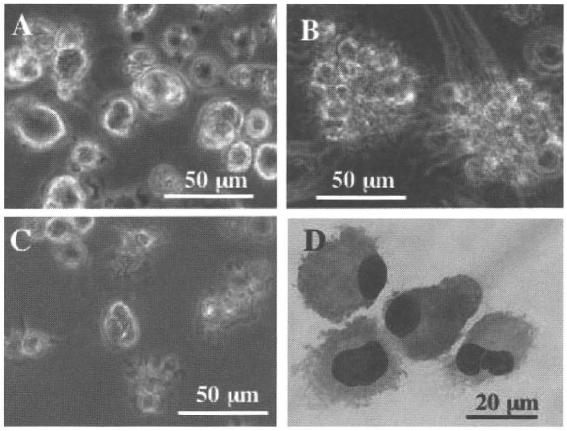


**Figure 1.** Morphology of mouse iPS–DC. Phase-contrast images of immature iPS-DC (A) and mature iPS-DC (B, C) are shown. (D) Mature iPS-DC on a glass slide stained with May-Grunwald-Giemsa are shown.

## 7. GENERATION OF DC FROM IPS CELLS

As described so far, we can generate ES-DC from both mouse and human ES cells. Mouse systems have demonstrated the induction of anti-cancer immunity and the prevention of autoimmune disease by *in vivo* administration of genetically engineered ES-DC.

Considering the future clinical application of ES-DC technology, however, the unavailability of human ES cells genetically identical to the patients to be treated is a problem. Alloreactivity caused by differences in the genetic background between human ES cell lines and the recipients is a critical problem in medical application of ES-DC. In addition, ethical concerns related to the use of human ES cells are anticipated to be serious obstacles, which will hinder the realization of the medical use of human ES-DC.

It was recently revealed that ES cell-like pluripotent stem cells, designated as induced pluripotent stem (iPS) cells, can be generated by the simultaneous introduction of several factors into somatic cells, for both mice and humans (32) (33) (34). The issue of histoincompatibility between patients to be treated and ES cells may be overcome by the generation of iPS cells from somatic cells of the patients such as fibroblasts or blood cells. The major ethical issues related to human ES cells may be avoided by the use of iPS cell-technology, because the use of human embryos is not necessary for the generation of iPS cells.

We recently reported the generation and characterization of DC derived from mouse iPS cells (35). The iPS cell-derived DC (iPS-DC) possessed the characteristics of DC including morphology (Figure 1), the capacity for T cell-stimulation, antigen-processing and presentation, and cytokine production. There was some delay in the kinetics of differentiation process of iPS cells, in comparison to the mouse ES cell lines that we have tested. On the other hand, the yield of differentiated cells was higher than that in the cases of most mouse ES cell lines. Genetically modified iPS-DC expressing antigenic protein primed T cells specific to the antigen *in vivo* and elicited efficient antigen-specific anti-tumor immunity.

Using the OP9-co-culture-based method, Choi and colleagues generated various myelomonocytic cells including DC, macrophages, osteoclasts, neutrophils, and eosinophils from human iPS cells (36). We have also recently generated iPS-DC from human iPS cells. Human iPS-DC exhibited characteristics of DC, as human ES-DC do, in morphology, surface molecules, and T cell-stimulating capacity. Theoretically, we can now generate an infinite number of DC genetically identical to the patients by using iPS cells generated from relatively small numbers of somatic cells of the patients, such as dermal fibroblasts or blood cells.

The iPS cell technology provides unlimited cell source of DC for immune therapy. However, safety issues related to tumor formation need to be clearly resolved before clinical applications. Currently, we are using mouse-derived OP9 feeder cells and culture media containing fetal calf serum in the differentiation culture to generate DC from human iPS cells or ES cells. For the clinical application of iPS-DC, it is also desirable to develop a fully xeno-free culture procedure to generate iPS-DC.

We will circumvent the issue of histocompatibility between patients and DC to be used for the treatment, if iPS cell lines are generated from somatic cells of the patients. However, generation of clinical grade iPS cells for individual patients may be too costly, time consuming, and labor-intensive to be broadly applied. This may be resolved by the establishment of banks of iPS cells covering major HLA-haplotypes in each ethnic group (37). A bank of iPS cells with specific HLA-A,HLA-B, and HLA-DR haplotypes is feasible and may be more realistic resolution of the issue of histocompatibility than "fully personalized" iPS cells. According to the calculation by Nakatsuji and colleagues, a cell bank size of 30 iPS cell lines homozygous for the HLA haplotype would be able to find a three-locus match in 82.2 % of the Japanese population. Their calculation also indicates that at least one homozygote for each of 30 different haplotypes can be identified by examining 15,000 individuals in an HLA-type database.

## 8. SUMMARY

Because of the activity of DC as immune-regulatory cells, immune-modulation therapy by transfer of DC is regarded as promising to treat various diseases. In particular, DC-based cellular vaccination is considered to be a powerful means for active immunization therapy for cancer. ES cells possess a potential to differentiate into various kinds of cells and also an unlimited propagation capacity. Several groups including us have established methods to generate DC from mouse or human ES cells. Using mouse models, we have demonstrated the usefulness of genetically modified ES cell-derived DC in the induction of anti-cancer immunity and also in the control of

autoimmune disease. Recently, it has been discovered that ES cell-like pluripotent stem cells can be generated by introduction of several reprogramming factors into somatic cells, yielding iPS cells. DC can be generated from mouse and human iPS cells by the methods developed for ES cells. By the use of iPS cells instead of ES cells as the cell source of DC, we can overcome the issues of histoincompatibility and resolve ethical concerns related to human ES cells. For the clinical application of iPS cell-derived DC, it is necessary to develop a xeno-free culture procedure to generate iPS-DC.

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**Key Words:** Dendritic Cells, Emboryonic Stem Cells, iPS Cells, Cell Therapy, Review

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Int J Hematol (2010) 91:392–400
DOI 10.1007/s12185-010-0520-1



# Pluripotent stem cells as source of dendritic cells for immune therapy

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Received: 29 October 2009 / Revised: 24 November 2009 / Accepted: 26 November 2009 / Published online: 13 February 2010


**Abstract** Dendritic cells (DC) are the most potent antigen-presenting cells. In vivo transfer of antigen-bearing DC has proven efficient in priming T cell responses specific to the antigen. DC-based cellular vaccination is now regarded as a powerful means for immunotherapy, especially for anti-cancer immunotherapy. Clinical trials of therapy with DC pulsed with peptide antigens or genetically modified to present antigens are currently carried out in many institutions. In addition, antigen-specific negative regulation of immune response by DC is considered to be a promising approach for treatments of autoimmune diseases and also for regulation of allo-reactive immune response causing graft rejection and GVHD in transplantation medicine. DC for transfer therapy are now generated by in vitro differentiation of peripheral blood monocytes of the patients. However, there is a limitation in the number of available monocytes, and the DC-differentiation potential of monocytes varies depending on the blood donor. Embryonic stem (ES) cells possess both pluripotency and infinite propagation capacity. We consider ES cells to be an ideal source for DC to be used in immunotherapy. Several groups, including us, have developed methods to generate DC from ES cells. This review introduces the studies on generation, characterization, and genetic modification of DC derived from ES cells or induced pluripotent stem (iPS) cells. The issues to be resolved before clinical application of pluripotent stem cell-derived DC will also be discussed.

**Keywords** Dendritic cells · Embryonic stem cells · iPS cells · Cell therapy

## 1 Introduction

Dendritic cells (DC) are the most potent antigen-presenting cells responsible for priming of naive T cells in the immune response. DC are also involved in the maintenance of immunologic self-tolerance in the periphery, inducing regulatory T cells or anergy of autoreactive T cells. We can say that DC physiologically play a central role in immune-regulation. Manipulation of functions of DC by genetic modification and in vivo transfer of DC with modified property is considered a promising means to therapeutically manipulate immune responses in an antigen-specific manner.

Embryonic stem (ES) cells are characterized by pluripotency and infinite propagation capacity. Non-virus-mediated methods for gene transfer, including targeted gene integration and procedures for isolation of appropriate transfectant cell clones, have been established for ES cells. Genetic modification of ES cells and their subsequent in vitro differentiation to DC would be an attractive strategy for genetic manipulation of DC and for analysis of gene functions in DC.

For hematopoietic differentiation of ES cells in vitro, embryoid body (EB)-mediated methods and the feeder cell-coculture method have been established. OP9 is a bone marrow stromal cell line that originated from macrophage

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colony stimulating factor-defective op/op mouse, and generation of various hematopoietic cells from ES cells using OP9 cells as feeder cells has been reported, including granulocytes, erythrocytes, and B lymphocytes [1]. The method has been applied to a number of molecular and cellular analyses for investigations of hematopoiesis.

We and others developed methods to generated DC from mouse ES cells, and the functions of ES cell-derived DC (esDC or ES-DC), including stimulation of allogeneic T cells, processing and presentation of antigenic proteins, and migration upon in vivo transfer, were comparable to those of DC generated in vitro from bone marrow cells [2, 3]. We have also established a strategy for the genetic modification of mouse ES-DC. Expression vectors were introduced into ES cells by electroporation and subsequently the transfectant ES cell clones were induced to differentiate to ES-DC. The studies using mice have demonstrated that in vivo transfer of genetically engineered mouse ES-DC is very effective for modulation of immune responses both positively and negatively. It is possible to induce anti-cancer immunity [4–9] and prevent autoimmune disease [10, 11] in mouse models with genetically engineered ES-DC. Looking toward the future clinical application of ES-DC technology, a method was developed to generate ES-DC also from human ES cells [12].

## 2 Generation of dendritic cells from mouse ES cells

### 2.1 Generation of dendritic cells from mouse ES cells by embryoid body-mediated hematopoietic differentiation

In 2000, Fairchild and his colleagues reported the first study on the generation of functional DC from mouse ES cells [2]. They used ESF116 mouse ES cell line derived from a CBA/Ca blastocyst. In their method, at first, EB were formed, and subsequently differentiation of DC was induced by addition of specific cytokines.

At the initiation of the differentiation, ES cells were grown in suspension to allow EB formation. After culture for 14 days, EB were cultured in medium supplemented with granulocyte/macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-3. Fairchild and colleagues observed that the combination of the cytokines uniquely supported the development of cells with an appearance suggestive of DC within 4 days of culture. These cells expanded rapidly over the following 7–10 days to form lightly adherent clusters, reminiscent of those observed in cultures of immature DC derived from bone marrow cells. Cells released from these clusters seeded uncolonised areas of the dish and displayed typical dendritic morphology, and they were designated as esDC.

Flowcytometric analysis showed that esDC expressed CD11c, MHC class II, CD40, CD54, CD80, and CD86. On the other hand, expression of CD8 and Dec-205 (CD205) was not observed, suggesting esDC were of myeloid lineage. RT-PCR analysis revealed that esDC expressed mRNA for IL-20, IL-18, and IL-1$\beta$ converting enzyme (ICE). They possessed a potent capacity to process a protein antigen, hen egg lysozyme (HEL), and present the resultant epitope in the context of H-2E$^k$, shown by the response of co-cultured antigen-specific T cell hybridoma cells. Upon co-culture with allogeneic T cells, esDC stimulated the T cells to proliferate vigorously, indicating strong T cell-stimulating capacity of esDC. Collectively, the ES cell-derived cells with DC-like morphology were fully functional in antigen-presentation and T cell-stimulation.

### 2.2 Generation of dendritic cells from mouse ES cells by aid of OP9-coculture-based hematopoietic differentiation

We established a method to generate DC from mouse ES cells using OP9 stromal cells and our protocol is shown in Fig. 1 [3]. Mouse ES cells, maintained on primary mouse embryonic fibroblast feeder layers in the presence of leukemia inhibitory factor (LIF), were transferred onto the OP9 cell layers and cultured in $\alpha$MEM medium containing fetal calf serum without exogenous cytokines. ES cell colonies changed their morphology from undifferentiated dome-like to a differentiated flattened one in 4–5 days (Fig. 2a, b). To induce differentiation to DC, the mesodermally differentiated ES cell-derived cells harvested from a 5 or 6-day culture on OP9 feeder layers were cultured on fresh OP9 cell layers in the presence of exogenous GM-CSF. In comparison with the culture without exogenous GM-CSF, addition of this cytokine resulted in appearance of a larger number of floating cells in a few days, indicating that cells grew responding to GM-CSF. Culture without GM-CSF at this step resulted in generation of relatively small numbers of various types of hematopoietic cells (Fig. 2c–e).

On days 8–9 (3 days after the transfer), we observed many round and relatively homogenous floating cells (Fig. 3a), and most of them expressed CD11b, suggesting their commitment to myeloid cell lineage. On day 10 or 11, we recovered the cells floating or loosely adherent to feeder cells and transferred to bacteriologic Petri dishes without feeder cells. After this passage, some of the transferred cells adhered to the dish surface and resembled macrophages. On days 15–18, floating cells could be divided roughly into 2 types, 1 with a round shape and of a larger size and the other smaller and irregularly shaped with protrusions (Fig. 3b–d). In addition, clusters of floating cells (Fig. 3e) of the latter type were observed after days

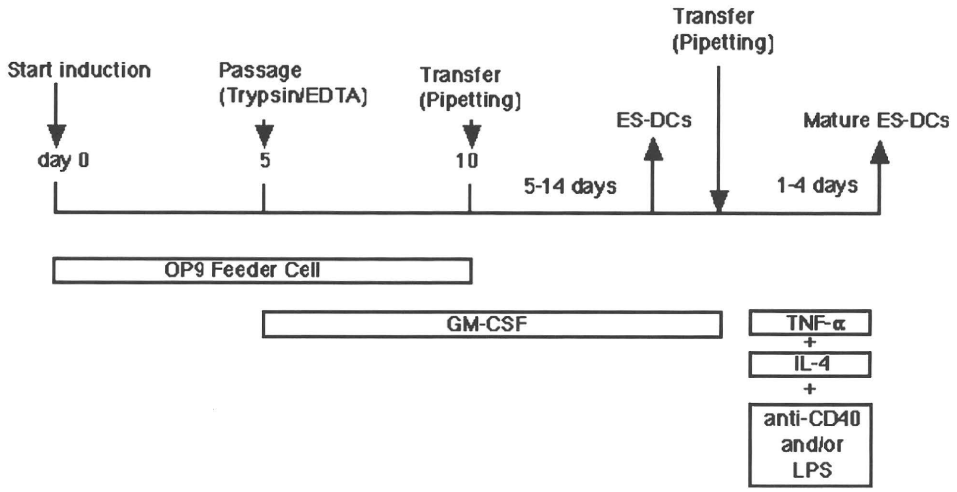


**Fig. 1** Schema of the culture protocol for generation of mouse ES-DC. To initiate differentiation, undifferentiated ES cells were suspended in α-MEM supplemented with 20% fetal calf serum (*FCS*) and seeded onto OP9 cell layers. On days 5–6, cells were harvested using trypsin/EDTA, reseeded onto fresh OP9 cell layers, and cultured in α-MEM supplemented with FCS and GM-CSF. 5–6 days after the transfer, floating cells were recovered by pipetting. At this step, 100–200 times more numbers of cells as compared with undifferentiated ES cells was recovered. The recovered cells were transferred to bacteriologic Petri dishes without feeder cells and cultured in RPMI-1640 medium supplemented with 10% FCS, GM-CSF. After 5–10 days, floating or loosely adherent cells were recovered by pipetting (ES-DC). To induce a complete maturation of ES-DC, cells cultured for longer than 10 days in Petri dishes were transferred to fresh Petri dishes and IL-4, TNF-α, plus anti-CD40 mAb, or IL-4, TNF-α, plus lipopolysaccharide were added

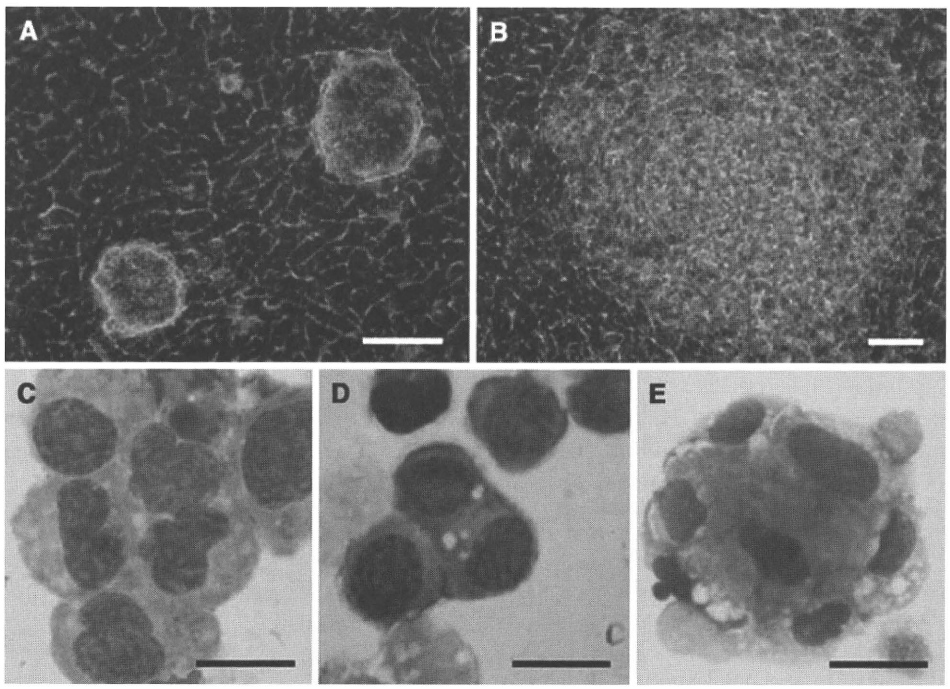


**Fig. 2** Hematopoietic differentiation of TT2 ES cells on feeder cell layers of OP9. Phase-contrast micrographs of TT2 ES cell colonies on OP9 feeder cell layers on day 3 (**a**) and day 5 (**b**) are shown. **c–e** May–Giemsa staining of cytospin specimens of hematopoietic cells derived from TT2 ES cells. TT2 cells were cultured on OP9 feeder cell layer for 15 days in total, without addition of exogenous cytokines. Floating cells were applied to cytospin preparations and stained with May–Giemsa. Cells of myeloid (**c**), erythroid (**d**), and megakaryocytic (**e**) lineages are shown. *Scale bars* represent 50 μm (**a**, **b**) and 20 μm (**c–e**) (the photo images were originally published in [3])

17–19, and the cell clusters gradually increased. Addition of maturation stimuli, IL-4, TNF-α, plus anti-CD40 antibody or LPS, to the cells resulted in typical morphology of mature DC (Fig. 3f–h).

Floating cells were positive for MHC class I, MHC class II, CD80, CD86, DEC205, and CD11c. Upon stimulation with LPS they matured and became highly competent to stimulate T cells. Characteristics of the EB-derived esDC seemed to be similar to those of our ES-DC. The cells had capacity to process and present protein antigen to T cells. They were very potent in stimulating allogeneic T cells to proliferate (primary mixed lymphocyte reaction). Based on the morphology, surface phenotype, and function, we referred to the cells with protrusions as ES-DC. ES-DC

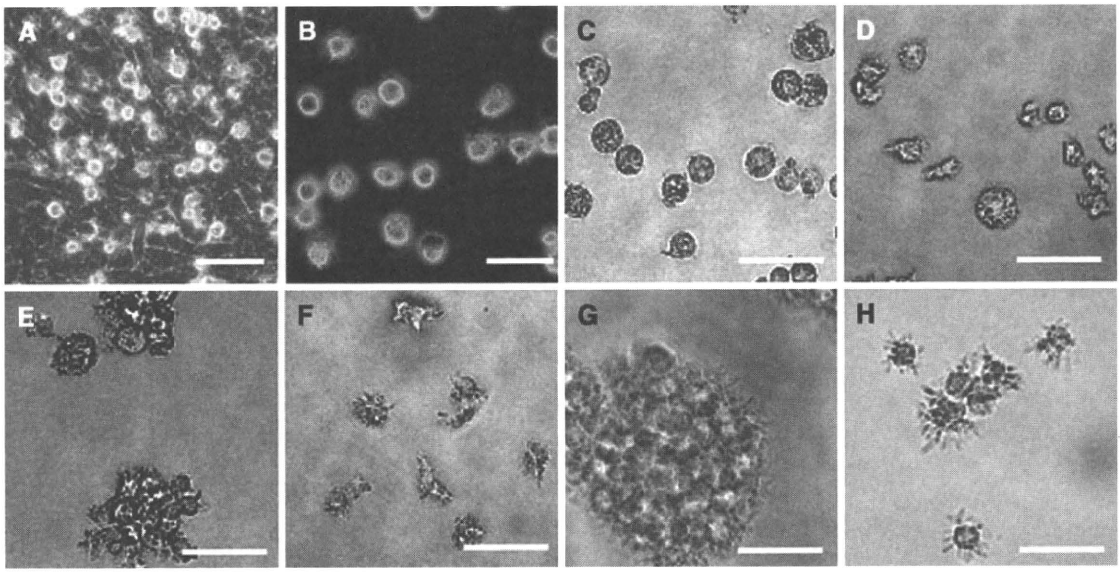


**Fig. 3** Morphology of ES–DC. ES cell-derived cells on day 8 (**a**), day 12 (**b**, **c**), day 17 (**d**, **e**), and day 27 (**f**) of differentiation culture are shown. Cells on day 24 were recovered and stimulated for 2 days with IL-4, TNF-α, plus agonistic anti-CD40 mAb (**g**), or with IL-4, TNF-α, plus LPS (**h**). **a**, **b** Phase-contrast micrographs. *Scale bars* represent 20 μm (the photo images were originally published in [3])

were positive for F4/80 and CD11b and negative for CD8, suggesting that they were of myeloid lineage.

On days 5–10 of the differentiation culture (2nd step), DC could also differentiate when PA6 or ST2 cells were used as feeder cells. However, the number of generated DC was fewer and the phenotype of the generated DC somewhat differed. With PA6 or ST2, generated DC did not express CD80 and CD205, and their activity to stimulate MLR was weaker than that of DC produced with OP9. The use of dishes treated for tissue culture after transfer from the OP9 feeder cell layer (after day 10 in Fig. 1) gave rise to a fewer number and a lower purity of ES-DC than did the use of bacterial-quality Petri dishes. If we used dishes of tissue-culture grade, many cells firmly adhered to the dish surface, resembling macrophages or fibroblasts, and inhibited the generation of ES-DC. The beneficial effect of bacterial-quality Petri dishes to DC-development has been noted also in generation of DC from mouse bone marrow cells. GM-CSF has been reported to be essential for in vitro generation of DC from hematopoietic cells and is also necessary for generation of ES-DC. We applied this culture protocol to various mouse ES cell lines, including TT2, E14, J1, D3, R1, CCE, and NOD mouse-derived ES cells, and observed that all of these lines also differentiated to ES-DC.

## 3 Studies on immune-therapy with ES-DC

### 3.1 Priming of antigen-specific cytotoxic T cells with genetically modified ES-DC

Genetic modification of ES-DC can readily be done by gene-introduction into undifferentiated ES cells, isolation of proper transfectant ES clones, and subsequent induction of differentiation into ES-DC. The capacity of TT2 ES cell-derived ES-DC introduced with an ovalbumin (OVA)-expression vector to prime OVA-specific T cells in vivo was analyzed [3]. ES-DC with or without OVA expression vector were injected intraperitoneally into syngeneic (C57BL/6 × CBA) F1 mice twice with a 7-day interval. Splenocytes were isolated 7 days after the second injection and cultured in vitro in the presence of OVA257–264 peptide, the major H-2K$^b$-restricted epitope derived from OVA protein. After 5 days, cultured cells were assayed for their capacity to kill EL-4 thymoma cells (H-2$^b$) prepulsed with the OVA peptide. The results shown in Fig. 4 indicated that cytotoxic T cells (CTL) specific to the OVA epitope were primed in vivo with ES-DC expressing OVA protein (ES-DC-OVA), but not with ES-DC without OVA expression. These results demonstrate that ES-DC genetically engineered to express an antigenic protein have the capacity to prime antigen-specific CTL in vivo.

### 3.2 Enhanced priming of antigen-specific CTL in vivo by ES-DC expressing chemokine along with antigenic protein

In immunotherapy by using DC, such as anti-cancer immunotherapy, the efficiency of T cell-priming in vivo by transferred DC is critical. T cell-priming in vivo by injected DC should depend on their encounter with T cells. When exogenous antigen was injected intracutaneously, about 25% of the DC capturing the antigen migrated to the T cell area of draining lymph nodes, where they present antigen to prime naive T cells specific to the antigen.

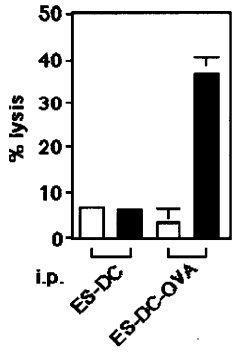


**Fig. 4** Priming of OVA-specific CTL by ES-DC expressing OVA in vivo. ES-DC with (ES-DC-OVA) or without (ES-DC) expression of OVA protein were injected intraperitoneally on days 0 and 7 into syngeneic F1 mice. Splenocytes from the injected mice were harvested on day 14 and cultured in the presence of OVA257–264 (0.1 μM) for 5 days. The resultant cells were assayed for the capacity to lyse EL-4 tumor cells either pulsed with 10 μM OVA peptide (*closed bars*) or left unpulsed (*open bars*)

On the contrary, when BM-DC or splenic DC are transferred exogenously by s.c. or i.p. injection, the absolute number of the DC found within the draining lymph nodes represented only a small proportion (0.1–1%). It has been also reported that almost all of transferred DC remained at the s.c. immunization site 24 h after transfer. The inefficient migration of the exogenous DC to lymphoid organs may lower the frequency of their encounter with T cells. It may be possible to improve the efficacy of exogenously transferred DC to prime immune responses by augmenting their encounter with T cells. For example, if transferred DC produce chemokines to intensively attract T cells, they may prime immune response efficiently, even though the DC do not migrate to lymphoid organs. We attempted to improve the capacity of DC to prime T cells in vivo by genetic modification to express chemokine with T cell-attracting property. We generated double-transfectant DC expressing a chemokine along with the model antigen, OVA, by sequential transfection of ES cells with 2 kinds of expression vectors and then inducing differentiation to DC [4].

Several kinds of chemokines with capacity to attract T cells are produced by different cell types. CCL21 (secondary lymphoid tissue chemokine, SLC) is produced in T cell regions of lymph nodes and spleen and also by high endothelial venules in lymph nodes. SLC chemoattracts T cells, NK cells, B cells, and DC. CXCL9 (monokine induced by IFN-$\gamma$, Mig) is produced by macrophages and binds to the chemokine receptor CXCR3, which mediates the recruitment of predominantly Th1 cells and activated NK cells. XCL1 (Lymphotactin, Lptn), produced by activated T cells, has chemoattractive properties on $CD4^+$ and $CD8^+$ T cells and on NK cells. We comparatively evaluated the effect of these 3 kinds of chemokines.

All three types of double transfectant ES-DC primed OVA-specific CTL in vivo more efficiently than did ES-DC expressing only OVA, and co-expression of SLC or Lptn was more effective than that of Mig. Immunization with DC expressing OVA plus SLC or Mig provided protection from OVA-expressing tumor cells more potently than that with OVA only, and SLC was more effective than Mig. On the other hand, co-expression of Lptn gave no additive effect on protection from the tumor. collectively, among the three chemokines, expression of SLC was the most effective in enhancing anti-tumor immunity by transferred ES-DC in vivo.

### 3.3 Negative manipulation of immune response for the treatment of autoimmune disease

For the treatment of subjects with autoimmune or inflammatory diseases, it is desirable to down-modulate immune responses in an antigen-specific manner and without causing systemic immune suppression. GVHD and graft rejection are the most serious problems in transplantation medicine, and control of allo-reactive immune response is the key to overcome them. Antigen-specific negative regulation by DC is considered to be promising means in these fields. We investigated whether we could negatively manipulate immune response with genetically modified ES-DC expressing specific antigen along with immune-suppressive molecules.

Experimental autoimmune encephalomyelitis (EAE), characterized by neurological impairment resulting from de-myelination in the central nervous system caused by activated myelin antigen-reactive $CD4^+$ T cells, is induced by immunization with myelin antigens such as myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein (MBP). We tried to prevent MOG-induced EAE by pre-treatment of mice with genetically modified DC presenting MOG peptide in the context of MHC class II molecules and simultaneously expressing molecules with T cell-suppressive property [10].

For efficient presentation of MOG peptide in the context of MHC class II molecules, we used a previously devised expression vector in which cDNA for human MHC class II-associated invariant chain (Ii) was mutated to contain antigenic peptide in the class II-associated invariant chain peptide (CLIP) region. An epitope inserted in this vector is efficiently presented in the context of co-expressed MHC class II molecules [13].

As T cell-suppressive molecules, we tested TNF-related apoptosis-inducing ligand (TRAIL) and programmed death-1 ligand (PD-L1). TRAIL as a member of the TNF superfamily is constitutively expressed in a variety of cell types, including lymphocytes, NK cells, and neural cells. $TRAIL^{-/-}$ mice are hypersensitive to collagen-induced

arthritis and streptozotocin-induced diabetes. PD-L1, a ligand for PD-1 and member of the CD28/CTLA-4 family, is expressed on DC, IFN-$\gamma$-treated monocytes, activated T cells, placental trophoblasts, myocardial endothelium, and cortical thymic epithelial cells. PD-$1^{-/-}$ mice spontaneously develop a lymphoproliferative/autoimmune disease, a lupus-like disease, arthritis, and cardiomyopathy [14]. Thus, abrogation of either of these two molecules makes mice autoimmune-prone, suggesting that these molecules play significant roles in maintaining immunological self-tolerance in physiological situations.

ES cells were sequentially transfected with an expression vector for TRAIL or PD-L1 and an MHC class II-associated invariant chain-based MOG epitope presenting vector. Subsequently, double-transfectant ES cell clones were induced to differentiate into ES-DC expressing the introduced genes, ES-DC-TRAIL/MOG, and PDL1/MOG.

Severity of MOG peptide-induced EAE was significantly reduced in mice pre- or post-treated with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG. The severity of MBP-induced EAE was also reduced by ES-DC-TRAIL/MOG but not PD-L1/MOG. In addition, adoptive transfer of $CD4^+$ T cells from ES-DC-TRAIL/MOG-treated mice protected the recipient mice from subsequent induction of EAE [11]. These results demonstrate the prevention of an autoimmune disease by the treatment with ES-DC expressing target antigen along with immune-suppressive molecules, and implicate future clinical application of this technology.

## 4 Generation of DC from human ES cells

### 4.1 Generation of DC from human ES cells based on EB formation

As for the generation of DC from human ES cells, the report by Zhan and colleagues [15] is the first. Similar to the method of generation of DC from mouse ES cells by Fairchild and colleagues, they induced formation of EB by suspension culture of human ES cells, and then cultured the EB in the presence of hematopoietic cytokines to generate leukocytes with antigen presenting function.

In their method, human ES cells were cultured at a highdensity onto Matrigel (Becton Dickson) before induction of differentiation. After reaching the full size or confluency, undifferentiated ES cell colonies were harvested with dispase. The cell colonies were cultured in medium for differentiation induction with fetal calf serum and without basic fibroblast growth factor (bFGF). To inhibit the adherence to the plastic surface of the culture plates, ES colonies were plated in special low-attachment plates. Cystic embryonic bodies emerged after 5–20 days in the suspension culture. When harvested on days 10–20, 50–80% of EB were cystic, each EB containing about 10,000 cells on average.

Whole EB formed in suspension were transferred onto tissue culture plates and allowed to differentiate into hemopoietic and other cell types. To stimulate production of hemopoietic progenitor cells and also DC, they added stem cell factor (SCF), FLT3-ligand, and thrombopoietin (TPO), cytokines widely used to maintain human postnatal haemopoietic stem cells. To expand DC-committed progenitor cells IL-3, GM-CSF, and IL-4 were added.

A part of the resulting leucocytes expressed MHC class II, CD80, CD86, CD40, and CD83. The MHC class $II^+$ leucocytes resembled DC and macrophages. They functioned as stimulator cells capable of eliciting allogeneic T cell responses in culture.

After this report, Su and colleagues [16] and also Tseng and colleagues [17] reported similar methods of EB-mediated generation of DC from human ES cells.

### 4.2 Generation of human ES-DC based on OP9-co-culture

Considering future clinical application of ES-DC, we also tried to generate ES-DC from human ES cells [12]. Based on experience in the generation of DC from mouse ES cells and also based on the findings in a preliminary study using cynomolgus monkey ES cells [18], feeder cell-co-culture method was adopted for the generation of DC from human ES cells, instead of the EB based method. The human ES cell line selected was KhES-1 which exhibited the highest growth rate and stability among the 3 lines of human ES cell lines established by Suemori and colleagues [19] at Kyoto University. For feeder cells, 3 lines of mouse stromal cell lines, ST2, OP9, and PA6 were evaluated for their capacity to induce hematopoietic differentiation of KhES-1 ES cells, and OP9 had the best yield among them.

The protocol for the differentiation culture to generate ES-DC from human ES cells developed in our study is composed of 3 steps. At the beginning of the differentiation culture, undifferentiated ES cells maintained on mouse PEF feeders were harvested using dissociation solution, CTK, and plated on OP9 feeder cell layers (step 1). Next, the ES cells grew and formed clusters composed mostly of epithelial cell-like large flat cells. Clusters of round, cobble stone-like cells also appeared on about day 8. The size and number of round cell clusters gradually increased, and, around day 15, covered 20–30% of the surface area.

On days 15–18 of the step 1 culture, cells were recovered from the dishes using trypsin/EDTA and isolated non-adherent cells, and then they were seeded onto freshly prepared OP9 cell layers, to begin step 2. Culture medium containing GM-CSF and M-CSF was used in this step.

Thereafter, small round cells, floating or loosely adhering to the feeder layer, appeared and gradually increased in number. The growth of the round cells depended primarily upon GM-CSF, thus suggesting that they grew in response to that factor. The cells expressed CD34 and CD45, thus indicating that they followed a hematopoietic cell lineage. They also expressed CD31, CD43, and CD11b, thus collectively indicating a commitment to a myeloid cell lineage.

On days 7–10 of the step 2 culture the floating or loosely adherent cells were harvested by pipetting, and they were transferred to Petri dishes without feeder cells and cultured in the presence of GM-CSF and IL-4 (step 3). Following this passage, the cells changed their morphology from round to irregular shape, and some had protrusions. Cells with protrusions gradually increased and more than 50% of the cells exhibited DC-like irregular shape after 2-3 days. The floating cells expressed CD86 and CD40, but scarcely expressed CD80 and CD83.

Simultaneous addition of TNF-α, LPS, soluble CD40-ligand, and IL-4 to the cells induced maturation. Many of the cells formed aggregates. Flowcytometric analysis showed the increased expression of CD86 and the expression of CD80, CD83, and HLA-DR. Collectively, the cells exhibited the characteristics of DC in their morphology and expression of surface molecules, and thus they were designated as human ES-DC. Production of TNF-α was profoundly induced by either LPS or OK432. OK432, but LPS did not induce the production of IL-12, consistent with the reports that OK432 is an efficient inducer of IL-12.

ES-cell-derived floating cells first appeared during the 2nd step of the culture for differentiation (pre-ES-DC) and could readily be isolated by pipetting procedure. Their morphology, pattern of expression of surface molecules, and T cell-stimulation capacity continuously changed until the final maturation. To determine the change in gene expression associated with such changes in the phenotypes, the gene expression profiles of pre-ES-DC, immature ES-DC, and mature ES-DC were analyzed using cDNA microarrays. Consistent with the results of flowcytometric analysis, up-regulation of the expression of genes encoding for cell surface molecules such as HLA class I, HLA class II, CD86, and CD40 along with differentiation of ES-DC was observed. In addition, expression of the genes related to DC function including CD74/invariant chain, CCR7, and CCL17/TARC was increased during the differentiation. Clustering analysis indicates similarity between change of the gene expression pattern from monocytes to immature Mo-DC and that from pre-ES-DC to immature ES-DC as well as that from immature Mo-DC to mature Mo-DC and that from immature ES-DC to mature ES-DC.

The protocol of differentiation culture described to this point was originally developed using the KhES-1 line of human ES cells. This differentiation procedure was also applied to KhES-3, another human ES cell line. KhES-3 differentiation was similar to KhES-1 except that KhES-3 differentiated slightly more quickly than KhES-1, and a first step culture of 14–15 days was sufficient for the differentiation of KhES-3. Generation of ES-DC from non-human primate, cynomolgus monkey, ES cells was also achieved by this method [12].

Slukvin and colleagues [20] also reported a method using OP9. Slukvin's method is also composed of 3 steps and is different from ours in that OP9 feeders were used only in the first step. Recently, extending this study, they demonstrated generation of various myelomonocytic cells including DC, macrophages, osteoclasts, neutrophils, and eosinophils from human ES cells or induced pluripotent stem (iPS) cells [21].

### 4.3 Function of human ES-DC as antigen presenting cells

The capacity of the human ES-DC to stimulate T cells was examined based on the proliferative response of allogeneic T cells co-cultured with ES-DC. ES cell-derived floating cells recovered from the second step (pre-ES-DC) showed little capacity to induce a response of T cells. In contrast, ES-DC following the third step before the addition of maturation stimuli (immature ES-DC) showed a weak but definite stimulation, and following exposure to the maturation stimuli (mature ES-DC) showed a strong capacity to stimulate allogeneic T cells to proliferate.

Next, the antigen-presenting capacity of ES-DC was examined. KhES-1 is positive for the HLA-DRB4*0103 gene encoding β chain of HLA-DR53 molecule. Presumably, ES-DC derived from KhES-1 should express the DR53 molecule, and their ability to present antigen to DR53-restricted $CD4^+$ T cells was determined. KhES-1-derived ES-DC pre-loaded with GAD65-derived synthetic peptide stimulated GAD65-specific DR53-restricted human T cell clone, SA32.5 [22], to proliferate. To examine the capacity to process antigenic protein and present epitope, recombinant protein was used as the antigen. The SA32.5 T cell clone co-cultured with the ES-DC in the presence of recombinant GAD65 protein also showed proliferative response, thus indicating that ES-DC processed the antigenic protein and presented the epitope derived from the protein in the context of HLA class II molecules.

### 4.4 Genetic modification of human ES-DC

We applied the strategy for the genetic modification of ES-DC established by using mouse ES cells to human ES cells. Briefly, the expression vectors were introduced into ES

cells by electroporation and subsequently the transfectant ES cell clones were induced to differentiate to ES-DC.

ES-DC carrying an epitope-presenting vector and expressing recombinant human invariant chain (Ii/CD74), which included GAD65p115-127 in the CLIP region, was generated. It was expected that the epitope could be efficiently targeted on MHC class II pathway [13]. The vector was introduced into KhES-1 ES cells, and a transfectant clone, KhES-1-Ii23, highly expressing transgene-derived recombinant CD74 was selected by a flowcytometric analysis at the pre-ES-DC stage. The expression of CD74 was detected even in the non-transfectant pre-ES-DC, reflecting intrinsic expression of CD74. The transfectant exhibited an increased expression of CD74 in comparison with the non-transfectants, thus indicating additional expression of the molecule derived from the transgene. The ability of the transfectant ES-DC, ES-DC-Ii23 to stimulate the GAD-epitope-specific T cell clone, SA32.5, in the absence of antigenic peptide or protein was next examined. As a result, ES-DC-Ii23 stimulated SA32.5 T cells and induced their proliferation, thus demonstrating functional expression of the epitope-presentation vector in the transfectant ES-DC. The in vivo transfer of ES-DC transfected with this antigen-presenting vector is therefore expected to be useful for controlling the immune response in an antigen-specific manner.

Su and colleagues [16] introduced mRNA for green fluorescence protein (GFP) or prostate-specific antigen (PSA) into DC generated from human ES cells. They showed induction of CTL specific to the antigens by in vitro stimulation of semi-allogeneic, HLA-A*0201$^+$, donor-derived T cells with the genetically modified DC.

## 5 Generation of DC from mouse iPS cells

As described so far, we can generate ES-DC from both mouse and human ES cells. Mouse systems have demonstrated the induction of anti-cancer immunity and the prevention of autoimmune disease by in vivo administration of genetically engineered ES-DC.

Considering the future clinical application of ES-DC technology, however, the unavailability of human ES cells genetically identical to the patients to be treated is a problem. Allogenicity caused by differences in the genetic background between human ES cell lines and the recipients is a critical problem in medical application of ES-DC. In addition, ethical concerns related to the use of human ES cells are anticipated to be serious obstacles, which will hinder the realization of the medical use of human ES-DC.

It was recently revealed that ES cell-like pluripotent stem cells, designated as iPS cells, can be generated by the simultaneous introduction of several genes for re-programming factors, such as Oct3/4, Sox2, Klf4, and c-Myc, into somatic cells, for both mouse and human [23–25]. The issue of histoincompatibility between patients to be treated and ES cells may be overcome by the generation of iPS cells from somatic cells of the patients such as fibroblasts or blood cells. The major ethical issues related to human ES cells would be avoided by aid of iPS cell-technology, because the use of human embryos is not necessary for the generation of iPS cells.

Differentiation of iPS cells into various cells belonging to the 3 germ layers has been demonstrated by the analysis of teratomas generated from mouse and human iPS cells. In addition, the pluripotency of iPS cells is obvious by the contribution of iPS cell-derived cells to various organs of the chimeric mice developed from iPS cell-introduced blastocysts. As for the in vitro generation of cells of mesodermal lineage from iPS cells, differentiation into cardiac myocytes and endothelial cells from mouse iPS cells has been reported.

We recently reported the generation and characterization of DC derived from mouse iPS cells [26]. The iPS cell-derived DC (iPS-DC) possessed the characteristics of DC including the capacity of T cell-stimulation, antigen-processing and presentation, and cytokine production. DNA microarray analyses revealed the up-regulation of genes related to antigen-presenting functions during differentiation into iPS-DC and similarity in gene-expression profile in iPS-DC and bone marrow cell-derived DC. There was some delay in the kinetics of differentiation process of iPS cells, as in comparison with most of mouse ES cell lines. On the other hand, the yield of differentiated cells was higher than that in the cases of most of mouse ES cell lines. Genetically modified iPS-DC expressing antigenic protein primed T cells specific to the antigen in vivo and elicited efficient antigen-specific anti-tumor immunity.

## 6 Future direction

We recently succeeded in generating iPS-DC from human iPS cells. Human iPS-DC exhibited characteristics of DC, as human ES-DC do, in morphology, surface molecules, and T cell-stimulating capacity. Theoretically, we can now generate infinite number of DC genetically matched to the patients by using iPS cells generated from relatively small number of somatic cells of the patients, such as dermal fibroblasts or blood cells.

Considering clinical application of iPS-DC, the technical issue to be resolved is that we are still using mouse-derived OP9 feeder cells and culture medium containing fetal calf serum for the differentiation culture. A xeno-free culture method to maintain human iPS cells has been already developed, and we should make considerable effort

to establish xeno-free differentiation culture. Other issues to be considered may be the time and cost for production of iPS cells specific to the individual patients. This is not the issue specific to iPS-DC therapy, and in this regard, establishment of a bank of iPS cells derived from somatic cells of HLA haplotype-homozygous donors would be profoundly valuable [27].

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Journal of Medical Virology 82:539–545 (2010)

# Incidence of Hepatocellular Carcinoma in Patients With Chronic Hepatitis B Virus Infection Who Have Normal Alanine Aminotransferase Values

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The importance of alanine aminotransferase (ALT) levels in the progression of hepatitis B virus (HBV) infection remains a subject of debate. This study sought to identify independent risk factors involved in development of hepatocellular carcinoma (HCC), particularly in patients with chronic HBV infection who have normal ALT values. Data from 381 consecutive hepatitis B patients were analyzed with average ALT integration values ≤40 IU/L and follow-up periods of >3 years. Integration values were calculated from biochemical tests, and serological markers associated with the cumulative incidence of HCC were analyzed. HCC developed in 17 of the 381 patients (4.5%) during the follow-up period. Male sex (hazard ratio, 6.011 [95% confidence interval: 1.353–26.710], $P=0.018$), high HBV-DNA levels (≥5.0 log copies/ml; 5.125 [1.880–13.973], $P=0.001$), low platelet counts ($<15.0\times10^4/mm^3$; 4.803 [1.690–13.647], $P=0.003$), and low total cholesterol levels (<130 mg/dl; 5.983 [1.558–22.979], $P=0.009$) were significantly associated with greater incidence of HCC development. High HBV-DNA levels and low platelet counts are associated with the development of HCC in patients infected with hepatitis B who have normal ALT values. Therefore, maintenance of low HBV-DNA levels is important for the prevention of HCC in patients with low platelet counts, particularly in patients whose ALT values fall within the current normal range. ***J. Med. Virol. 82:539–545, 2010.*** © 2010 Wiley-Liss, Inc.

**KEY WORDS:** hepatitis B virus (HBV); HBV-DNA; normal alanine aminotransferase; platelet counts; hepatocellular carcinoma

## INTRODUCTION

Worldwide, an estimated 350 million individuals are infected chronically with hepatitis B virus (HBV), and 1 million die each year from HBV-related liver disease [EASL Jury, 2003]. Chronic HBV infection is a major risk factor for the development of hepatocellular carcinoma (HCC) [Beasley, 1988; EASL Jury, 2003]. Patients who test positive for the hepatitis B surface antigen (HBsAg) have a 70-fold greater risk of developing HCC compared with HBsAg-negative patients [Szmuness, 1978; Beasley et al., 1981]. HBV infection is endemic in Southeast Asia, China, Taiwan, Korea, and sub-Saharan Africa, where up to 85–95% of patients with HCC are HBsAg-positive [Rustgi, 1987]. HCC is the third and fifth leading cause of death from malignant neoplasms in Japanese men and women, respectively, and the death rate from HCC has increased markedly in Japan since 1975 [Kiyosawa et al., 2004]. Hepatitis C virus (HCV)-related HCC accounts for 75% of all cases of HCC in Japan, while HBV-related HCC accounts for 15% of such cases [Kiyosawa et al., 2004].

Although an increasing body of epidemiological and molecular evidence suggests that HBV is associated with the development of HCC, the exact role of HBV in carcinogenesis is unclear [Ikeda et al., 2005; Wong et al., 2006]. HBV elicits a chronic necroinflammatory hepatic disease [Yu and Chen, 1994], and liver injury associated with HBV infection is mediated by viral factors in addition to the host immune response. Patients who are positive for the hepatitis B e antigen (HBeAg) commonly have increased hepatic inflammatory activity and an increased risk of developing HCC [Yang et al., 2002]. HBeAg-negative HBsAg carriers who retain high levels of HBV-DNA and show persistent necroinflammation of the liver have an increased risk of acquiring HCC [Yu et al., 2005; Chen et al., 2006].

The authors report no conflicts of interest.

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Accepted 10 September 2009
DOI 10.1002/jmv.21686
Published online in Wiley InterScience (www.interscience.wiley.com)

Alanine aminotransferase (ALT) activity is the most widely used laboratory test for the evaluation of necroinflammatory activity in liver disease [Prati et al., 2002]; however, it is well known that HCC occurs in some HBsAg carriers with normal ALT values. Recently, Chen et al. [2006] conducted a large cohort study in Taiwan and found that elevated serum HBV-DNA levels are strong predictive factors for the development of HCC, independent of the ALT values. It is an important problem for early detection of HCC that general practitioners are sometimes unaware of those patients with normal ALT as high-risk subjects for HCC. There is little information about how many patients with normal ALT develop HCC. It is important that ALT values should be expressed with integration values to ensure a valid analysis, since ALT values fluctuate frequently [Kumada et al., 2007]. Therefore, this study sought to identify the independent risk factors, involving mainly serological markers, associated with the development of HCC in patients infected chronically with HBV with average ALT integration values $\leq$40 IU/L.

## MATERIALS AND METHODS

### Patient Selection

A total of 1,861 consecutive patients who were positive for HBsAg visited the Department of Gastroenterology at Ogaki Municipal Hospital, Japan, between September 1994 and August 2003. After assessing each patient's long-term prognosis, 381 consecutive patients were selected for further study who (1) were positive for HBsAg for at least 6 months; (2) displayed no evidence of HCV infection; (3) had no other possible causes of chronic liver disease (i.e., alcohol consumption lower than 80 g/day, no history of hepatotoxic drug use, and negative tests for autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease); (4) had a follow-up period of >3 years; (5) had no evidence of HCC for at least 3 years from the start of the follow-up period; (6) had no history of therapy involving interferons, nucleosides, or nucleotide analogues; (7) had ALT measurements taken more than twice in a year; and (8) had average ALT integration values $\leq$40 IU/L (Fig. 1).

Patients were evaluated at the hospital at least every 6 months. During each follow-up examination, platelets, ALT, aspartate aminotransferase (AST), gamma glutamyl transpeptidase (gamma-GTP), total bilirubin, cholinesterase, alkaline phosphatase (ALP), albumin, total cholesterol, HBeAg, anti-HBe, HBV-DNA, and alpha-fetoprotein (AFP) were measured at least every 6 months. Commercial radioimmunoassay kits were used to test blood samples for HBsAg, HBeAg, and anti-HBe (Abbott Japan Co., Ltd, Tokyo, Japan). Before July 2001, serum HBV-DNA concentrations were monitored using the amplification-hybridization protection assay (DNA probe, Chugai-HBV; Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) with a lower detection limit of ~5,000 viral genome copies/ml (3.7 log copies/ml). After August 2001, serum HBV-DNA levels were monitored using the polymerase chain reaction (PCR) (COBAS Amplicor HBV monitor test, Roche Diagnostics K.K., Tokyo, Japan) with a lower detection limit of ~400 viral genome copies/ml (2.6 log copies/ml). HBV genotyping was carried out as described previously [Kato et al., 2001]. ALT, AST, gamma-GTP, ALP, and AFP were expressed as integration values [Kumada et al., 2007]. When ALT was used as an example, the integration value of ALT was calculated as follows: $(y_0+y_1)\times x_1/2+(y_1+y_2)\times x_2/2+(y_2+y_3)\times x_3/2+(y_3+y_4)\times x_4/2+(y_4+y_5)\times x_5/2+(y_5+y_6)\times x_6/2+(y_6+y_7)\times x_7/2+(y_7+y_8)\times x_8/2$ (Fig. 2). The area of a trapezoid with ALT value was calculated and the measurement interval and added the values. The

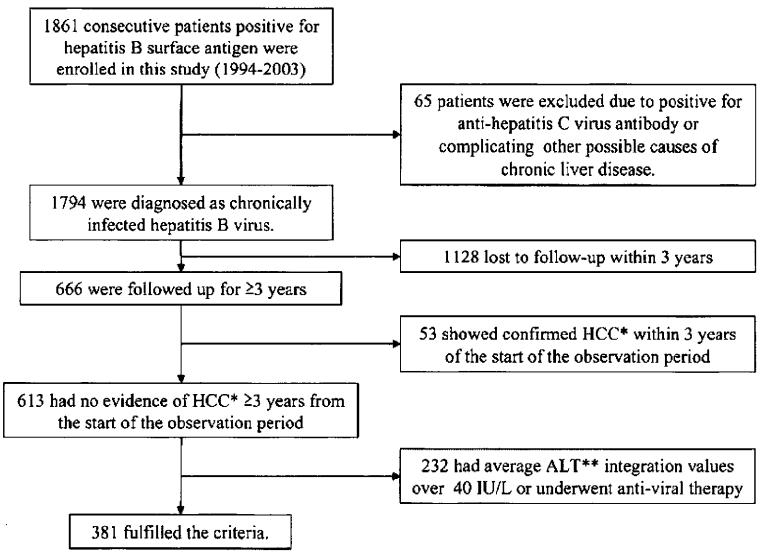


Fig. 1. Schematic flowchart of enrolled patients. *, hepatocellular carcinoma (HCC); **, alanine aminotransferase (ALT).