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Differential Induction from X-irradiated Human Peripheral Blood Monocytes to Dendritic Cells

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Dendritic cell/Monocyte/X-irradiation.

Dendritic cells (DCs) are a type of antigen-presenting cell which plays an essential role in the immune system. To clarify the influences of ionizing radiation on the differentiation to DCs, we focused on human peripheral blood monocytes and investigated whether X-irradiated monocytes can differentiate into DCs. The non-irradiated monocytes and 5 Gy-irradiated monocytes were induced into immature DCs (iDCs) and mature DCs (mDCs) with appropriate cytokine stimulation, and the induced cells from each monocyte expressed each DC-expressing surface antigen such as CD40, CD86 and HLA-DR. However, the expression levels of CD40 and CD86 on the iDCs derived from the 5 Gy-irradiated monocytes were higher than those of iDCs derived from non-irradiated monocytes. Furthermore, the mDCs derived from 5 Gy-irradiated monocytes had significantly less ability to stimulate allogeneic T cells in comparison to the mDCs derived from non-irradiated monocytes. There were no significant differences in the phagocytotic activity of the iDCs and cytokines detected in the supernatants conditioned by the DCs from the non-irradiated and irradiated monocytes. These results suggest that human monocytes which are exposed to ionizing radiation can thus differentiate into DCs, but there is a tendency that X-irradiation leads to an impairment of the function of DCs.

INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells which stimulate naïve T cells and regulate immune responses.[1–3] There are 3 stages of differentiation for DCs: DCs precursors, immature DCs (iDCs), and mature DCs (mDCs).[1–3] iDCs locate in various tissues, where they capture antigens such as invading bacteria, viruses, or damaged tissue, and then process the antigens for presentation on major histocompatibility complex molecules (MHC). The pathogen-derived components or pro-inflammatory cytokines induce the DCs maturation. During the maturation process, the mDCs acquire a high antigen-presenting capacity instead of losing their phagocytotic activity, and vigorously stimulate T-cell responses.[2] This interaction results in immune activation or tolerance, depending on the phenotype and functional state of the involved DCs. In general, DCs are broadly classified into two types, plasmacytoid DCs and myeloid DCs. Plasmacytoid DCs are found primarily in blood and lymphoid organs and have the capacity to produce type I interferon (IFN).[3] On the other hand, myeloid DCs are found in many tissues.

Various applications of DCs to immunotherapy are now being investigated. The most striking application of DCs is the immunotherapy for cancer.[4–7] This immunotherapy utilizes the immunostimulatory ability of DCs for inducing antitumor immunity. On the other hand, DCs have also been applied to induce graft tolerance in hematopoietic stem cell transplantation or to induce tolerance in patients with autoimmune disease.[3] The tolerogenic or regulatory DCs, which cause immune tolerance, have been used to treat acute graft-versus-host disease and systemic inflammatory response.[8–10]

For these applications, DCs are prepared from $CD34^+$ hematopoietic progenitors or $CD14^+$ peripheral blood monocytes ex vivo.[11–16] Especially, $CD14^+$ peripheral blood monocytes are relatively easy to obtain in large quantities. However, it appears that the DCs derived from the monocytes of cancer patients are phenotypically and functionally inefficient in comparison to the DCs derived from the monocytes of healthy donors.[17,18] This is crucial problem when DCs are prepared from cancer patients. On the other hand,

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although radiotherapy and chemotherapy have been established as effective cancer therapies, whether the differential process for DCs is affected by these therapies remains to be elucidated.[19] Some previous reports have described that DCs derived from peripheral blood monocytes of healthy donors are resistant to ionizing radiation-induced cell death, but the DCs exposed to ionizing radiation are functionally weakened.[20–23] However, the influences of ionizing radiation on the DCs precursors have not yet been reported. This issue is important to consider the immune system of patients undergoing radiotherapy and the prognosis of patients who have undergone radiotherapy. In addition, the influences of ionizing radiation on the DCs precursors may also involve in the efficacy of immunotherapy using monocyte-derived DCs when monocytes are prepared from patients who have already undergone radiotherapy.

In this study, in order to clarify the influence of radiation on the differentiation in DCs, we focused on peripheral blood monocytes and investigated whether or not X-irradiated monocytes can differentiate into normal DCs.

MATERIALS AND METHODS

Reagents

The recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), interleukin-4 (rhIL-4) and tumor necrosis factor-α (rhTNF-α) were purchased from PeproTech Inc. (Rocky Hill, NJ, USA). The fluorescence-labeled monoclonal antibodies (mABs), anti-human cluster of differentiation 1a-phycoerythrin (CD1a-PE), CD2-fluorescein isothiocyanate (CD2-FITC), CD40-FITC, human leukocyte antigen-ABC-FITC (HLA-ABC-FITC) and mouse IgG_{2a}-PE were purchased from Becton Dickenson (San Jose, CA, USA). Anti-human CD8-FITC, CD19-FITC, CD80-FITC, CD4-PE, CD14-PE, CD11c-PE, CD83-PE, CD86-PE, mouse IgG_1-FITC, mouse IgG_{2b}-PE were purchased from Beckman Coulter (Fullerton, CA, USA), and anti-human CD3-PE, HLA-DR-PE were purchased from eBioscience (San Diego, CA, USA) to use for the flow cytometer analysis. FITC-dextran was purchased from Molecular Probes, Inc. (Eugene, OR, USA) for the analysis of phagocytosis.

Collection of Monocytes from Buffy-Coat

This study was approved by the Committee of Medical Ethics of Hirosaki University School of Medicine (Hirosaki, Japan). After obtaining informed consent from all of the normal human blood donors (n = 18), peripheral blood was collected by the Aomori Red Cross Blood Center. The buffy-coat was prepared from whole-blood (400 ml) by the above-mentioned facility and was supplied to our research team. The peripheral blood mononuclear cells (PBMCs) were separated from the buffy-coat by centrifugation for 30 min at 400 × *g* on a cushion of Lymphosepar I (1.077 g/ml; Immuno-Biological Laboratories Co. Ltd., Takasaki, Japan). After centrifugation, the PBMCs were washed three times with phosphate-buffered saline (PBS) containing 5 mM ethylenediaminetetraacetic acid (EDTA). The monocytes were separated from the PBMCs according to the percoll density gradient-protocols published by Repnik *et al.*[24] or adhering. When the PBMCs were more than 2.0×10^8 cells, the monocytes were separated from the PBMCs by centrifugation for 15 min at 580 × *g* on a cushion of the hyper-osmotic percoll solution.[24] After centrifugation, the monocyte fractions were collected, and washed with PBS containing 5 mM EDTA. Thereafter, the number of these cells was counted. In order to remove any obstacle platelets and dead cells, the monocytes fraction were centrifuged for 15 min at 350 × *g* on a cushion of the iso-osmotic percoll solution.[24] After centrifugation, the monocytes were collected and washed with PBS containing 5 mM EDTA. The purity of $CD14^+$ monocytes was 64.6 ± 7.6% by a flow cytometer. On the other hand, when the PBMCs were less than 2.0×10^8 cells, the PBMCs were resuspended in PBS and allowed to adhere plastic dish (up to 1.0×10^7 cells per 60 mm dish) at 37°C for 1.5–2 h in a humidified atmosphere containing 5% CO_2. Thereafter, non-adherent cells were washed out twice with PBS.

The $CD14^+$ monocytes or adherent monocytes were incubated in RPMI 1640 (GIBCO, Grand Island, NY, USA) supplemented with 2% heat-inactivated human AB serum (GEMINI BIO-PRODUCTS, Woodland, CA, USA) and 1% antibiotic-antimycotic (which hereafter we refer to as the medium). The concentration of $CD14^+$ monocytes was up to 2.0×10^6 cells/ml.

In Vitro Irradiation of Monocytes

The radiation of X-rays (150 kVp, 20 mA, 0.5 mm Al and 0.3 mm Cu filters) was performed with an X-ray generator (MBR-1520R, Hitachi Medical Corporation, Tokyo, Japan) at a distance of 45 cm from the focus at a dose rate of 80.0–98.0 cGy/min.

In Vitro Generation and Culture of Human DCs

The DCs were generated from the monocytes according to previously published protocols[14] with some modification. In brief, non-irradiated and X-irradiated monocytes were cultured in the medium at 37°C overnight in a humidified atmosphere containing 5% CO_2. On the next day, 50 ng/ml rhGM-CSF and 50 ng/ml rhIL-4 were added to the medium and the monocytes were cultured to prepare the iDCs at 37°C for 5 days in a humidified atmosphere containing 5% CO_2. Because there was a possibility that some factors induced by X-irradiation may be removed from the culture medium by changing the culture medium with new medium, the culture medium was not changed with new medium during this 5-day culture. After 5 days of culture, the cells were harvested and an aliquot of the culture supernatants were

collected and kept frozen at –85°C for the cytokine and zymography assay. Half of the culture medium was changed with new medium and the cells were re-cultured in the presence of 50 ng/ml rhTNF-α at 37°C for an additional 4 days in a humidified atmosphere containing 5% CO_2. After an additional 4 days of culture, the culture supernatants were also collected and kept frozen at –85°C for the cytokine and zymography assay.

Phenotypic Analysis

For the surface marker analysis of the iDCs and mDCs, the cells were stained with mABs conjugated to FITC or PE for 30 min at 4°C in the dark. The cells were also stained with corresponding FITC- or PE-conjugated isotype control mouse IgG. After 30 min, the cells were washed with cold PBS and analyzed by a flow cytometer (Epics XL, Beckman Coulter). The induced cells from monocytes could be distinguished from debris and lymphocytes using a region established by their high forward and side scatter signals. The cell surface phenotype of 1.0×10^4 cells within the region were analyzed. The data were expressed as the percent of positive cells or the mean fluorescence intensity (MFI).

Phagocytosis Assay

In order to determine the phagocytotic activity of the iDCs, FITC-dextran was added in a final concentration of 91 μg/ml to the cells (5.0×10^6 cells/ml), and the cells were incubated for 60 min at 37°C. As a negative control, the cells were put on ice in the presence of FITC-dextran. After incubation, the cells were washed twice with cold PBS and the FITC-dextran uptake of the iDCs was analyzed by a flow cytometer.

Allogeneic Mixed Leukocytes Reaction

Allogeneic 1.0×10^5 $CD4^+$ T cells (> 98% $CD4^+$ T cells), which were purified from PBMCs of three different individuals with the use of Human CD4 T lymphocyte Enrichment Set-DM (BD Bioscience), were co-cultured in 96-well flat bottom microplates (Asahi Techno Glass Co. Ltd., Chiba, Japan) with different numbers of mDCs. In order to prevent the proliferation of mDCs, the mDCs were 20 Gy-irradiated with an X-ray generator prior to co-culture with allogeneic $CD4^+$ T cells. Both cells were co-cultured for 3 days in RPMI 1640 supplemented with 2% heat-inactivated human AB serum at 37°C in a humidified atmosphere containing 5% CO_2. After 3 days of culture, the cells were incubated for additional 20 h in the presence of [^{3}H]-thymidine (1 μCi/well, specific activity, 5 Ci/mmol; Moravek Biochemicals Inc, CA, USA). To determine $CD4^+$ T cells proliferation, the cells were harvested onto glass fiber filters (Whatman, England) with a semiautomatic harvester (Labo Mash, Labo Science, Tokyo, Japan), and then the amount of [^{3}H]-thymidine incorporation was measured by a liquid scintillation counter (LSC-5100, Aloka Co. Ltd., Tokyo, Japan). The $CD4^+$ T cells alone were cultured as a negative control. The experiment was performed in triplicate wells.

Cytokines Measurements

The cytokines in the culture supernatants released from the iDCs and mDCs were measured using the Bio-Plex protein array system (Bio-Rad Laboratories, Hercules, CA, USA). The Bio-Plex cytokine assay is designed for the multiplexed quantitative measurement of multiple cytokines in a single well using as little as 50 μl of sample.[25] For the cytokine assays, we used premixed multiplex beads of the Bio-Plex human cytokine Th1/Th2 panel (Bio-Rad Laboratories), which included nine cytokines [IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, GM-CSF, IFN-γ, TNF-α]. Samples were analyzed in duplicate wells. The data were analyzed using the Bio-Plex Manager 4.0 software (Bio-Rad Laboratories). The detectable concentration of each cytokine was 2.0 pg/ml – 32 ng/ml.

Analysis of secreted MMP-9

The total matrix metalloproteinase-9 (MMP-9) in the culture supernatants of the iDCs and mDCs was analyzed by substrate zymography.[26] The electrophoresis of each culture supernatant was carried out on a sodium dodecyl sulfate–polyacrylamide gel (10% acrylamide) containing 0.1% gelatin. Proteinases separated in the gels were renatured by gentle shaking in 50 mM Tris–HCl buffer (pH 7.5, containing 0.1 M NaCl and 2.5% Triton X-100) at room temperature for 1.5 h. The gels were then incubated in 50 mM Tris–HCl buffer (pH 7.5, containing 10 mM $CaCl_2$) at 37°C for 15 h, and stained with Coomassie brilliant blue R-250. The bands were quantified by Image J analysis (1.37).

Statistical Analysis

The data are expressed as the mean values ± SD. The comparisons of non-irradiation *vs* X-irradiation were performed by two-sided Mann-Whitney's *U*-test. The *p*-value was considered to be statistically significant if $p < 0.05$. The statistical analysis was performed using the Excel 2003 software program (Microsoft, USA) with the add-in software Statcel 2.[27]

RESULTS

Phenotype of DCs

To clarify whether X-irradiated monocyte can differentiate into DCs, we attempted to induce iDCs from the human peripheral blood non-irradiated and 5 Gy-irradiated monocytes with a combination of rhGM-CSF plus rhIL-4. The cells derived from the non-irradiated monocytes had a typical dendritic morphology, which show a very large contact surface to their surroundings in comparison to the overall cell volume with the dendrites, under a microscope (data not shown). To analyze the cell surface phenotype of the iDCs,

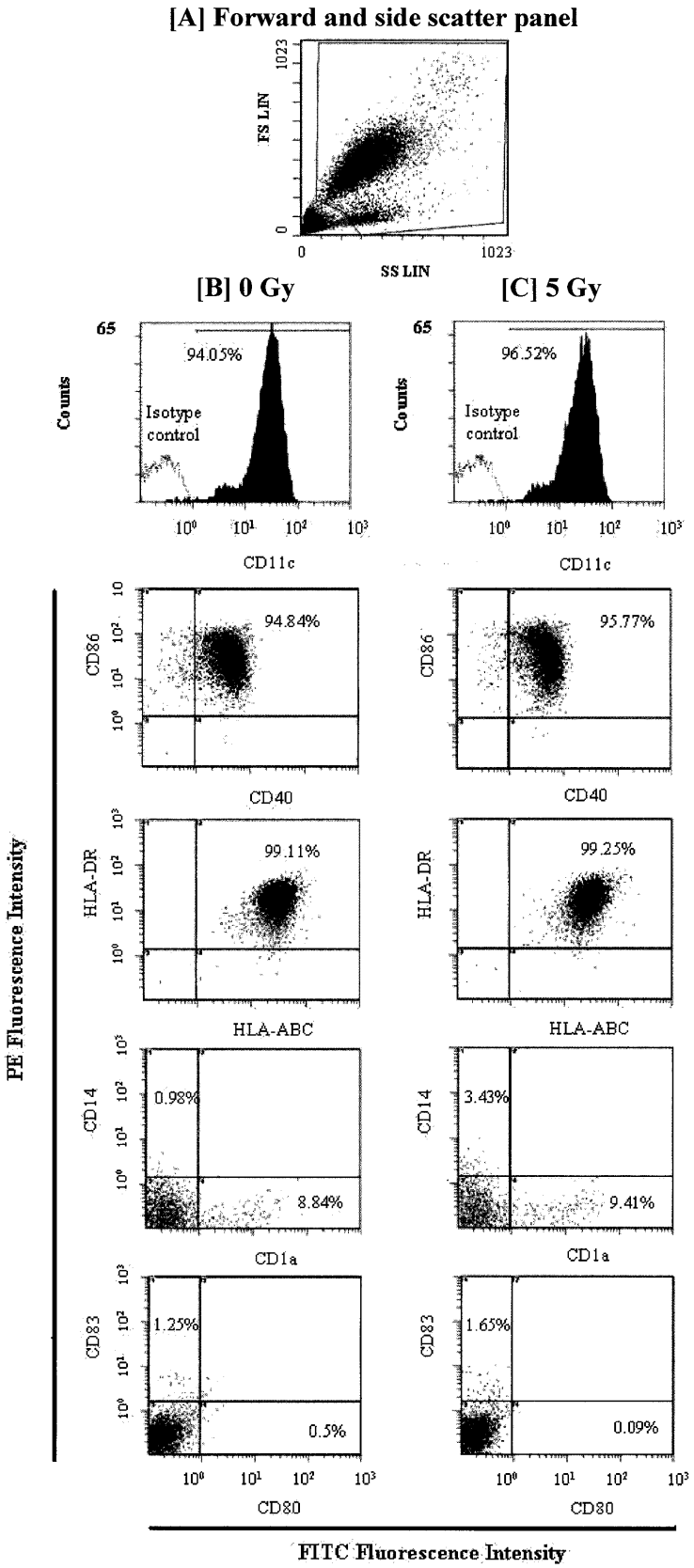

Fig. 1. Phenotypic characterization of iDCs by a flow cytometer. The non-irradiated and 5 Gy-irradiated monocytes were cultured with rhGM-CSF (50 ng/ml) plus rhIL-4 (50 ng/ml) for 5 days. After gating as indicated in the forward scatter (FS) and side scatter (SS) panel [A], the surface molecule expression of the cells derived from the non-irradiated [B] and 5 Gy-irradiated [C] monocytes were analyzed by a flow cytometer. The values in each quarter are the positive percentages of cell populations. The representative cytograms are shown.

the induced cells were measured after gating on forward and side scatter by a flow cytometer (Fig. 1-[A]). The cells within this region showed no expression of any lineage-markers such as CD3 (T cells) and CD19 (B cells) (data not shown). Furthermore, these cells expressed high levels of CD11c, which is a myeloid-DCs marker (Fig. 1-[B]). These cells also expressed high levels of CD40, CD86 and HLA-DR, low levels of CD1a, and trace levels of CD14 (Fig. 1-[B]). Based on these results, the induced cells were thus determined to not be monocytes/macrophages, but DCs. Furthermore, these DCs also expressed low levels of CD80 and showed no expression of CD83 (Fig. 1-[B]), thus showing the characteristics of iDCs. The cells derived from the 5 Gy-irradiated monocytes also showed similar phenotype to the cells derived from the non-irradiated monocytes (Fig. 1-[C]).

Next, both types of iDCs were induced to mDCs in the culture with rhTNF-α. As shown in Fig. 2, both the cells induced from the non-irradiated and 5 Gy-irradiated monocytes expressed CD83, which is a DC's maturation marker.[28] In addition, the expression of CD80, CD86 and HLA-DR on the stimulated cells were higher than those on the iDCs, showing the characteristics of mDCs. These results indicate that the induced cells from 5 Gy-irradiated monocytes are phenotypically iDCs and mDCs.

In our preliminary experiments, we observed that 2, 5 or 10 Gy-irradiated monocytes were able to differentiate into DCs and that X-irradiation influenced the CD40 expression on iDCs (data not shown). X-irradiation to monocytes

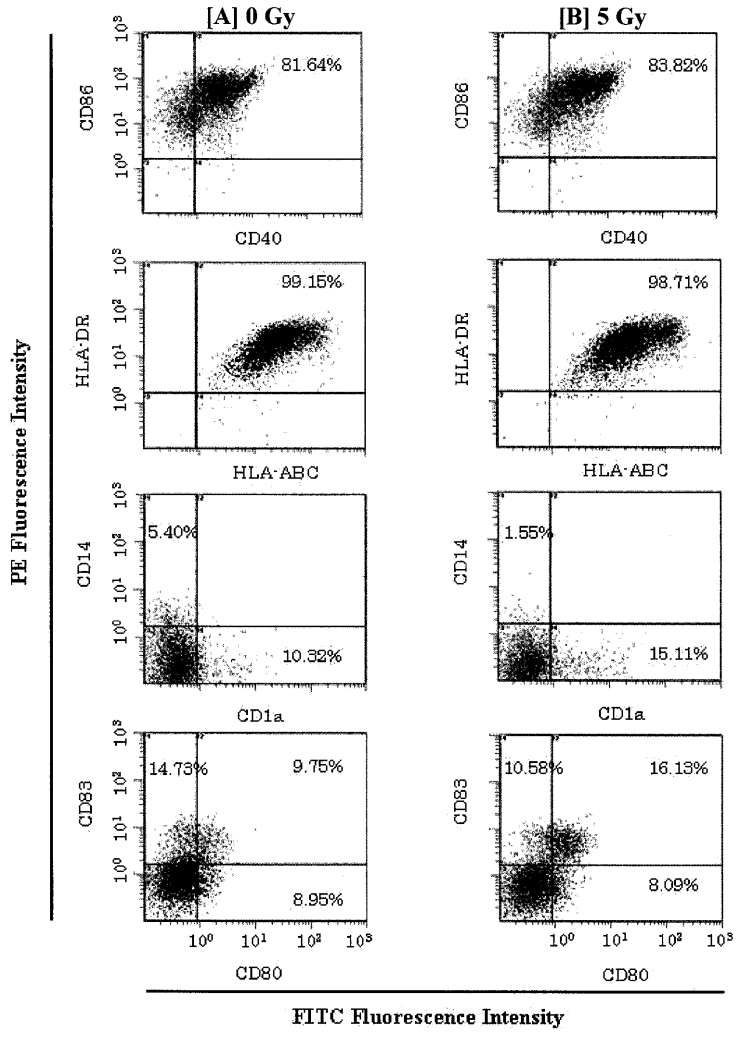

Fig. 2. Phenotypic characterization of mDCs by a flow cytometer. The cells, which were cultured with rhGM-CSF plus rhIL-4 for 5 days, were re-cultured in the presence of 50 ng/ml rhTNF-α for an additional 4 days. For determination of surface molecule expression, the cells derived from the non-irradiated [A] and 5 Gy-irradiated [B] monocytes were analyzed by a flow cytometer after forward and side scatter gating as indicated in Fig. 1-[A]. The values in each quarter are the positive percentages of cell populations. The representative cytograms are shown.

overall enhanced the CD40 expression on the induced-iDCs, but 2 Gy-irradiation to monocytes didn't always enhance the CD40 expression on the induced-iDCs in all individuals that we preliminarily tested (data not shown). Furthermore, the enhancement of the CD40 expression by 10 Gy-irradiation was similar to that by 5 Gy-irradiation (data not shown). Based on these results, we determined that 5 Gy-irradiation was a relevant dose to investigate the influence of ionizing irradiation on the differentiation into DCs from monocytes, and chose 5 Gy-irradiation for the further experiments.

We investigated the influences of 5 Gy-irradiation to monocytes on the cell surface antigen expression in detail.

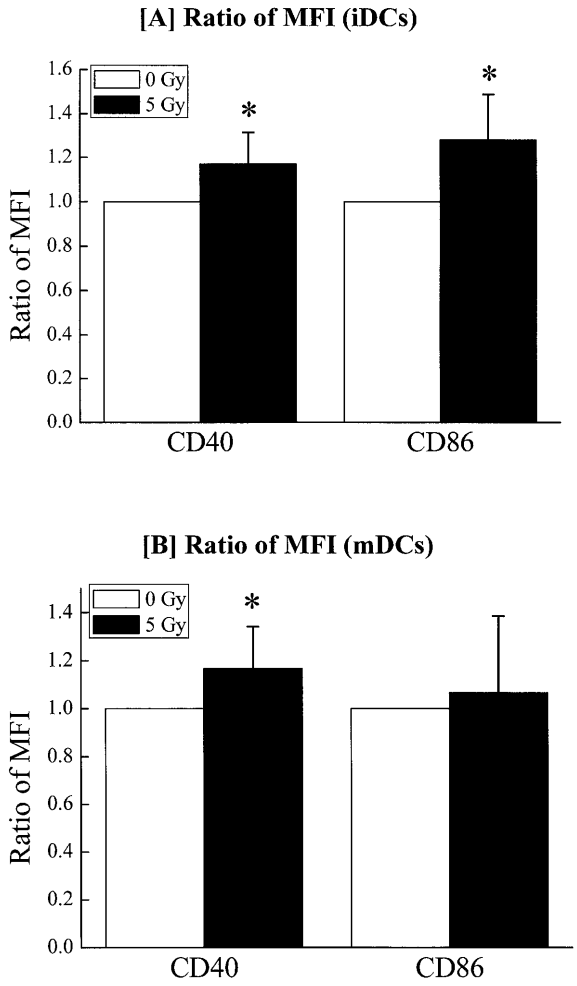

Fig. 3. Differences in the expression levels of the cell surface antigens between the DCs derived from non-irradiated and 5 Gy-irradiated monocytes. Ratios of CD40 and CD86 MFI, respectively, were obtained by calculating a ratio of the MFI of DCs derived from 5 Gy-irradiated monocytes to that of DCs derived from non-irradiated monocytes (5 Gy/ 0 Gy) in each individual. [A] The ratio of MFI on iDCs are expressed as the mean + SD of nine different individuals. [B] The ratio of MFI on mDCs are expressed as the mean + SD of 13 different individuals. *$p < 0.005$ by two-sided Mann-Whitney's *U*-test.

As shown in Fig. 3-[A], the MFI of CD40 and CD86 on the iDCs derived from 5 Gy-irradiated monocytes was higher than that on the iDCs derived from non-irradiated monocytes (n = 9, $p < 0.005$, respectively). Although the MFI of CD40 on the mDCs derived from 5 Gy-irradiated monocytes was higher than that on the mDCs derived from non-irradiated monocytes (n = 13, $p < 0.005$), there was no significant difference in the CD86 expression on mDCs between non-irradiation and 5 Gy-irradiation (Fig. 3-[B]).

Function of DCs

The functional characteristics of the DCs were analyzed. As one of the characteristics of iDCs, it is known that iDCs have a high phagocytotic activity which thus allows them to capture antigens.[1,2] In the present study, the uptake of FITC-dextran was measured by a flow cytometer. The iDCs

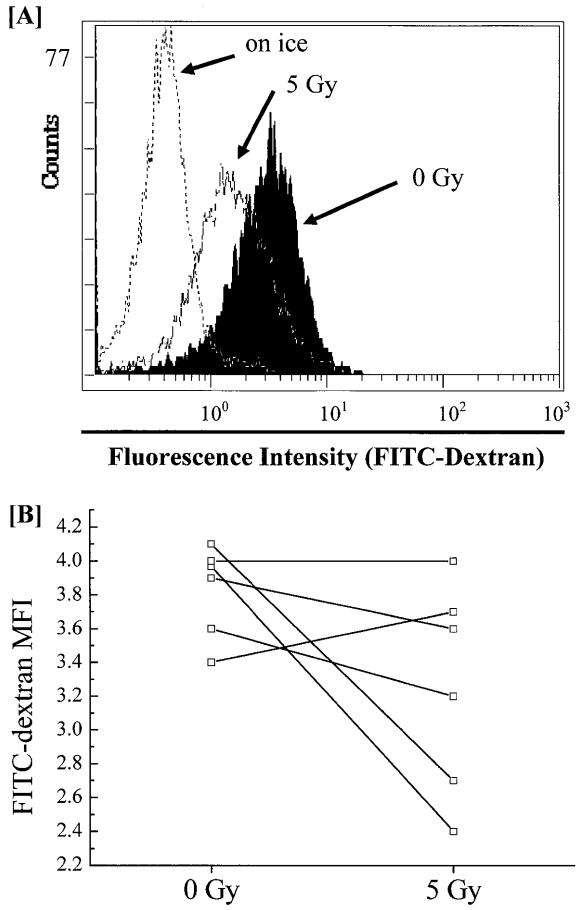

Fig. 4. The phagocytotic activity of iDCs. Phagocytosis was measured by the uptake of FITC-dextran. [A] A representative cytogram is shown. The uptake of FITC-dextran by the iDCs derived from the non-irradiated (filled gray histogram) and 5 Gy-irradiated (broken line histogram) monocytes was quantified by a flow cytometer. The dotted line histogram represents the negative control. [B] The data of six different individuals are shown as mean fluorescence intensity (MFI).

derived from non-irradiated monocytes showed an increase in fluorescence intensity, thus demonstrating phagocytotic activity (Fig. 4-[A]). The iDCs derived from 5 Gy-irradiated monocytes showed less phagocytotic activity than the iDCs derived from non-irradiated monocytes in four out of six different individuals, and the large decrease (34% and 40%) was observed in two out of them (Fig. 4-[B]). However, there was no statistically significant difference in the phagocytotic activity between the non-irradiation and 5 Gy-irradiation. These results suggest that X-irradiation to monocytes relatively attenuate the ability of iDCs to capture antigens.

Since mDCs can stimulate the proliferation of allogeneic leukocytes and this ability is often used as a surrogate marker of their activation state, we next investigated the ability of the mDCs in allogeneic mixed leukocytes reaction (MLR) in seven different individuals. In order to quantify the $CD4^+$ T cells proliferation, we measured the incorporation of [^{3}H]-thymidine by $CD4^+$ T cells. Both mDCs stimulated the proliferation of allogeneic $CD4^+$ T cells (Fig. 5-[A]), although there was the large difference in the incorporation of [^{3}H]-thymidine by $CD4^+$ T cells among individuals. Overall, the mDCs derived from 5 Gy-irradiated

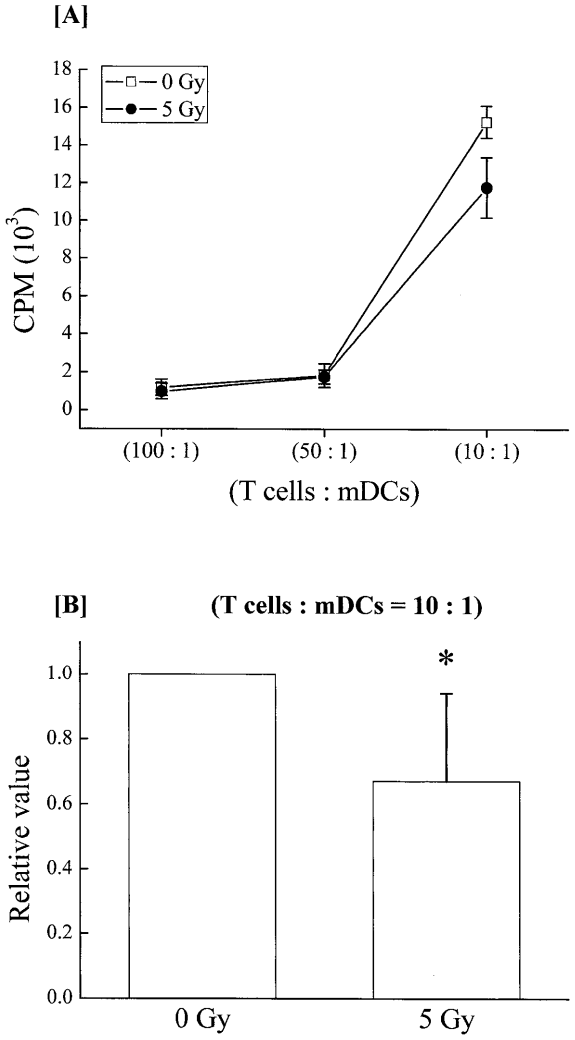

Fig. 5. The stimulating ability of mDCs on the allogeneic lymphocytes in a MLR. As we described in the materials and methods, each mDC was co-cultured with allogeneic $CD4^+$ T cells, and the proliferation of $CD4^+$ T cells was determined by the incorporation of [^{3}H]-thymidine. [A] The representative result of seven different individuals is shown. Values are the mean ± SD obtained from triplicate cultures. The incorporation of [^{3}H]-thymidine by only $CD4^+$ T cells was less than 300 cpm. [B] A relative value of the proliferation (mean cpm obtained from triplicate cultures) was obtained by calculating the ratio of 5 Gy-irradiation to non-irradiation (5 Gy/ 0 Gy) in each individual. The data when mDCs were cultured with allogeneic $CD4^+$ T cells at a ratio of 1: 10 are shown and expressed as the mean + SD of seven different individuals. *$p < 0.05$ by two-sided Mann-Whitney's *U*-test.

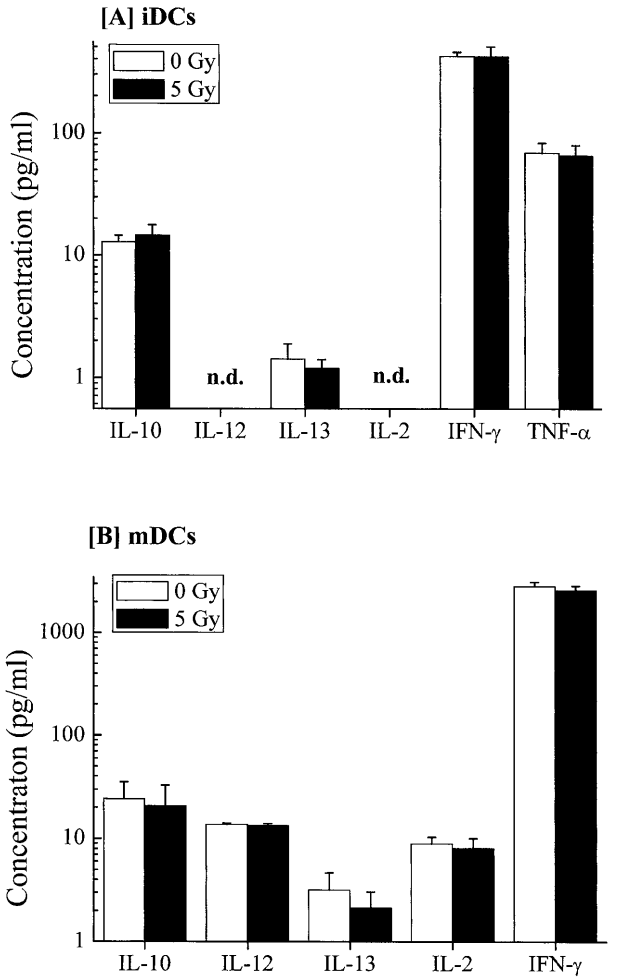

Fig. 6. Cytokines concentration detected in the culture supernatants of iDCs and mDCs. Each cytokine concentration in the culture supernatants of the iDCs [A] and mDCs [B] was measured using the Bio-Plex cytokine protein array system. The data are represented as the mean + SD of three different individuals. n.d. = not detectable (< 2 pg/ml).

monocytes had about 32% less ability to stimulate allogeneic $CD4^+$ T cells compared with mDCs derived from non-irradiated monocytes ($n = 7, p < 0.05$) when mDCs were co-cultured with $CD4^+$ T cells at a ratio of 1 : 10 (Fig. 5-[B]). These results suggest that X-irradiation to monocytes attenuate the interaction between mDCs and T cells.

Cytokines Production by DCs

mDCs produce various types of cytokines, which affect the T cell-mediated immune system. We thus next measured the cytokine production of the DCs in three different individuals. The cytokines released in the culture supernatants were assayed using premixed multiplex beads, as described in the materials and methods. The culture supernatants of both iDCs contained large amounts of GM-CSF and IL-4 (data not shown). As shown in Fig. 6, IFN-γ, TNF-α and IL-10 were detected in the medium conditioned by both iDCs and mDCs derived from the non-irradiated monocytes. The medium conditioned by both iDCs and mDCs derived from the 5 Gy-irradiated monocytes also contained those cytokines (Fig. 6). The concentration of TNF-α in the culture supernatants of mDCs was more than 32 ng/ml (data not shown). Although IL-2 and IL-12 were not detected in the culture supernatants of the iDCs, they were detected in the culture supernatants of both mDCs (Fig. 6-[B]). However, no significant difference in the all cytokines concentrations tested in this study was observed between the non-irradiation and the 5 Gy-irradiation. These results indicate that DCs derived from 5 Gy-irradiated monocytes retain the capacity to produce cytokines tested here.

Total MMP-9 in the Culture Supernatants of DCs

It is reported that DCs express several MMPs including MMP-9.[29,30] MMP-9, which is gelatinase B, cleaves type IV collagen and contributes to DCs migration through the extracellular matrix.[29,31] We therefore investigated total MMP-9 in four different individuals. As shown in Fig. 7-[A], MMP-9 was presented in the culture supernatants of each DC. In agreement with previous reports,[29] the total MMP-9 increased in the culture supernatants of the mDCs in comparison to the iDCs (Fig. 7-[B]). There was about 19% less total MMP-9 in the culture supernatants of iDCs derived from 5 Gy-irradiated monocytes compared with non-irradiation ($n = 4, p < 0.05$) (Fig. 7-[B]). However, when the iDCs were induced to mDCs with rhTNF-α, this reduction disappeared.

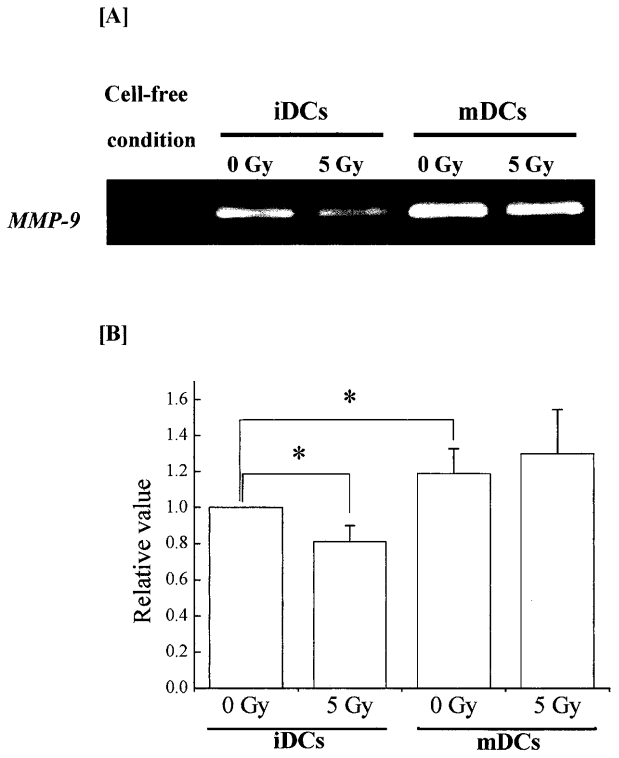

Fig. 7. Total MMP-9 in the culture supernatants of iDCs and mDCs. The total MMP-9 in the culture supernatants of iDCs and mDCs derived from non-irradiated and 5 Gy-irradiated monocytes was analyzed by substrate zymography. [A] The data are representative of similar results obtained from four different individuals. [B] Quantification of bands was performed using Image J analysis. Each of data was divided by the value of iDCs derived from non-irradiation monocytes, respectively. The data are represented as the mean + SD of four different individuals. *$p < 0.05$ by two-sided Mann-Whitney's *U*-test.

DISCUSSION

In this study, we evaluated the influences of X-irradiation on the differentiation from peripheral blood monocytes to DCs. We showed that X-irradiated monocytes can differentiate into iDCs and mDCs. We also showed that iDCs derived from 5 Gy-irradiated monocytes expressed higher levels of CD40 and CD86 compared with iDCs derived from non-irradiated monocytes (Fig. 3). Although few reports have described the influences of irradiation on the differential pathway from monocytes to DCs, previous studies have reported that ionizing radiation to iDCs caused a change in the expression of the surface antigens.[21–23] Cao *et al.*[21] and Reuben *et al.*[22] demonstrated that gamma irradiation reduced the expression of the CD80, CD86 and HLA-DR molecules on iDCs induced from human peripheral blood monocytes. On the other hand, Merrick *et al.* reported that the CD86 expression of iDCs was significantly up-regulated after gamma irradiation.[23] Our results are consistent with Merrick's report regarding the up-regulation of co-stimulatory molecule expression, although there is a difference whether irradiation to monocytes or to iDCs. McBride *et al.*[19] suggest the possibility that the "danger" signals induced by ionizing radiation may cause the maturation of DCs. Their suggestion led us to consider that the various "danger" signals, which were induced either in the irradiated

DCs-precursors or released from the irradiated bystander cells (including DCs-precursors and others), might influence the differentiation into DCs. TNF-α is an inflammatory cytokine and is one of the danger signals. Krivenko *et al.* reported that irradiation increased TNF production by human monocytes.[32] One possibility is that X-irradiation performed on peripheral blood monocytes may induce TNF-α production, which directly influences the differentiation into DCs from monocytes. However, there is no significant difference between the tested cytokines including TNF-α in the supernatant conditioned by the non-irradiated and the 5 Gy-irradiated monocytes (Fig. 6-[A]), thus indicating no involvement of TNF-α. Further studies regarding other "danger" signals, for example reactive oxygen species produced by irradiated-cells[19] and the high-mobility-group box 1 protein released from dying tumor cells,[33] are needed to clarify the mechanisms involved in the relationship between X-irradiation and the up-regulation of co-stimulatory molecule expression on iDCs. In this study, we used rhTNF-α as the maturation stimuli, and the mDCs derived from 5 Gy-irradiated monocytes expressed higher levels of CD40 compared with mDCs derived from non-irradiated monocytes (Fig. 3-[B]). However, this up-regulation of CD40 expression by 5 Gy-irradiation was not observed when we used lipopolysaccharide (LPS) as maturation stimuli in our progressing experiments (data not shown). These results indicate that the influences of X-irradiation to monocytes on the maturation of DCs depend on the types of maturation stimulus. It is thought that X-irradiation may influence the intracellular signal transduction induced by maturation stimuli, because the receptor of LPS is Toll-like receptor 4 and different from TNF receptor. Further studies regarding other maturation stimuli including LPS are now in progress.

In a functional analysis of the monocyte-derived DCs, no significant difference in the phagocytotic activity was observed between the non-irradiation and 5 Gy-irradiation, although this activity decreased by 5 Gy-irradiation in four out of six different individuals (Fig. 4-[B]). The result that the reduction of phagocytotic activity was not observed in all individuals may be owing to individual difference in the radiosensitivity. Regarding the stimulating ability of the mDCs on allogeneic T cell proliferation, although there was no difference in the MHC class II (HLA-DR) expression, which is involved in allogeneic MLR,[28,34] the T cell proliferation by mDCs induced from the irradiated monocytes was reduced (Fig. 5). This result is consistent with previous studies that the stimulating capacity of DCs on T cell proliferation is reduced by applying ionizing radiation to the DCs,[21,23] although there is a difference whether irradiation to monocytes or to DCs. Merrick *et al.* indicated that irradiated DCs were less effective in a MLR, and upon maturation produced significantly less IL-12 in comparison to the non-irradiated controls, while IL-10 secretion was maintained.[23] On the other hand, Chauveau *et al.*[35] demonstrated that human iDCs express heme oxygenase-1 (HO-1), which is an intracellular antioxidant enzyme to degrade heme and inhibit immune responses,[36] and that HO-1 expression is down-regulated by maturation stimuli. In addition, the induction of HO-1 not only inhibited the allostimulatory capacity of DCs but also suppressed IL-12 secretion of DCs, while it didn't affect IL-10 secretion of DCs.[35] These two reports led to a hypothesis that oxidative stress such as ionizing radiation induce HO-1 in the monocytes and/or iDCs, and HO-1 suppress IL-12 secretion and allostimulatory ability of DCs. However, no detectable change in the IL-12 production by 5 Gy-irradiation was observed in our study (Fig. 6-[B]). Therefore, we think that other factors such as chemokine may be controlled by X rays-induced HO-1 in this case.[37] In order to clarify these precise mechanisms, further experiments about the involvement of HO-1 are now in progress. On the other hand, zymographic analysis revealed that 5 Gy-irradiation induced a decrease of total MMP-9 during differentiation into iDCs from monocytes (Fig. 7). In contrast, 5 Gy-irradiation increased the ratio of mDCs to iDCs in the total MMP-9 activity. Lu *et al.* reported that addition of H_2O_2 to LPS-activated monocytes, but not naïve monocytes, caused a significant enhancement of MMP-1 production.[38] We think that the reactive oxygen species generated by ionizing radiation enhance production of MMP-9 in the TNF-α-stimulated mDCs. However, it is unclear how X-irradiation induced a decrease of total MMP-9 during the differentiation into iDCs from monocytes.

In conclusion, the present data demonstrate that human peripheral blood monocytes exposed to ionizing radiation can differentiate into DCs, but some differences in their characteristics are observed between the DCs from non-irradiated monocytes and those from irradiated monocytes. Since the DCs derived from the monocytes of patients are phenotypically and functionally inefficient in comparison to healthy donors,[17,18] the immune system in many cancer sufferers is thus considered to possibly be more severely damaged by radiotherapy or other therapies, such as chemotherapy during or after therapy. Furthermore, the efficacy of immunotherapy using monocyte-derived DCs against malignant tumors may be attenuated after undergoing radiotherapy. Further understanding about the influences of ionizing radiation on immune cells including DCs will allow combination of radiotherapy and immunotherapy.[39] At the start of radiotherapy or chemotherapy, the degree of damage to the DCs or DC precursors by various types of oxidative stress, including ionizing radiation, should therefore be monitored.

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