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CD40 Activation Protects Dendritic Cells from Anticancer Drug-Induced Apoptosis

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Dendritic cells (DCs) play a critical role in various immune responses involving $CD4^+$ T cells and have been used to generate anti-tumor immunity. Chemotherapy induces severe side effects including immunosuppression in patients with cancer. Although immunosuppression has been studied, the effects of anticancer drugs on DCs are not fully determined. In this study, we demonstrated that CD40 activation strongly protected DCs from 5-fluorouracil (5-FU) or mitomycin C-induced apoptosis. DC-specific surface markers, including CD11c and major histocompatibility complex (MHC) class II, were used for identifying DCs. CD 40 activation with anti-CD40 mAb significantly enhanced the viability of DCs treated with 5-FU or mitomycin C, assayed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Fluorescence staining and analysis clearly confirmed the enhancing effect of anti-CD40 mAb on the viability of DCs, suggesting that CD40 activation may transduce critical signals for the viability of DCs. Annexin V staining assay showed that CD40 significantly protected DCs from 5-FU or mitomycin C-induced apoptosis. Taken together, this study shows that CD40 activation with anti-CD40 mAb has strong anti-apoptosis effect on DCs, suggesting that CD40 activation may overcome the immunosuppression, especially downregulation of number and function of DCs in chemotherapy-treated cancer patients.

Key Words: Dendritic cells, CD40 activation, Apoptosis, 5-FU, Mitomycin C

INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs). DCs originated from bone marrow circulate in peripheral tissues and organs, and take up, process antigen and present antigenic peptides to nave T lymphocytes, stimulating their proliferation. Thus, DCs play an essential role in initiating immune responses (Banchereau & Steinman, 1998; Mellman & Steinman, 2001).

CD40, lipopolysaccharide (LPS), and tumor necrosis factor (TNF)-alpha induce the maturation of DCs, resulting in the upregulation of immunomodulatory molecules, including major histocompatibility complex (MHC) class I, II, CD40, CD54, CD80, and CD86, on DCs and the production of various cytokines, including interleukin (IL)-12 (Mackey et al, 1998; Labeur et al, 1999). Interestingly, a recent study demonstrated that the reagents for maturation enhanced the viability of DCs against a variety of apoptotic signals. Especially, LPS and TNF-alpha induced the maturing signals via p38 kinase in DCs (Arrighi et al, 2001).

It has been well known that patients with advanced cancer are in severe immunosuppressive condition due to tumor burden. A recent study shows that the supernatants of murine tumor cells increase ceramide, which in turn induces apoptosis of bone marrow-derived DCs (Kanto et al, 2001). Furthermore, cancer patients often receive chemotherapy, however, anticancer drugs used for chemotherapy remove both cancer cells as well as normal cells, including immune cells (Garewal, 1988), and chemotherapy also enhances myelosuppression of cancer patients (Smith et al, 1980). It is believed that the function and numbers of DCs in cancer patients are downregulated by tumor cell and chemotherapy as one part of myelosuppression. Therefore, therapeutic methods that can overcome the immunosuppression in chemotherapy-treated cancer patients may provide an essential clinical benefit.

Anti-CD40 monoclonal antibody (mAb), but not LPS or TNF-alpha, protects DCs from MHC class II-mediated apoptosis (McLellan et al, 2000). Although the effect of CD40 activation on the viability of DCs has been reported, the anti-apoptosis effect of CD40 activation on DCs treated with anticancer drugs for chemotherapy has not been studied. In the present study, we demonstrated that CD40 activation protected DCs from anticancer drug-induced apoptosis. It is, therefore, suggested that signals involved in CD40 activation may provide a target to enhance the viability of DCs in host immune system of cancer patients.

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ABBREVIATIONS: DCs, dendritic cells; 5-FU, 5-fluorouracil; MHC, major histocompatibility complex; mAb, monoclonal antibody; TNF, tumor necrosis factor.

METHODS

Animals and reagents

Female 6- to 7-week-old C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were kept in the laboratory animal facility and used at the age of 7- to 10-weeks. Purified anti-mouse CD8, CD19, and Gr-1 mAbs (BD PharMingen, San Diego, CA) were used for the detection of $CD8^+$ T lymphocytes, B lymphocytes, and granulocytes in bone marrow-derived DCs. Cells were stained with trypan blue solution (Sigma, St. Louis, MO) and counted for viable and dead cells.

Preparation of DCs

DCs from bone marrow cells of mice were cultured, using an established method (Inaba et al, 1992). Briefly, bone marrow cells harvested from tibia and femur were cultured at 2×10^6 cells/ml in 6-well culture plates. RPMI-1640 medium containing 5% fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin (all from Life Technologies Inc, Gaithersburg, MD), and 10 ng/ml mouse granulocyte-macrophage colony stimulating factor (GM-CSF, Biosource International, Camarillo, CA) were used. The culture medium was replaced with fresh medium every two days. To increase the purity of $CD11c^+$ DCs, floating cells including T, B lymphocytes, and granulocytes were thoroughly removed at 2 and 4 days of culture. At 6~10 days of culture, 70% (v/v) of the medium was replaced by fresh medium, and floating cells were used for experiments as DCs. DCs in this study were over 85% $CD11c^+$ DCs, based on fluorescence-activated cell-sorter (FACS) analysis.

CD40 activation and anticancer drug treatment on DCs

Anti-CD40 mAb (clone HM40-3, BD PharMingen) at 1 μg/ml concentration was used for CD40 activation. DCs were harvested at 6~10 days after culture and washed twice with Hanks balanced salt solution (HBSS, Sigma) before experiments. Cells were seeded at a concentration of 5×10^5 cells/ ml/well in 24-well culture plates. Anti-CD40 mAb, 5-fluorouracil (FU), and mitomycin C (Sigma) were directly added into culture medium. 5-FU and mitomycin C were dissolved in dimethyl sulfoxide (DMSO, Sigma) and water, respectively. The final concentration of DMSO in culture medium did not exceed 0.1% (v/v), and control cultures received the same dose of DMSO.

Assessment of cytotoxicity by MTT assay

To measure the viability of DCs, cells were seeded at a concentration of 5×10^4 cells/well in 96-well culture plates and treated with 5-FU, mitomycin C, and anti-CD40 mAb. After 48 hr culture, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added at a concentration of 0.5 mg/ml for 4 hr. Viable DCs generate insoluble crystal, and float and loosely attach on the surface of culture plates. So, 100 μl/well 10% SDS solution containing 0.01 N HCl was directly added into wells to avoid the potential loss of and dissolve the insoluble crystal generated by DCs. After 2 hr, the absorbance of the sample at 570 nm and 630 nm as reference was measured by using microplate reader (Molecular Devices, Sunnyvale, CA).

Morphological analysis of apoptotic cells

Morphological changes in nuclear chromatin of cells undergoing apoptosis were detected by staining with 2.5 μg/ml of Hoechst 33342 fluorochrome and 2.5 μg/ml propidium iodide (PI, Sigma), followed by examination on a fluorescence microscope (Olympus optical, Tokyo). Intact blue nuclei, condensed/fragmented blue nuclei, condensed/ fragmented pink nuclei, and intact pink nuclei were considered as viable, early apoptotic, late apoptotic, and necrotic cells, respectively (Shimizu et al, 1996).

Flow cytometry analysis

To block Fc receptors, cells were incubated with purified anti-mouse CD16/CD32 mAb (BD PharMingen) at a concentration of 1 μg/100 μl/10^6 cells for 15 min at 4°C. The cells were incubated with each mAb at a concentration of 1 μg/100 μl for 30 min at 4°C and washed twice with HBSS containing 5% FBS and 0.1% sodium azide. Fluorescein isothiocyanate (FITC)-labeled anti-mouse $I\text{-}A^b$ mAb and phycoerythrin (PE)-labeled anti-mouse CD11c mAb were used for direct staining. FITC- or PE-labeled isotype-matched mAb (all from BD PharMingen) was used as control. The cells were stained with 2 μl/sample annexin V-FITC (Biosource International) at 4°C to measure apoptosis of cells. After staining, the cells were analyzed with FACSCaliber flow cytometer (Becton Dickinson, Mountain View, CA) and CellQuest software.

Statistical analysis

In MTT assay, the result of each sample is expressed as mean±standard deviation (SD) from three independent wells. Most of data are representatives of three individual experiments with similar results. The statistical significance of experimental data was evaluated by the Student's *t*-test. $P<0.05$ was considered as statistically significant.

RESULTS

Generation and characterization of bone marrow-derived DCs

Bone marrow-derived DC precursor cells attach to the bottom of culture plates and grow as floating cells after 4 days of culture. Therefore, floating cells at 6~10 days of culture were used as DCs. As seen in Fig. 1A, cells have the dendritic process, a typical morphological characteristics for DCs on their surface. MHC class II and CD11c were used as surface markers for DCs, and the DC preparation was found to be composed of over 80% MHC class II^+ and $CD11c^+$ cells. Interestingly, CD40 activation by anti-CD40 mAb revealed a rapid homotypic aggregation as a morphological character of DCs (Fig. 1B).

CD40 activation strongly enhances the viability of DCs

The viability of DCs was measured by MTT assay. 5-FU or mitomycin C treatment significantly decreased the viability of DCs in a concentration-dependent manner, and CD40 activation significantly enhanced the viability of DCs ($P<0.05$, Fig. 2). These data strongly suggest that CD40

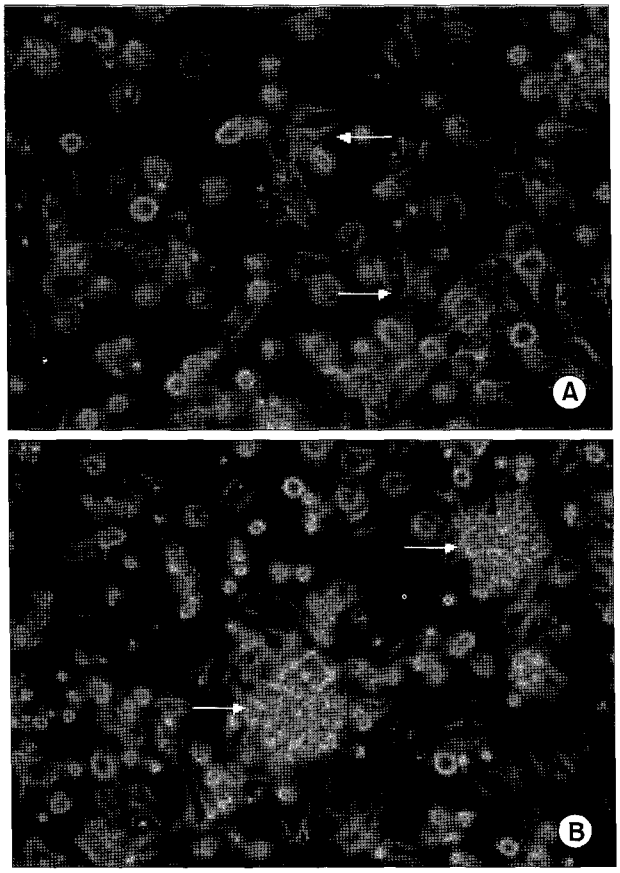

Fig. 1. Photomicrographs of mouse bone marrow-derived DCs. Bone marrow cells were cultured in complete medium containing GM-CSF for 6 days. Cells were treated with medium alone (A) or anti-CD40 mAb (B) for 48 hr. Floating cells have the dendritic process (white arrow), a typical morphological character of DCs on their surface (A). Homotypic aggregation of DCs was observed in anti-CD40 mAb-treated DCs (B).

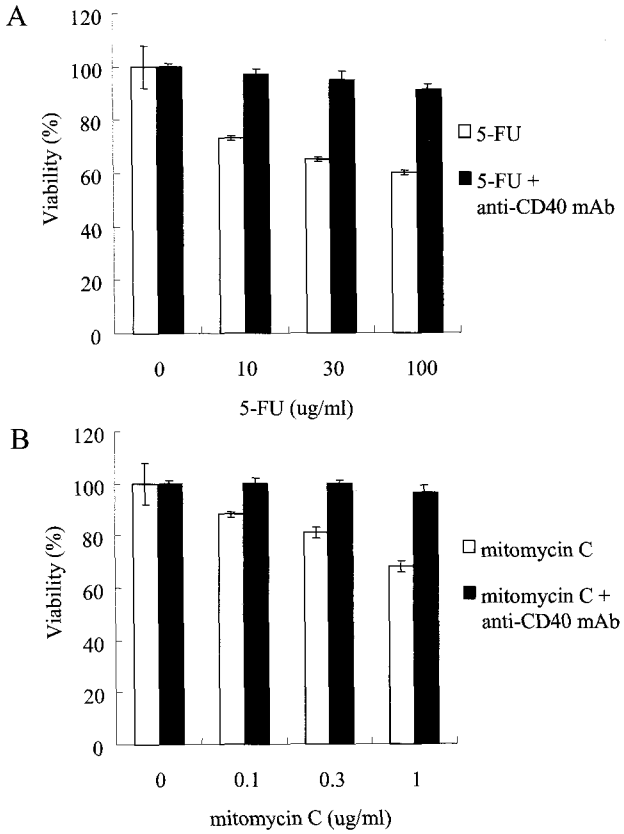

Fig. 2. Viability of anticancer drug-treated DCs was enhanced by CD40 activation. DCs were seeded at a concentration of 5×10^4 cells/well in 96-well culture plates. Cells were cultured in a combination of 5-FU (A), mitomycin C (B), or anti-CD40 mAb for 48 hr. After adding MTT reagent, insoluble crystals were dissolved with 10% SDS solution. Absorbance at 570 nm was measured using ELISA reader, and the OD of DCs treated with DMSO (5-FU) or medium (mitomycin C) alone was set to 100%. Results are means ±SD from three independent wells and a representative of three individual experiments.

activation may protect DCs from the anticancer drug-induced cell death and immunosuppression under physiological as well as pathological conditions.

CD40 activation recovers the expression of MHC class II molecules on DCs

Dead cells are gated based on cell size and also determined by PI staining as hypoploid apoptotic cells (data not shown). Since MHC class II is a critical immunomodulatory molecule for the APC function of DCs, the expression of MHC class II molecules on DCs was measured. As shown in Fig. 3, the expression of MHC class II molecules on DCs treated with 5-FU or mitomycin C was significantly decreased, but recovered by CD40 activation.

Fluorescence analysis confirms the enhancing effect of CD40 activation on the viability of DCs

5-FU significantly increased the number of DCs containing condensed/fragmented pink nuclei and intact pink nuclei, indicating apoptotic or necrotic cells (Fig. 4). The percentage of cells containing intact blue nuclei, viable cells, was increased by CD40 activation. Taken together, fluorescence analysis revealed that CD40 activation enhanced the viability of DCs treated with 5-FU.

CD40 activation protects DCs from the anticancer drug-induced apoptosis

Annexin V-FITC staining was performed to confirm by quantitative analysis whether cell death was apoptosis. The optimal period of 5-FU or mitomycin C treatment for annexin V-FITC staining was determined in a preliminary study, since annexin V specifically binds to apoptotic cells at early stage of apoptosis (data not shown). Thus, cells were stained with annexin V-FITC and PI to distinguish early and late apoptotic cells by PI negative/annexin V positive and PI positive/annexin V positive, respectively. Indeed, 5-FU or mitomycin C consistently enhanced the binding of annexin V to DCs, indicating the increase of apoptotic cells and CD40 activation dramatically inhibited the binding (Fig. 5A). The viability assay using trypan blue staining further confirmed the enhancing effect of CD40 activation on the survival of DCs (Fig. 5B). Anti-CD40 mAb

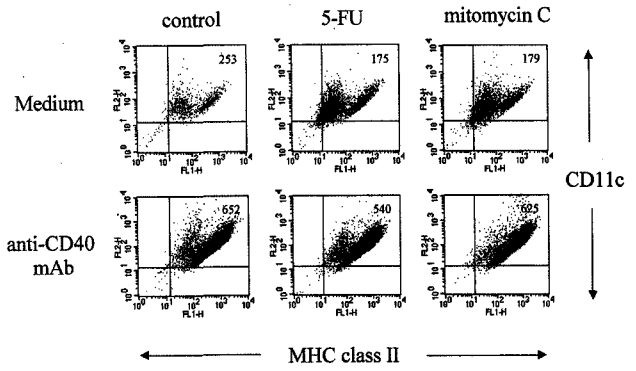

Fig. 3. CD40 activation enhances the expression of MHC class II on DCs. After 6~8 days of culture, DCs were seeded in 24-well culture plates at a concentration of 5×10^5 cells/ml. Cells were incubated with a combination of 1 mg/ml 5-FU, 10 μg/ml mitomycin C, or 1 μg/ml anti-CD40 mAb for 48 hr. The expressions of MHC class II and CD11c molecules were analyzed using flow cytometry. Result is a representative of three individual experiments.

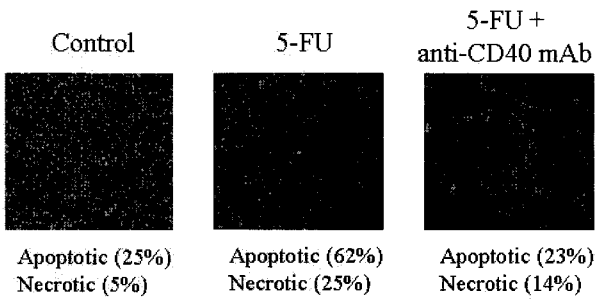

Fig. 4. Morphological changes in the nuclear chromatin of DCs. Cells were harvested and seeded as described in Fig. 3. Cells were treated with control (DMSO only), 1 mg/ml 5-FU, or 1 mg/ml 5-FU +1 μg/ml anti-CD40 mAb, and stained with Hoechst 33342 and PI for nuclear staining. Stained cells were examined under a fluorescence microscope. Result is a representative of three individual experiments.

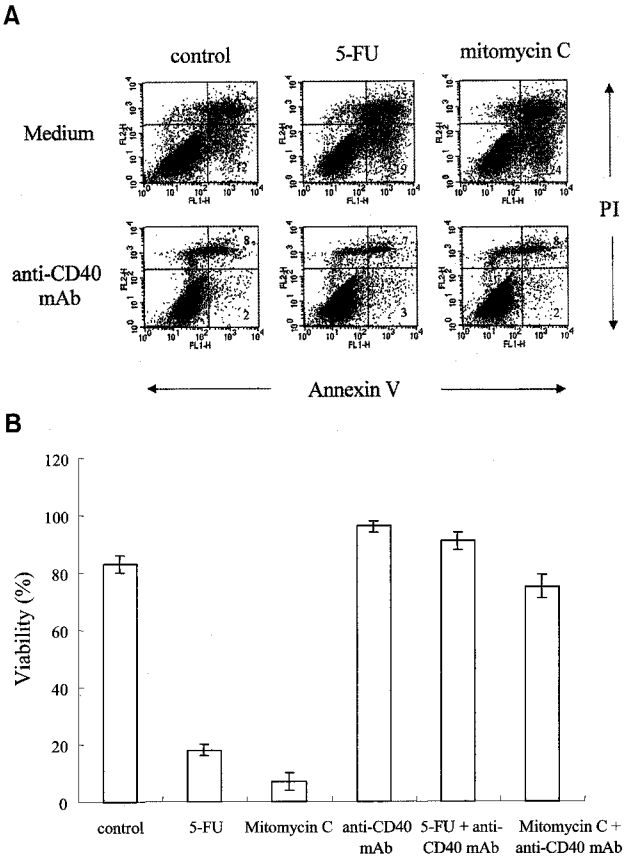

Fig. 5. CD40 activation protects DCs from the anticancer drug-induced apoptosis. After culture of DCs for 6 days, DCs were treated with medium alone, 1 mg/ml 5-FU, 10 μg/ml mitomycin C, 1 μg/ml anti-CD40 mAb, 5-FU+anti-CD40 mAb, mitomycin C+ anti-CD40 mAb for 48 hr. Cells were stained with Annexin V-FITC and PI, and then analyzed using flow cytometry (A). To confirm the viability, cells were stained by trypan blue and counted for viable and dead cells (B). Result is a representative of three individual experiments.

significantly improved the viability of DCs treated with 5-FU or mitomycin C ($P<0.01$).

DISCUSSION

DCs play an essential role in initiating the antigen-specific proliferation of nave T lymphocytes. To enhance tumor-specific immune responses, DCs have been used in various cancers including lymphoma, melanoma, and renal cancer (Hsu et al, 1996; Nestle et al, 1998), suggesting that DC therapy may provide an efficient clinical tool. Chemotherapy is the major therapy for cancer patients, however, it results in severe immunosuppression including myelosuppression. Therefore, we investigated if CD40 activation can protect DCs from chemotherapy-induced apoptosis.

Bone marrow-derived DCs (BMDCs) were used in this study, since only small number of nonproliferating DCs can be harvested from mouse spleen and thymus. In addition, BMDCs have been used as standard DCs for various purposes, because a generating method of large number of DCs has been established (Inaba et al, 1992). 5-FU and mitomycin C were used as representative anticancer drugs for chemotherapy, since two drugs have clinically been used for many years (Moertel, 1975; DeKernion, 1977). Low-dose chemotherapy, especially when combined with 5-FU, is known to be one of the effective chemotherapies for patient with a variety of cancers (Keane et al, 1994; Macdonald et al, 2002). It is a valuable therapy, however, generates some extent of myelosuppression in cancer patients. Therefore, it is very important to develop a therapeutic method to protect host immune system including DCs. In this study, we demonstrated that CD40 activation protected anticancer drug-induced apoptosis of DCs.

It was of interest to observe that CD40 activation by anti-CD40 mAb revealed a rapid homotypic aggregation of DCs (Fig. 1B). Although LPS is also a representative maturation agent for DCs, it did not cause any aggregation in our study (data not shown), in agreement with recent observation, which demonstrated that cross-linking of MHC class II molecules onto human monocyte-derived DCs generated a rapid homotypic aggregation, accompanied by

the increase of some adhesion molecules (Lokshin et al, 2002). It is, therefore, suggested that CD40 activation may transduce signals involved in some adhesion processes of DCs.

The survival signal in DCs has partly been studied. PI3 kinase/Akt, but not the other pathways, is important in maintaining survival of LPS-stimulated monocyte-derived DCs, whereas inhibition of p38 SAPK reduced the LPS-induced upregulation of CD80, CD86, and CD83 molecules (Ardeshna et al, 2000). However, CD40-mediated protection of DC apoptosis was reduced by SB202190, the specific inhibitor of mitogen-activated protein kinase p38, but not related to ERK or NF-κB activation (McLellan et al, 2000). And also, CD40 activation induced an anti- apoptotic molecule, cellular inhibitor of apoptosis protein-2, via p38 kinase in DCs (Aicher et al, 1999). It is likely that the maturation process of DCs may have differential signal pathways for their survival and other function. We are in a process to investigate the signal pathway of CD40 activation on DCs treated by anticancer drugs.

Taken together, our data demonstrated that 5-FU or mitomycin C induced severe cell death, apoptosis of DCs, and CD40 activation effectively ameliorated the apoptosis of DCs. It is suggested that CD40 activation may provide cancer patient with a valuable therapeutic way.

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