大韓獸醫學會誌(2003) 第43卷 第4號
Korea J Vet Res(2003) 43(4) : 607~613

수지상세포에서 GM-CSF의 항암제유도 세포사멸 방지효과에 관한 연구

주 홍 구*
제주대학교 수의학과
(게재승인: 2003년 11월 20일)

Granulocyte-macrophage colony stimulating factor protects dendritic cells from anticancer drug-induced apoptosis

Hong-Gu Joo*
Department of Veterinary Medicine, Cheju National University, Jeju 690-756, Korea
(Accepted: November 20, 2003)

Abstract : Dendritic cells (DCs) play an essential role in a variety of immune reactions involving $CD4^+$ T cells and have been used to enhance tumor-specific immune responses. Immunosuppression in patients with cancer includes the downregulation of function and number of DCs. Although DCs have been studied, the apoptosis of DCs induced by anticancer drugs for chemotherapy remains largely uncharacterized. This study demonstrated that GM-CSF protects DCs from 5-fluorouracil (5-FU) or mitomycin C-induced apoptosis. After 6 - 10 days culture, DCs were characterized by specific surface marker, CD11c and MHC class II. MTT assay revealed that GM-CSF significantly enhanced the viability of DCs treated with 5-FU or mitomycin C. The percentage of dead cells of DCs was determined by cell size using FACScan and GM-CSF was clearly effective. However, GM-CSF did not increase the expression of MHC class II on viable DCs gated, suggesting that GM-CSF may differentially regulate critical factors involved in the function of DCs. For the quantitative analysis of apoptosis, annexin V-FITC staining was performed. 5-FU induced the apoptosis of DCs and GM-CSF significantly protects DCs from 5-FU-induced apoptosis. Taken together, the results in this study that GM-CSF has an anti-apoptosis effect on DCs may provide patients with cancer with clinical benefits to overcome the immunosuppression induced by the decrease of number and functional insufficiency of DCs.

Key words : dendritic cells, apoptosis, 5-FU, GM-CSF

Introduction

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) [1]. DCs originated from bone marrow migrate to peripheral tissues and organs. DCs take up, process antigen and present antigenic peptides to naive T lymphocytes, stimulating their proliferation. Thus, DCs play a critical role in host immune system.

Various cytokines including GM-CSF, IL-4, and TNF-alpha, have been applied to proliferate DCs from their progenitor cells. Especially, GM-CSF has been used for the proliferation of human monocyte-derived, rat or mouse bone marrow-derived DCs since the generating method of large numbers of DCs had been developed [3, 13].

It is well known that tumor burden induces the immunosuppression in patients with advanced cancer. Recent study demonstrated that tumor-derived soluble factors promote altered maturation and early apoptosis of monocyte-derived DCs [6]. Furthermore, patients with cancer often receive chemotherapy, but anticancer drugs used for chemotherapy kill both cancer cells and

*Corresponding author: Hong-Gu Joo
Department of Veterinary Medicine, Cheju National University, Ara 1 dong, Jeju city, Jeju 690-756, Korea
[Tel: 82-64-754-3379, Fax: 82-64-756-3354, E-mail: jooh@cheju.ac.kr]

normal cells including immune cells. Chemotherapy thus escalates the immunosuppression of cancer patients. The function and numbers of DCs in patients with cancer are presumably downregulated by tumor cell and chemotherapy [2]. Therefore, therapeutic methods that can overcome the immunosuppression in chemotherapy -treated cancer patients may provide an essential clinical benefit.

Although the effect of GM-CSF on the proliferation of hematopoietic cells including DCs has been studied [8] and a variety of clinical trials for cancer patients include GM-CSF [10], the anti-apoptosis effect of GM-CSF on DCs treated with anticancer drugs for chemotherapy has not been studied.

The results of this study demonstrated that GM-CSF protects DCs from anticancer drug-induced apoptosis. It is thus suggested that GM-CSF can be used as an anti-apoptotic agent for DCs in host immune system of cancer patients.

Materials and 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 maintained in the lab animal facility for breeding and used at the age of 7- to 10-weeks. Purified anti-mouse CD8, CD19, Gr-1 monoclonal antibodies (mAbs, BD PharMingen, San Diego, CA) were used for the detection of $CD8^+$ T lymphocytes, B lymphocytes, 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 were cultured from bone marrow of mice using a general method that was initially established by Inaba *et al* [3]. Briefly, bone marrow cells were harvested from tibia and femur of mice by flushing with PBS. Cells were cultured at a concentration of 2×10^6 cells/ml in 6-well culture plates. RPMI-1640 medium including 5% fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin (all from Life Technologies Inc., Gaithersburg, MD), and 10 ng/ml mouse GM-CSF (Biosource International, Camarillo, CA) were used. The culture medium was replaced with fresh medium at 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 day of culture. At 6-10 day of culture, 70% (v/v) of the medium was replaced by fresh medium and floating cells were used as DCs for experiments. DCs in this study were over 85% $CD11c^+$ DCs based on FACS analysis.

Assessment of cytotoxicity by MTT assay

The viability of DCs was measured by using MTT assay. Briefly, cells were seeded at a concentration of 5×10^4 cells/ml in 96-well plates and treated with the combination of GM-CSF and/or anticancer drug, 5-fluorouracil (5-FU) or mitomycin C (Sigma). 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 and followed by incubation at 37°C in CO_2 incubator for 24 hr. Viable DCs generate insoluble crystal, but DCs are floating and loosely attached 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 24 hr, the absorbance of sample was measured at 570 nm, 630 nm as reference 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 Hoechst 33342 (Sigma) and followed by examination on a fluorescence microscope (Olympus Optical, Tokyo). In some experiments, cells were double stained with 2.5 μg/ml propidium iodide (PI, Sigma) and 2.5 μg/ml Hoechst 33342 to distinguish apoptotic cells from necrotic cells. 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 [11].

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. Cells were incubated with each mAb at a concentration of 1 μg/100 μl for 30 min at 4°C and washed twice with Hanks' balanced salt solution (HBSS, Sigma) containing 5% FBS and 0.1% sodium azide. FITC-labeled anti-mouse $I\text{-}A^b$ mAb, PE-labeled

anti-mouse CD11c mAb (BD PharMingen) were used for direct staining. FITC- or PE-labeled isotype-matched mAb (BD PharMingen) was used as control, respectively. Cells were stained with 2 µl/sample annexin V-FITC (Biosource International) at 4°C for measuring apoptosis of cells. After staining, 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 mean± standard deviation (SD) from three independent wells. Most of data are the representative 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.

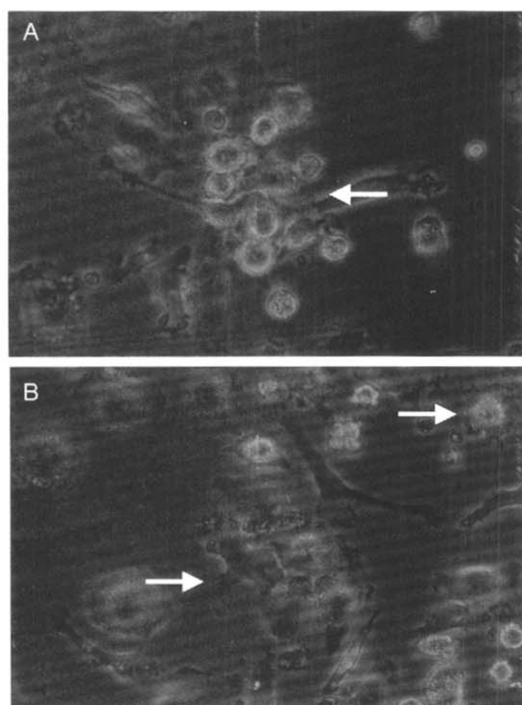

Fig. 1. Photomicrographs of mouse bone marrow-derived DCs. Bone marrow cells were harvested from tibia and femur of mice and then cultured in complete medium containing GM-CSF. Clusters of proliferating cells (white arrow) were observed in the cultures after 48 hr (A). Some floating cells have the dendritic process (white arrow), a typical morphological character for DCs on their surface after 96 hr culture (B). The detailed culture conditions and characterization of DCs are described in Materials and Methods.

Results

Generation and characterization of bone marrow-derived DCs

To investigate if GM-CSF protects DCs from anticancer drugs, we cultured bone marrow-derived DCs. Bone

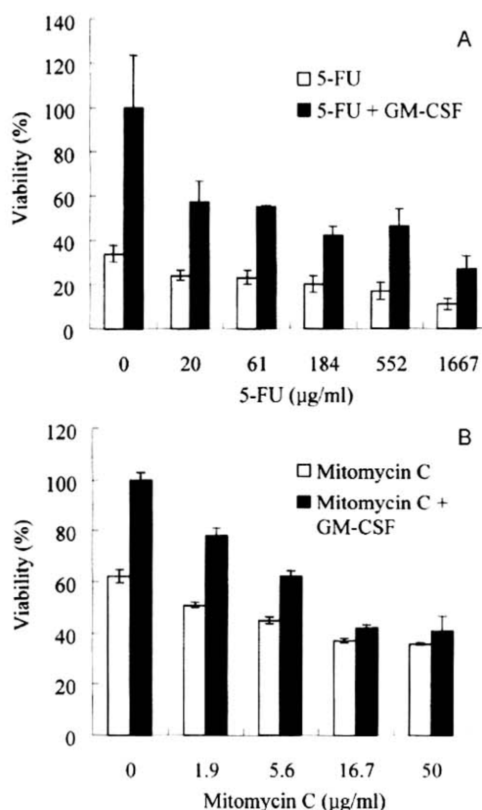

Fig. 2. The viability of anticancer drugs-treated DCs was enhanced by GM-CSF. DCs were harvested at 6-8 days after culture. Cells were washed with HBSS twice before experiment and seeded at a concentration of 5×10^4 cells/well in 96-well culture plates. Cells were cultured in a combination of 5-FU, mitomycin C, or GM-CSF for 48 hr. Then, MTT reagent was added into wells for 24 hr and insoluble crystals were dissolved with 10% SDS solution. The absorbance was measured at 570 nm using ELISA reader and the absorbance value of the GM-CSF-treated cells was set to 100%. Results are means±SD from three independent wells and a representative of three individual experiments.

marrow cells were harvested from tibia and femur of mice. Cells were cultured in complete medium (CM) containing 5% FBS and 10 ng/ml GM-CSF. Bone marrow-derived DC precursor cells were attached to the bottom of culture plates (Fig. 1A) and grown as floating cells after 4 days culture. Floating cells were harvested by centrifugation after 6-10 days culture and used as DCs for experiments. Cells have the dendritic process, a typical morphological character for DCs on their surface (Fig. 1B). MHC class II and CD11c were used as surface markers for DCs. Cells used as DCs in this study were over 80% MHC class II^+ and $CD11c^+$ cells. The yield of DCs was approximately 4 to 5×10^6 DCs from one mouse.

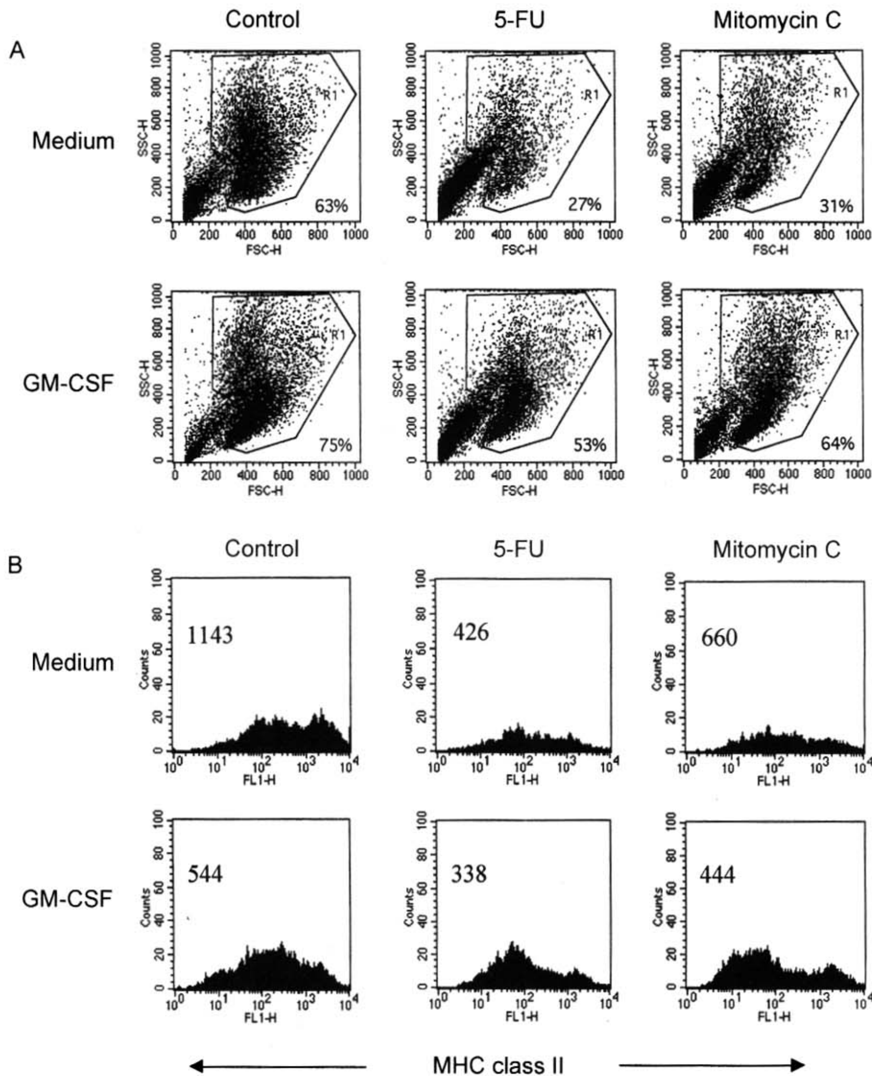

Fig. 3. GM-CSF enhances the viability of DCs. After 6-8 days, DCs were seeded in 24-well culture plates at a concentration of 5×10^5 cells/ml. Cells were incubated with a combination of 1 mg/ml 5-FU, 10 μg/ml mitomycin C, or 10 ng/ml GM-CSF for 48 hr. After washing twice with HBSS, cell size and the expression of MHC class II molecules were analyzed using flow cytometry. Viable cells were gated as R1 based on cell size (A). The expression of MHC class II expression of viable cells gated was measured (B). Result is a representative of three individual experiments.

The cytotoxicity of DCs treated by anticancer drug was decreased by GM-CSF

MTT assay was performed for measuring the viability of DCs. 5-FU or mitomycin C treatment decreased the viability of DCs in a concentration-dependent manner and GM-CSF enhanced the viability of DCs. Cells were cultured in 96-well culture plates and treated with a combination of 5-FU, mitomycin C, or GM-CSF. Cells were treated by 3-fold serial diluted 5-FU, mitomycin C in the absence or presence of GM-CSF. The optimal concentration of GM-CSF was determined based on its biological activity on DCs in a preliminary study (data not shown). The viability of cells treated with 5-FU or mitomycin C was significantly enhanced by 10 ng/ml GM-CSF (Fig. 2). This data suggest that GM-CSF may protect DCs from the anticancer drug-induced cell

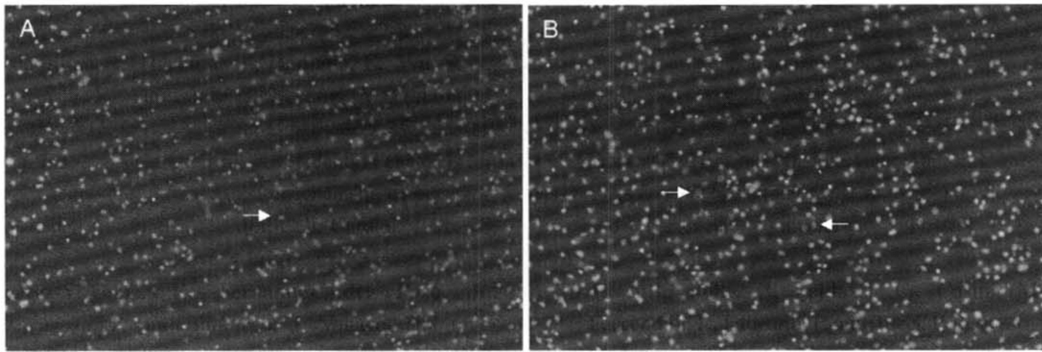

Fig. 4. Morphological changes in the nuclear chromatin of DCs. Cells were harvested and seeded as described in Fig. 3. Cells treated with 1 mg/ml 5-FU (A) or 1 mg/ml 5-FU +10 ng/ml GM-CSF were stained with Hoechst 33342 and PI for nuclear staining. Stained cells were examined on fluorescent microscope. Result is a representative of three individual experiments.

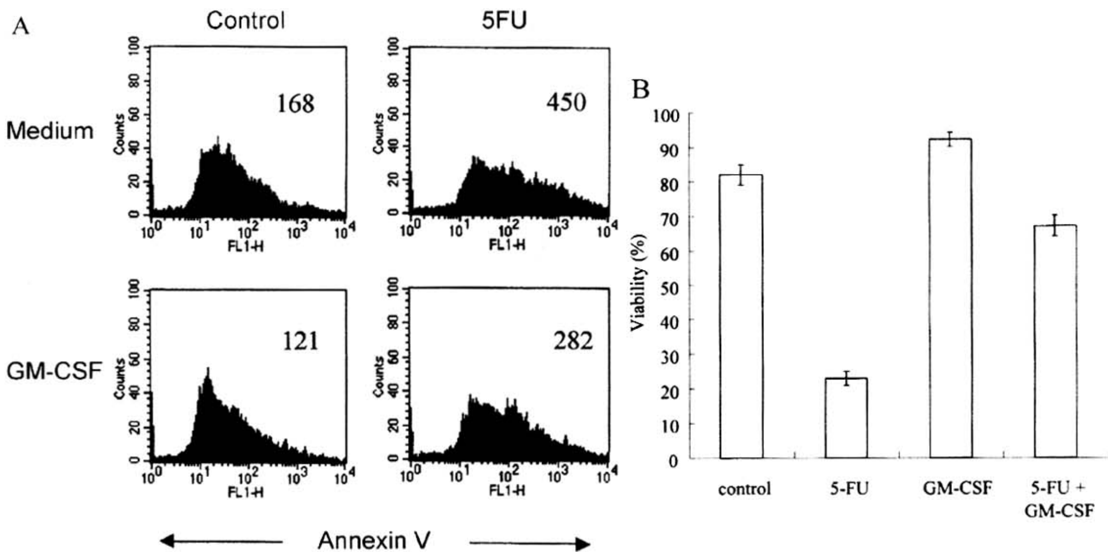

Fig. 5. GM-CSF protects DCs from the anticancer drug-induced apoptosis. After culture of DCs for 6 days, DCs were treated with medium alone, 1 mg/ml 5-FU, 10 ng/ml GM-CSF, 5-FU+GM-CSF for 48 hr. Cells were stained with Annexin V-FITC and analyzed using flow cytometry (A). To confirm the viability, cells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

death and immunosuppression.

GM-CSF enhances the viability of DCs, but not the expression of MHC class II

Dead cells were gated based on cell size and also determined by PI staining as hypoploid apoptotic cells (data not shown). Based on cell size, GM-CSF strongly enhanced the viability of DCs treated with 5-FU or mitomycin C (Fig. 3A). Since MHC class II is a critical immunoregulatory molecule for the APC function of DCs, the expression of MHC class II molecules on DCs was measured in this study. Total expression of MHC class II on DCs treated with 5-FU or mitomycin C was increased by GM-CSF (data not shown), however, the expression of MHC class II on viable DCs gated was marginally decreased by GM-CSF (Fig. 3B). It is possible that GM-CSF may differentially regulate essential factors related to the function of DCs.

Fluorescence analysis revealed enhancing effect of GM-CSF on the viability of DCs

To confirm the enhancing effect of GM-CSF on the cell death of DCs treated with 5-FU, cells were seeded in 24-well culture plates. PI staining and Hoechst 33342 were used for determining necrotic or apoptotic cells, and viable cells respectively [12]. Fluorescence analysis revealed that GM-CSF enhanced the viability of DCs treated by 5-FU (Fig. 4).

GM-CSF protects DCs from the anticancer drug-induced apoptosis

Annexin V-FITC staining was performed to check if cell death was apoptosis and for quantitative analysis. Annexin V is a 35-36 kDa calcium-dependent phospholipid binding protein with high affinity for phosphatidylserine, which is found in outer cell membrane beginning early in the process of apoptosis [7]. In a preliminary study, the duration of 5-FU treatment was optimized for annexin V-FITC staining since annexin V specifically binds to apoptotic cells at early stage of apoptosis (data not shown). Indeed, 5-FU repeatedly enhanced the binding of annexin V to DCs and GM-CSF decreased the binding (Fig. 5A). The viability assay using trypan blue staining confirmed the enhancing effect of GM-CSF on the survival of DCs (Fig. 5B). This result strongly suggests that GM-CSF may provide clinical benefit, the protection of DCs from anticancer drug-induced apoptosis in patients with cancer.

Discussion

DCs play a critical role in initiating the antigen-specific proliferation of naive T lymphocytes. Recent study demonstrated that DCs have an additional function, such as cytotoxic activity, as well as APC function [4]. To enhance tumor-specific immune responses, DCs have been used in various cancers including melanoma, renal cancer [9], suggesting that DC therapy may be a feasible clinical approach with promising results. However, chemotherapy is a representative therapy for patients with cancer, but resulting in severe immunosuppression. It was investigated in this study if GM-CSF has a clinical benefit for protecting DCs from chemotherapy-induced apoptosis.

Bone marrow-derived DCs (BMDCs) were used as DC source in this study because only a small number of nonproliferating DCs could be isolated from mouse spleen and thymus [15]. And also, BMDCs have been used as representative or standard DCs for multiple purposes since the generation method of DCs in large scale was established [3]. 5-FU and mitomycin C were selected as representative anticancer drugs for chemotherapy since two reagents have been used clinically for many years. Low-dose chemotherapy, especially when combined with 5-FU, is known to be one of effective chemotherapies for patient with a variety of cancers including bladder cancer [5].

The expression of MHC class II on viable DCs gated was marginally decreased by GM-CSF, whereas total expression of MHC class II on DCs treated with 5-FU or mitomycin C was increased by GM-CSF. It is possible that in case of DCs treated with anticancer drugs, only DCs with high survival activity might be alive in stringent environment, such as 5-FU or mitomycin C containing medium and therefore the expression of MHC class II on viable cells be affected by the survival activity. The exact mechanism of this observation remains to be investigated in further experiments.

Chemotherapy is one of widespread therapies and DCs, as alone or in combination, are applied for various immunotherapy. Recent study showed that the intratumoral injection of DCs after treatment of anti-cancer drugs induced tumor-specific antitumor effect in tumor-bearing mice [14]. With regard to this, the information to protect DCs may be essential and useful to inhibit the immunosuppression in cancer patients.

Taken together, results in this study demonstrated that GM-CSF protects DCs from chemotherapy-induced apoptosis, suggesting that GM-CSF may provide an additional clinical benefit as well as proliferating effects on hematopoietic cells in patients with cancer.

References

1. **Banchereau, J. and Steinman, R. M.** Dendritic cells and the control of immunity. Nature 1998, **392**, 245-252.
2. **Gabrilovich, D. I., Chen, H. L., Girgis, K. R., Cunningham, H. T., Meny, G. M., Nadaf, S., Kavanaugh, D. and Carbone, D. P.** Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nature Medicine 1996, **2**(10), 1096-1103.
3. **Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S. and Steinman, R. M.** Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 1992, **176**(6), 1693-1702.
4. **Joo, H.-G., Fleming, T. P., Tanaka, Y., Dunn, T. J., Linehan, D. C., Goedegebuure, P. S. and Eberlein, T. J.** Human dendritic cells induce tumor-specific apoptosis by soluble factors. Int. J. Cancer 2002, **102**(1), 20-28.
5. **Keane, T. E., Gingrich, J. R., Rosner, G., Webb, K. S., Poulton, S. H. and Walther, P. J.** Combination versus single agent therapy in effecting complete therapeutic response in human bladder cancer: analysis of cisplatin and/or 5-fluorouracil in an *in vivo* survival model. Cancer Res. 1994, **54**(2), 475-481.
6. **Kiertscher, S. M., Luo, J., Dubinett, S. M. and Roth, M. D.** Tumors promote altered maturation and early apoptosis of monocyte-derived dendritic cells. J. Immunol. 2000, **164**(3), 1269-1276.
7. **Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M. and Green, D. R.** Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med. 1995, **182**(5), 1545-1556.
8. **Morstyn, G. and Burgess, A. W.** Hematopoietic growth factors: a review. Cancer Res. 1988, **48**(20), 5624-5637.
9. **Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G. and Schadendorf, D.** Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nature Medicine 1998, **4**(3), 328-332.
10. **Pinedo, H. M., Buter, J., Luykx-de Bakker, S. A., Pohlmann, P. R., van Hensbergen, Y., Heideman, D. A. M., van Diest, P. J., de Gruijl, T. D. and van der Wall, E.** Extended neoadjuvant chemotherapy in locally advanced breast cancer combined with GM-CSF: effect on tumor-draining lymph node dendritic cells. Eur. J. Cancer 2003, **39**(8), 1061-1067.
11. **Shimizu, S., Eguchi, Y., Kamiike, W., Itoh, Y., Hasegawa, J., Yamabe, K., Otsuki, Y., Matsuda, H. and Tsujimoto, Y.** Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by Bcl-2 and Bcl-XL. Cancer Res. 1996, **56**(9), 2161-2166.
12. **Suk, K., Chang, I., Kim, Y.-H., Kim, S., Kim, J. Y., Kim, H. and Lee, M.-S.** Interferon γ (IFNγ) and tumor necrosis factor α synergism in ME-180 cervical cancer cell apoptosis and necrosis: IFNγ inhibits cytoprotective NF-κB through STAT1/IRF-1 pathways. J. Biol. Chem. 2001, **276**(16), 13153-13159.
13. **Talmor, M., Mirza, A., Turley, S., Mellman, I., Hoffman, L. A. and Steinman, R. M.** Generation or large numbers of immature and mature dendritic cells from rat bone marrow cultures. Eur. J. Immunol. 1998, **28**(3), 811-817.
14. **Tanaka, F., Yamaguchi, H., Ohta, M., Mashino, K., Sonoda, H., Sadanaga, N., Inoue, H. and Mori, M.** Intratumoral injection of dendritic cells after treatment of anticancer drugs induces tumor-specific antitumor effect *in vivo*. Int. J. Cancer 2002, **101**(3), 265-269.
15. **Vremec, D., Pooley, J., Hochrein, H., Wu, L. and Shortman, K.** CD4 and CD8 expression by dendritic cells subtypes in mouse thymus and spleen. J. Immunol. 2000, **164**(6), 2978-2986.