A Mouse Thymic Stromal Cell Line Producing Macrophage-Colony Stimulating Factor and Interleukin-6

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A thymic stromal cell line, TFGD, was established from a thymic tumor mass developed spontaneously in p53 knock out mouse, and was found to produce cytokines that could induce bone marrow hematopoietic stem cells (HSCs) to differentiate into macrophages. The cytokines produced by the TFGD line were assessed by immunoassays. High level of macrophage-colony stimulating factor (M-CSF) and interleukin (IL)-6 was detected in the TFGD-culture supernatant, whereas granulocyte/macrophage-colony stimulating factor (GM-CSF), IL-3, IL-4, IL-5, IL-13, or interferon (IFN)-γ was undetectable. Blocking experiments showed that anti-M-CSF monoclonal antibody could neutralize the differentiation-inducing activity shown by the TFGD-culture supernatant. Dot blot analysis of the total RNA isolated from the cultured fetal thymic stromal cells showed that M-CSF transcripts were expressed in the normal thymus. These observations, together with the earlier finding that M-CSF plus IL-6 is the optimal combination of cytokines for the induction of macrophage differentiation from HSCs *in vitro*, may indicate that thymic macrophages could be generated within the thymus by cytokines involving M-CSF.

Key words: Thymic stromal cell line, Macrophage-colony stimulating factor, Thymic macrophages

INTRODUCTION

T cells develop in the thymus from precursors originating in the fetal liver or in the bone marrow (for a review see Scollay *et al.*, 1986). T cell precursors enter the thymus, proliferate, and rearrange T cell receptor (TCR) genes. Once TCRs are expressed on the surface, thymocytes must pass through a series of positive and negative selection events (reviewed by Amsen and Kruisbeek, 1998). The selection processes are based on the TCRs expressed on the thymocytes, and thus TCRs play a central role in determining cell proliferation or cell death. Thymic dendritic cells (DCs) and thymic macrophages are also indispensable in the selection processes because TCRs can only "see" antigens presented by these cell types (for reviews see Kiesielow and von Boehmer, 1991; Fowlkes and Schweighoffer, 1995). The origin of these cells, however, is less defined yet, although the T cell differentiation pathways have been extensively studied in terms of the molecular and cellular events.

An important attribute of mature DCs and macrophages in the periphery is their motility and migratory capacity. However, it has not been established that thymic DCs and thymic macrophages are immigrants from peripheral tissues. Instead, recent studies showed that thymic DCs could be generated from the same early T cell progenitors that generate T cells. The earliest intrathymic T progenitors found within the thymus are $CD4^{lo}CD8^{-}CD3^{-}CD44^{+}CD25^{-}$ (pro-T1) cells (Godfrey *et al.*, 1993), and were shown to generate, as well as T cells, thymic DCs, B cells and NK cells when reconstituted into irradiated mouse embryonic thymus lobes or mice (Wu *et al.*, 1991; Ardavin *et al.*, 1993; Lee *et al.*, 1998; Marquez *et al.*, 1998). The next stage cells in maturation pathway are $CD4^{-}CD8^{-}CD3^{-}CD44^{+}CD25^{+}$ (pro-T2) cells (Godfrey *et al.*, 1993). Pro-T2 cells were recently shown to generate thymic DCs, as well as T cells, by transfer to irradiated recipients or by mixture of cytokines (Wu *et al.*, 1996; Saunders *et al.*, 1996). From these observations, it has been suggested that thymic DCs are developmentally lymphoid-related rather than myeloid-related (Shortman *et al.*, 1998).

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Thymic macrophages, like thymic DCs, have been postulated to be generated within the thymus, based on the observations that fetal thymocytes generate macrophages *in vitro* (Peault *et al.*, 1994). In recent study, evidence of myelopoiesis has also been documented in the thymus (Kendall *et al.*, 1999). The thymic microenvironments that induce myelopoiesis, however, have not been elucidated yet. Here, we report that a thymic stromal cell line produces cytokines, M-CSF and IL-6, which have been shown to be the optimal combination of cytokines for the induction of macrophage differentiation from hematopoietic progenitor cells. Our finding supports the hypothesis that thymic macrophages are generated within the thymus.

MATERIALS AND METHODS

Cell line and cell culture

The TFGD cell line was established from a thymic tumor mass developed spontaneously in a p53$^{-/-}$ mouse. Briefly, a single cell suspension of the tumor mass was prepared by treatment with trypsin, and then cultured in RPMI-1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Hyclone, USA) in a tissue culture plate. The cell line was established by selecting for adherence by routinely removing nonadherent cells for a period of 2 months. Conditioned medium of the TFGD cells was prepared from confluent cultures of TFGD.

Flow cytometry

The monoclonal antibodies recognizing murine cell surface markers, anti-I-A^b (clone AF6-120.1), anti-H2-K^b (clone AF6-88.5), anti-ICAM-1 (clone 3E2), anti-B7-1 (clone 16-10A1) and anti-B7-2 (clone GL1) were purchased from Pharmingen (San Diego, CA). For staining, the TFGD cells were resuspended in a staining solution of PBS containing 5% FBS and 0.1% NaN_3, mixed with 50ul of pre-diluted antibody solution, and then incubated for 20 min on ice. Unbound antibodies were removed by washing the cells twice with staining buffer. If biotinylated antibody was used, the cell pellet obtained from the second wash was resuspended in 50 μl of PE- or FITC-conjugated avidin, incubated for 10 min on ice, and then washed twice with staining buffer. After staining, cells were fixed in 1% p-formaldehyde in PBS and flow cytometric analysis was performed on a FACStar Plus (Becton-Dickinson Immunocytometry System). Dead cells were gated out by their low forward angle light scatter intensity. In most analysis, 10,000 cells were scored.

Quantitation of cytokines

The amounts of cytokines contained in the culture supernatant of TFGD were measured by commercial immunoassay kits purchased from Endogen (IL-3, IL-4, IL-5, IL-6, IFN-g, GM-CSF) and R&D System (IL-13). As a negative control, the culture supernatant of NIH3T3 fibroblast cells was also included in the immunoassays. The amount of M-CSF was estimated by a bioassay using mouse BM cells. Briefly, 100 μl of the serially diluted human M-CSF (PeproTec Inc.) or the culture supernatant of TFGD cells was added to mouse BM cell cultures (5×10^4 cells/well) in a volume of 100ml, and the plate was incubated for 5 days. The amount of adherent cells developed from BM cells was assessed by XTT assay after removal of nonadherent cells by washing the plate with PBS. The amount of M-CSF in the culture supernatant was calculated by a parallel line analysis using a standard curve obtained from cultures added with known amounts of recombinant human M-CSF.

Blocking experiment

Anti-mouse M-CSF neutralizing antibody was purchased from R&D System. For neutralization, the culture supernatant of TFGD cells was first diluted with complete media (1:3 or 1:7), mixed with an equal volume of serially diluted anti-GM-CSF antibody solution, and then incubated for 1 h at 37°C. The incubated mixtures were then added to wells of a 96-well tissue culture plate (100 μl/well) in triplicate. Mouse BM cells were prepared from femurs, and then added to each well (5×10^4 cells/well) in a volume of 100 μl. The cultures were incubated for 5 days, and the amount of adherent cells developed from BM cells wase assessed by XTT assay after removal of nonadherent cells by washing the plate with PBS. The neutralization efficiency was calculated as follows: [1-(O.D.test-O.D.control)/(O.D.max-O.D.control)] × 100. O.D. max was defined as the optical density of the cultures added with the TFGD-culture supernatant without blocking, and O.D.control was defined as the optical density of BM cell only cultures.

Dot blot analysis

Fetal thymocyte suspension was prepared from d.16 fetal thymus lobes as previously described (Lee *et al.*, 1998), and cultured overnight in a tissue culture plate. Nonadherent cells were then washed out after shaking, and the adherent cells were lysed with guanidine isothiocyanate solution (4M guanidium isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1M 2-mercaptoethanol). The lysate was extracted with phenol and chloroform mixture, and then the RNA was precipitated by addition of an equal volume of isopropanol at -20°C. RNA samples were denatured by heating for 15 min at 60°C, cooled on ice, serially diluted with 20X SSC, and then spotted onto nitrocellulose filter using a Dot Blot Apparatus (Bio-Rad) attached to vacuum. The filter was removed, dried, and

baked in an oven for 2 hr at 80°C. The filter was prehybridized (40% formamide, 5X Denhardts solution, 5X SSPE, 0.1% SDS, 150 μg/ml herring testis DNA) for 16 hr at 42°C, and then hybridized with the murine M-CSF cDNA probe which was labeled with ^{32}P-dCTP by an Oligo-label kit (Pharmacia).

RESULTS AND DISCUSSION

We established several mouse thymic stromal cell lines from thymic tumor masses developed spontaneously in p53 knock out mice. The TFGD cell line is one of them, and was selected for further characterization because it was identified to produce cytokines that could induce BM cells to differentiate into macrophages (data not shown). Phenotypic analysis of the TFGD cells showed that they expressed class I MHC antigens, but not class II MHC antigens (Fig. 1). The TFGD cells also expressed ICAM-1. However, the TFGD cells did not express other co-stimulatory molecules such as B7-1 and B7-2.

To identify the cytokines responsible for the generation of macrophages from BM cells, the cytokines in the TFGD-culture supernatant were determined by immunoassays. As shown in Table I, the TFGD cells produced high levels of M-CSF and IL-6, but not GM-CSF, IL-4, IL-5, IL-9, IL-13 or IFN-γ. M-CSF was the most predominant cytokine in the TFGD-culture supernatant, reaching to 2.45 ng/ml concentration. The amount of IL-6 in the TFGD-culture supernatant was much lower than M-CSF, but was present in a significant level (492 pg/ml).

M-CSF is a hematopoietic glycoprotein that stimulates the proliferation and differentiation of BM progenitor cells into myeloid cells (Tushinski *et al.*, 1982; Wong *et al.*, 1987; Metcalf, 1986). In addition, earlier studies have shown that M-CSF synergies with IL-6 in the generation of macrophages from BM progenitor cells (Jansen *et al.*, 1992). Based on these findings and the fact that TFGD cells produce high levels of M-CSF and IL-6, it is reasonable to speculate that the macrophage differentiation-inducing activity shown by the culture supernatant of TFGD cells is due to M-CSF+IL-6.

To confirm that M-CSF is involved in the induction of macrophage differentiation from BM cells, neutralization experiments were performed with polyclonal anti-M-CSF antibodies. In these experiments, the culture supernatant of the TFGD cells was first diluted with a culture medium (1:7, and 1:15), mixed with different amounts of polyclonal anti-M-CSF antibodies, pre-incubated, and then tested for the remaining BM cell differentiation-inducing activity. As shown in Fig. 2, addition of polyclonal anti-M-CSF antibodies to the culture supernatant of TFGD cells

Table I. Production of cytokines by the TFGD cells

Cytokines[a]	TFGD supernatant (pg/ml)	NIH 3T3 supernatant(pg/ml)
IL-3	≤3.9	≤3.9
IL-4	≤5.9	≤5.9
IL-5	≤5.0	≤5.0
IL-6	492.0	19.0
IL-13	≤7.8	7.8
IFN-γ	≤3.9	3.9
GM-CSF	≤3.9	7.0
M-CSF	2,450.0	ND[b]

[a] The amount of the cytokines was measured by commercial immunoassay kits except that of M-CSF, which was estimated by a biological assay.
[b] Not determined

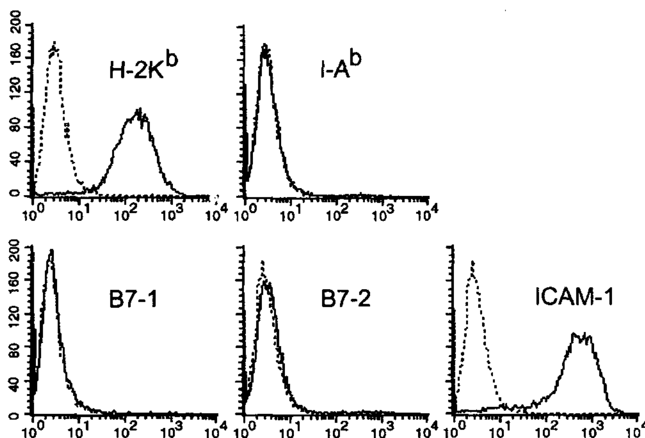

Fig. 1. Phenotypic analysis of the TFGD cells. Cells were stained for the indicated markers, and analyzed by flow microfluorimetry. Immunophenotypic profiles are shown compared to isotype controls (dotted lines)

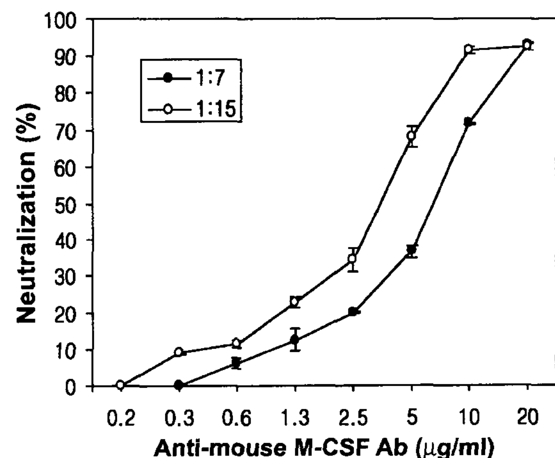

Fig. 2. Neutralization of the BM cell differentiation-inducing activity by polyclonal anti-mouse M-CSF antibodies. The culture supernatant of the TFGD cells was diluted as indicated with a culture medium, preincubated with the indicated amounts of polyclonal anti-mouse M-CSF antibodies, and then assayed for the residual BM cell differentiation-inducing activity.

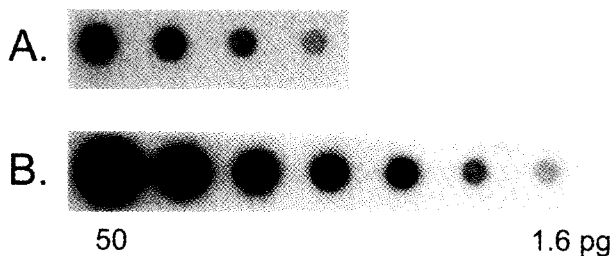

Fig. 3. Expression of M-CSF transcripts by thymic stromal cells. A: Total RNA isolated from cultured mouse thymic stromal cells was serially diluted in 2-fold, and then blotted on a nitrocellulose filter. The filter was hybridized with oligolabeled M-CSF cDNA probe. B: Known amounts of the M-CSF cDNA were treated by the same methods, and then hybridized with the same probe

neutralized the macrophage differentiation-inducing activity in a dose-dependant manner. Almost complete inhibition of macrophage differentiation was observed in cultures containing 10-20 μg/ml of anti-M-CSF antibodies. Thus, we concluded that the macrophage differentiation-inducing activity shown by the culture supernatant of TFGD cells is primarily due to M-CSF.

Expression of M-CSF in the normal thymus tissue was then examined by dot blot analysis. In this experiment, thymic stromal cells were first enriched by plastic adherence, and then total RNA was isolated from the adherent cells by a guanidium isothiocyanate method. As shown in Fig. 3, dot blot analysis detected approximately 6.0 pg of M-CSF transcripts from 0.5 μg of the total RNA, confirming the expression of M-CSF transcripts in the normal thymic stromal cells.

Thymic macrophages have been shown to play an important role in eliminating apoptotic bodies generated from dead thymocytes (Surh and Sprent, 1994). The origin of thymic macrophages, however, has not been established; they could derive from blood monocytes, or they might develop within the thymus (Kendall *et al*, 1999). For more complexity, thymic macrophages were shown to be heterogeneous in surface markers (Vicente *et al*., 1995). If thymic macrophages were generated within the thymus, it would be expected that they are generated from hematopoietic progenitor cells. And, there should be a thymic microenvironment that supports differentiation of hematopoietic progenitor cells into macrophages. The present study suggests that thymic stromal cells could provide the thymic microenvironment, M-CSF+IL-6, that is necessary for the induction of macrophage differentiation from hematopoietic progenitor cells.

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