Glia maturation factor produced by thymic epithelial cells plays a role in T cell differentiation in the thymic microenvironment

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Abstract

In order to determine molecules on thymic epithelial cells which play an important role in the process of T cell differentiation, mAb recognizing thymic epithelial cells were made using stromal cells of the embryonic thymus at 15-gestation date. Among many mAb, a specific one was selected in terms of its inhibition of T cell development in an in vitro culture system of the embryonic thymus. cDNA of the protein recognized by one of the mAb was obtained by a panning method. Sequence analysis revealed that the protein was identical to glia maturation factor (GMF)-β. Northern blot analysis confirmed the expression of GMF-β mRNA in the thymus and brain. Furthermore, immunoblotting analysis identified the production of GMF-β protein in the thymus, the brain and a thymic epithelial cell line. GMF-β protein prepared by a glutathione-S-transferase gene fusion system greatly influenced T cell development in the in vitro culture system of the embryonic thymus in favor of a significant increase of CD4+ T cells with expression of TCRβ. These data taken together suggest that GMF-β protein is produced by thymic epithelial cells and plays a role in T cell development in favor of CD4+ T cells.

Introduction

T precursor cells from the fetal liver or bone marrow vigorously start to proliferate in the thymic microenvironment and proceed to complicated differentiation and selection steps, eventually leaving the thymus as mature T cells. During the maturation steps, multiple interactions between immature T cells and the thymic microenvironment take place in the presence of various factors, which have not been fully determined as yet. IL-7 is known to be necessary for lymphoid progenitors to proliferate and differentiate into cells expressing the pre-TCR complex (1). MHC molecules expressed on thymic epithelial cells play a major role in positive and negative selection during T cell development.

Many investigators are still using fetal thymic organ cultures for their study of T cell development (2,3). This model is useful to analyze various differentiation steps of T cells from the precursor to mature phase, but numerous factors produced by the thymic environment are left in the black box.

Thymic epithelial cells are the major component constituting the thymic environment and we postulate that thymic epithelial cells produce many more as yet unknown factors which play an important role in T cell development. In order to explore these unknown factors, we first produced mAb reacting with thymic epithelial cells by immunohistologic staining. Then, we selected mAb which inhibited T cell differentiation from CD4+CD8+ through CD4+CD8+ to CD4+ or CD8+ thymocytes in a fetal organ culture system. Finally, we determined the molecules reacting with one of the selected mAb using an expression cloning method.
We report that glia maturation factor (GMF)-β (4) is produced by thymic epithelial cells and plays an important role in the process of T cell development.

Methods

Animals

Animals employed were male Wister rats (3 months old), male C57BL/6 mice and pregnant C57BL/6 mice. They were purchased from SLC Japan (Shizuoka, Japan).

Production of mAb

Thymic lobes were collected from C57BL/6 embryos at 15-gestation date (15 gd). They were cultured on a filter (Nucleopore, St Louis, MO; pore size 8 μm) in complete medium supplemented with 1.35 mM 2-deoxyguanosine (2DG) at 37°C in 7.5% CO2 for 7 days to remove thymocytes. The embryonic thymic tissue treated with 2DG as described above is hereafter referred to as 2DG-treated embryonic thymus.

Wistar rats (3 months old) were immunized with 2DG-treated embryonic thymus homogenized and emulsified in an equal amount of Freund’s complete adjuvant. Spleen cells obtained from the immunized rats were hybridized with plasmacytoma cells and hybridoma clones were prepared as mentioned previously (5).

The detection of specific antibodies and cloning of hybridoma cells were performed by immunohistological methods. Wells containing specific antibodies to thymic stromal tissues and showing a negative reaction to thymocytes were selected and used for the next steps. The isotypes of the antibodies were examined with a rat monoclonal typing kit (The Binding Site, Birmingham, UK). As negative control, rat IgM mAb (R4-22; PharMingen, San Diego, CA) was used for immunohistology and flow cytometry.

Immunohistology

An indirect horseradish antibody method using frozen sections was employed for screening of the hybridoma cell lines. mAb Th-3 (5) was also used to observe localization of the cortical thymic epithelial cells. A double-color fluorescent antibody method was carried out using PT-73 and rabbit anti-keratin antibody (Dako, Glostrup, Denmark). Secondary antibodies were FITC-labeled anti-rat Ig antibody and Alexa Fluor 594-labeled anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands).

Effect of mAb on T cell development in the organ culture system

T cell development was carried out in a high oxygen submersion organ culture (HOS-OC) (6). Briefly, 2DG-treated embryonic thymus (15 gd) was submerged in 0.2 ml of a mixture of complete medium (0.1ml) and culture supernatant of mAb (0.1ml) using a 96-well U-bottom plate (Costar, Cambridge, MA). Fresh thymocytes (2 × 10^6) obtained from a 15 gd fetus were inoculated into each well. The plates were incubated for 7 days and the cells collected from each well were analyzed by three-color flow cytometry (FITC, PerCP and phycoerythrin).

Flow cytometric analysis

Analysis was performed on a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest software. The mAb employed were anti-Thy-1.2 (30-H12), anti-CD4 (GK1.5), anti-CD8 (53-6.7) and anti-TCRβ (H57-597), purchased from PharMingen.

RNA isolation and cDNA library construction

Total RNA was isolated from fresh mouse tissue by the acid guanidinium isothiocyanate–phenol–chloroform extraction single-step method (7). Poly(A)+ RNA was purified using a fastTrack mRNA isolation kit (Invitrogen, Carlsbad, CA).

Preparation of a cDNA library of mouse newborn thymus was performed from the SuperScript plasmid system (Gibco/BRL, Gaithersburg, MD). The cDNA thus obtained was fractionated by column chromatography and unidirectionally inserted into T4 ligase into the BstXI–NotI site of pME18S mammalian cell expression plasmid vector (8).

Recovery of cDNA clones by a panning method

COS-7 cells in 6-cm dishes were transfected with the cDNA library of newborn mouse thymus using TransFast transfection reagent (Promega, Madison, WI). The cells were allowed to attach to a bacteriological culture dish (Falcon 1007; Becton Dickinson, Franklin Lakes, NJ). Plasmid DNA was recovered according to the panning method described in (9) and amplified in Escherichia coli to obtain material for the next round of panning. This step was repeated 3 times and the cDNA obtained was used for sequence analysis.

DNA sequence analysis

Sequencing was performed according to the dideoxy chain termination method using a Thermo Sequenase 7-deaza-dGTP kit (Amersham Biosciences, Piscataway, NJ) and ALF II sequencer (Amersham Biosciences). The cDNAs were cloned into pBluescript II plasmid vector and the DNA sequencing was carried out for both strands using M4 universal, reverse primer (Takara, Tokyo, Japan). Sequences were analyzed for homology to known cDNA sequences using the GenBank database.

Northern blot analysis

Total RNA isolated by the method mentioned above was electrophoresed on a 1% agarose/formaldehyde gel, transferred to a nylon membrane (Hybond N+; Amersham Biosciences) and hybridized to 32P-labeled DNA probe. Mouse GMF-β cDNA (1.1 kb) was labeled by the random priming method (Roche, Mannheim, Germany) and used as a hybridization probe. The washed filters were exposed to imaging plates and analyzed with a BAS 2000 system image analyzer (Fuji Photo Film, Tokyo Japan).

Preparation of recombinant GMF-β protein

GMF-β cDNA was subcloned into pGEX-6P-1 (Amersham Biosciences). GMF-β as a glutathione-S-transferase (GST)-fusion protein was prepared using GST purification modules (Amersham Biosciences). Then, the recombinant GMF-β protein was obtained by treating the fusion protein with PreScission protease.
Preparation of cell lysates

Tissues were solubilized in cold cell lysis buffer [20 mM Tris-HCl (pH 7.4) containing 250 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 μg/ml aprotinin and 1 mM PMSF]. The cell lysates were centrifuged and the supernatants were used for Western blotting.

Immunoblotting

Tissue or cell lysates and a recombinant GMF-β protein were analyzed by SDS-PAGE under reducing conditions, and then transferred to a nitrocellulose microporous membrane. The membrane was blocked in Blocking Ace (Dainippon Pharmaceutical, Osaka, Japan) and incubated with PT-73 mAb. Immunoblots were washed with TBS containing 0.05% Tween 20 and then incubated with horseradish peroxidase-conjugated anti-rat Ig. After incubation, the membrane was washed with TBS containing 0.05% Tween 20 and detected with the ECL system (Amersham Biosciences).

Results

Production of mAb staining thymic epithelial cells

mAb produced against thymic tissue from 15 gd mouse embryos showed various staining patterns by immunohistochemistry (data not shown). Among many mAb, we selected one mAb (PT-73) by the immunohistologic staining pattern of the thymus and flow cytometric profile when added to the in vitro fetal thymus culture system described below. PT-73 was IgM class and immunohistologically showed a fine meshwork pattern (Fig. 1a) in both the cortex and the medulla (Fig. 1b). Th-3 mAb immunohistologically showed a fine meshwork pattern only in the cortical regions (Fig. 1c) (5). By two-color immunofluorescence, PT-73 was positive in the thymic epithelial cells, which were positive with keratin in both the cortex and the medulla (Fig. 1d and e). By flow cytometric analysis (Fig. 1f and g), PT-73 was negative for thymocytes.

Effect of PT-73 on T cell development in culture

Culture supernatant of the mAb PT-73 was added to the HOS-OC system containing 2DG-treated embryonic thymic tissue and thymocytes at 15 gd. Seven days later, the pattern of T cell development was observed in terms of CD4 and CD8. Thymocytes at 15 gd were mostly composed of CD4-CD8- double-negative (DN) cells (Fig. 1h). In normal conditions, these DN cells showed differentiation into four subpopulations, DN cells (mean 4.5%), CD4+CD8+ double-positive cells (DP), DN cells (mean 4.5%), CD4+CD8- double-negative cells (DN), and CD4- single-positive cells (8SP, mean 3.7%) in 7 days (Fig. 1i). In the presence of PT-73, the flow cytometric profile changed with a significant decrease in DP cells and an increase in DN cells (Fig. 1j).

Identification of the molecule binding with PT-73

By the panning method using the mAb PT-73 we recovered one cDNA from Cos 7 transfected cells with a cDNA library of mouse newborn thymus. Sequence analysis of the cDNA revealed 1940 bp nucleotides. The sequence was analyzed for homology to known cDNA sequences using the GenBank database. The search revealed that the obtained cDNA contained 426 bp of the complete coding region of the GMF-β protein. The amino acid sequence of mouse GMF-β, determined in the present study, was almost identical to that of mouse (10), human (11), bovine (12) and rat (13), as shown in Fig. 2.

Expression of GMF-β mRNA in the thymus and brain

Using cDNA of GMF-β protein, the expression of GMF-β mRNA was examined in the thymus, spleen, brain and liver. As shown in Fig. 3, GMF-β mRNA expression was observed in the brain and thymus. Very faint expression was observed in the spleen, but none in the liver.

Preparation of recombinant GMF-β protein

GST–GMF-β fusion protein was prepared by the GST gene fusion system as described in Methods. The recombinant GMF-β protein was obtained by cleaving the fusion protein with PreScission protease. The protein was subjected to SDS-PAGE and a specific band was confirmed at 19 kDa by immunoblotting as shown in Fig. 4.

Presence of GMF-β protein in the thymus, brain and thymic epithelial cell lines

Using mAb PT-73, we examined the production of GMF-β protein by an immunoblotting method. As shown in Fig. 4, GMF-β protein was detected at 23 kDa in the thymus, brain and a thymic epithelial cell line (TEC 3-10). The proteins detected in these organs and the cell line were slightly larger than the recombinant GMF-β protein.

Expression of GMF-β on the cell surface

We employed Cos 7 cells transfected with GMF-β cDNA and two thymic epithelial cell lines (TEC 1-6 and TEC 1-4C) (14) for the detection of GMF-β on the cell surface. These cells, without fixation, were stained with mAb PT-73 and examined by flow cytometry. As shown in Fig. 5, the expression of GMF-β was observed in most of the Cos 7 cells (Fig. 5a). It was important to note that PT-73 was positive in ~20% of TEC 3-10 cells (Fig. 5b), but not in TEC 1-4C. As observed by the immunoblotting method (Fig. 4), the production of GMF-β was detected in TEC 3-10 cells, but not in TEC 1-4C. These findings indicated that GMF-β was expressed on the cell surface of TEC 3-10 cells.

Promotion of CD4+ T cell development by recombinant GMF-β

The purified GMF-β protein (10 ng/well) was added to the HOS-OC system composed of 2DG-treated embryonic thymic tissue and thymocytes at 15 gd. The influence of GMF-β on T cell development was observed 7 days later. The results indicated that a significantly larger number of CD4+ T cells developed in the presence of GMF-β protein, as compared with control culture (Fig. 6a and b). Many of the CD4+ T cells generated in the presence of GMF-β expressed TCRβ (Fig. 6f).

Total cell number slightly increased in the culture with GMF-β, compared with control (Table 1). The effect of GMF-β protein was examined at different doses of 10 ng, 100 ng and 5 μg/well, but the results were almost the same regardless of concentration. The same concentration of GMF-β protein alone, without 2DG-treated embryonic thymic tissue, did not
Fig. 1. Immunohistological staining with mAb PT-73 in a newborn thymus (a) and in a young adult thymus from a 4-week-old mouse (b). (c) Immunohistological staining with mAb Th-3 in a neighboring section of (b). Th-3 is specific to cortical epithelial cells. (d and e) Two-color immunofluorescent staining of a young adult thymus by PT-73 (green) and anti-keratin antibody (red) in the cortex and the medulla. (f) Flow cytometric analysis of thymocytes showing positive (anti-Thy1.2 mAb solid line) and negative control (dotted line). (g) Thymocytes are negative for PT-73 by flow cytometric analysis. Flow cytometric profiles (CD4/CD8) of thymocytes in HOS-OC at 0 day (h), and 7 days without (i) and with (j) PT-73.
support maintenance, proliferation and differentiation of the embryonic thymocytes. As another control, the effect of IL-7 was examined on the pattern of T cell development. In contrast to GMF-β, IL-7 favored the development of CD8+ T cells and gave rise to a greater number of total cells (Fig. 5c and Table 1). These CD8+ T cells generated in the presence of IL-7 were composed of TCRβ+ and TCRβ± populations (Fig. 6i).

Discussion

GMF-β was first found and purified by Lim et al. (4). Since then have been many reports on the structure, biological function and localization in the brain (11,12,15,17). GMF is known to be produced by glia cells in the brain, and to play an important role in the growth and differentiation of glia cells in an autocrine fashion.

In the present study, we first produced many mAb to thymic epithelial cells and selected mAb inhibiting T cell development in an in vitro culture system of the mouse embryonic thymus. Eventually, we succeeded in determining a cDNA encoding a protein which was reactive with a mAb inhibiting T cell development. Sequence analysis revealed that the protein obtained from mouse was almost identical to GMF-β reported in mouse (10), human (11), rat (13) and bovine (12).

Using the cDNA obtained in the present study, we performed Northern blot analysis, and found that mRNA expression was detected in the thymus and brain. Very faint expression was observed in the spleen, but none in the liver.

We prepared recombinant GMF-β protein using the GST gene fusion system and examined the effect of GMF-β protein in an in vitro culture system of the embryonic thymus. We found that many CD4+ T cells developed with a concomitant decrease in CD4+CD8+ T cells in the presence of GMF-β. Most of these CD4+ T cells were positive for TCRβ and were considered to be the same as those observed in the adult thymus. However, embryonic thymocytes did not survive in the culture containing GMF-β protein alone. Thus, the presence of a thymic microenvironment was necessary for GMF-β protein to promote T cell differentiation.

One point to be investigated is the GMF-β receptor issue. Identification of the receptor is important to confirm that the action of GMF-β on the cell is specific or not. In this respect, however, no report is found in the literature. We are now conducting an experiment to determine the GMF-β receptor molecule.
Fig. 5. Flow cytometric analysis with mAb PT-73 in Cos 7 cells transfected with GMF-β cDNA and two thymic epithelial cell lines (TEC 1-6 and TEC 1-4C) for the detection of GMF-β on the cell surface. These cells, without fixation, were stained with mAb PT-73 and examined by flow cytometry. (a) The expression of GMF-β is observed in most of the Cos 7 cells compared with control. (b) GMF-β is positive in ~20% of TEC 3-10 cells. (c) GMF-β is mostly negative in TEC 1-4C.

Fig. 6. Effect of GMF-β and IL-7 on T cell development in the culture system using thymocytes at 15 gd and embryonic thymic stromal cells for 7 days. (a) Control culture, without additional factor. (b) A significant increase in CD4+ T cells in the presence of GMF-β. (c) A significant increase in CD8+ T cells in the presence of IL-7. Flow cytometric measurement of the expression of TCRβ in CD4+ T cells (d–f) and CD8+ T cells (g–i). (d and g) Thymocytes from an adult thymus are positive controls. (e and h) Control culture without additional factor. (f) CD4+ thymocytes recovered from the culture in the presence of GMF-β mostly consist of TCRβ+ cells. (i) CD8+ cells recovered from the culture in the presence of IL-7 mostly consist of TCRβ+ cells.
In the same culture system of the embryonic thymus, we also examined the effect of IL-7. In contrast to GMF-β, many CD8+ T cells developed with a concomitant decrease in CD4+CD8+ and CD4+ T cells in the presence of IL-7. Many of these CD8+ T cells expressed a low level of TCRβ and were considered to be of the immature type.

In order to observe the localization of GMF-β within the cell, we examined Cos 7 transfected GMF-β cDNA and thymic epithelial cell lines (TEC 3-10 and TEC 1-4C) by flow cytometry. The expression of GMF-β was observed in most of the Cos 7 cells and in ~20% of TEC 3-10 cells, but not in TEC 1-4C. Immunoblotting analysis indicated that GMF-β was detected in TEC 3-10 cells, but not in TEC 1-4C. These findings indicated that GMF-β was partly expressed on the cell surface of TEC 3-10 cells.

In fact, some cytokines such as IL-1 are secreted, although the protein is lacking a signal sequence (18).

Among various cytokines, those (IL-2, IL-4, IL-7, IL-9 and IL-15) dependent on a common γ chain are known to regulate T cell development (19). IL-2 plays a role in maintaining the functional microenvironment that is necessary to support thymocyte growth, development and selection (20). The present study reports that GMF-β plays a role in T cell development in favor of CD4+ T cells.

Table 1. The percentage of subsets of fetal thymocytes and total cell number after 7 days in HOS-OC

<table>
<thead>
<tr>
<th>CD4+CD8- (%)</th>
<th>CD4+CD8+ (%)</th>
<th>CD4+CD8- (%)</th>
<th>CD4+CD8+ (%)</th>
<th>Cell number (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.99 ± 0.65</td>
<td>68.20 ± 4.80</td>
<td>13.13 ± 2.99</td>
<td>6.68 ± 1.63</td>
</tr>
<tr>
<td>GMF-β</td>
<td>29.37 ± 3.44</td>
<td>40.95 ± 3.78</td>
<td>22.99 ± 2.16</td>
<td>6.69 ± 0.96</td>
</tr>
<tr>
<td>IL-7</td>
<td>5.02 ± 0.62</td>
<td>39.22 ± 4.51</td>
<td>26.17 ± 1.96</td>
<td>29.81 ± 1.19</td>
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Data indicate the mean ± SEM, n = 5–7 experiments. Concentration of GMF-β = 10 ng/culture. Concentration of IL-7 = 10 ng/culture.

Acknowledgements

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Abbreviations

2DG 2-deoxyguanosine  
DN double negative  
DP double positive  
gd gestation date  
GMF glia maturation factor  
GST glutathione-S-transferase  
HOS-OC high oxygen submersion organ culture  
PE phycoerythrin  
SP single positive

References