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Effects of thymic polypeptides on the thymopoiesis of mouse embryonic stem cells

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Abstract

The thymus provides a unique cellular and hormonic microenvironment for the development of immunocompetent T cells. Thymic polypeptides have been widely used clinically for the treatment of tumors, infectious diseases and immune deficiency diseases. They have already shown the ability to stimulate the maturation of hematopoietic stem cells towards the CD3+CD4+ T cell lineage. However, their effects on the thymopoiesis of embryonic stem cells are still unexplored.

In this paper, we compared the effects of three thymic polypeptides, thymopentin (TP5), thymosin alpha-1 (Tα-1) and thymopeptides on the *in vitro* thymopoiesis of mouse embryonic stem (ES) cells. Using the embryoid body induction system, we found that both Tα-1 and thymopeptides effectively induced ES cells to differentiate sequentially into the CD3+ and CD4+/CD8+ T cells. These T cells had T cell receptor (TCR) Vβ gene rearrangement and most were TCRαβ T cells. We also found that the expression of the Notch receptor and its ligands Delta-like-1 and Delta-like-4 gradually increased during the induction. However, TP5 failed to induce the T cell differentiation of the ES cells.

In summary, this is the first report to demonstrate that Tα-1 can stimulate the T cell early stage differentiation from ES cells using the embryoid body protocol. These findings provide a powerful model for studying T cell development and may open new venues for the clinical application of Tα-1.

Keywords: Embryonic stem (ES) cells; T cells; Thymopentin (TP5); Thymosin alpha-1 (Tα-1); Thymopeptides

1. Introduction

The thymus-derived T cells are essential to the normal functions of the immune system. As is true with other blood cells, T cells develop from multipotent hematopoietic stem cell (HSC) derived progenitors. T cell progenitors undergo a complicated program, involving proliferation, differentiation and selection, to produce a functionally diverse and largely non-autoreactive population of T cells. This process is restricted almost exclusively to the thymus because the development of T cells depends on their interactions with a complexity of thymic stromal cells and stromal-derived chemokines which attract T cell precursors to the thymus and direct maturing thymocytes to appropriate niches for their further maturation (Anderson and Jenkinson, 2001; Gray et al., 2005).

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos. The ability of ES cells to undergo indefinite self-renewal *in vitro* and to produce derivative lineages of all three embryonic germ layers *in vitro* and *in vivo* makes them a highly prized reagent

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in both clinical and research settings (Wobus and Boheler, 2005). Although most blood cells can be generated through *in vitro* ES cell differentiation, very few successful attempts to generate T cells have been reported. The reason might be that the thymic environment and the immigration of developing T cells in the thymus during lymphopoiesis are hard to mimic (Lehar and Bevan, 2002; Schmitt et al., 2004).

Many previous studies have provided evidence that thymic epithelial cells secrete thymic hormones that act at various stages of T cell differentiation and maturation, thereby inducing and maintaining immune functions. In the past decade, a variety of short peptides of thymic origin have been studied for their immunomodulatory effects and used for the treatment of immunodeficiency, malignant, and autoimmune diseases (Bodey et al., 2000; Lin et al., 2004). Although it was reported that thymosin alpha-1 (Tα-1) could stimulate maturation of hematopoietic stem cells to CD3+CD4+ T cells (Knutsen et al., 1999) *in vitro*, the role of Tα-1 on T cell lineage differentiation from ES cells is still unknown.

Thymopentin (TP5), Tα-1 and thymopeptides are three drugs that are currently licensed for the treatment of immune dysfunction related to impaired T cell activity. TP5 is a synthetic pentapeptide having biological activities similar to the native hormone, thymopoietin (Goldstein et al., 1979). Tα-1 is a protein of 28 amino acids which is derived from a 113 amino acid precursor protein, prothymosin-α (Goldstein et al., 1977). Thymopeptides are composite peptides extracted from animal thymus glands. Besides the major component, Tα-1, thymopeptides contain some other small peptides (Zhong, 2005). Although the three thymic peptides are different, they can all enhance the function of T cells and augment the response of T cells to interleukins and mitogens.

We investigated the effects of these three thymic polypeptides, TP5, Tα-1 and thymopeptides, on the *in vitro* differentiation of the T cell lineage from mouse ES cells using the embryoid body induction system. The results revealed that TP5 could not induce CD3+, CD4+CD8+ double positive T cell differentiation and the rearrangement of the TCR β locus from ES cells whereas Tα-1 and thymopeptides could.

2. Materials and methods

2.1. Cell culture

The E14.1 murine embryonic stem cells were maintained at 37 °C in humidified air with 5% CO_2 in ES media comprised of high glucose Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 50 U/ml penicillin (Invitrogen), 50 μg/ml streptomycin (Invitrogen), 0.1 mM nonessential amino acids (Sigma, St. Louis, MO), 0.1 mM 2-mercaptoethanol (Sigma), and 1000 U/ml recombinant murine leukemia inhibitory factor (LIF, Chemicon). Culture flasks were prepared prior to cell seeding by coating with a solution of 2% gelatin from porcine skin (Sigma) in phosphate buffered saline.

2.2. Choice of appropriate embryoid bodies (EBs)

ES cells were dissociated into single cells with 0.25% trypsin–EDTA (Sigma) and cultured at 1×10^4 cells/ml in EB medium comprised of high glucose DMEM (Invitrogen) supplemented with 15% fetal bovine serum (FBS, Hyclone), 50 U/ml penicillin (Invitrogen), 50 μg/ml streptomycin (Invitrogen), 0.1 mM nonessential amino acids (Sigma), and 0.1 mM 2-mercaptoethanol (Sigma). LIF was not added to the EB forming culture system. ES cells aggregated and spontaneously formed EBs in 10 cm petri dishes (Greiner) that prevented attachment of EBs to the plastic bottom. After 24 h culture, the EBs were transferred into 15 ml conical tubes. After sedimentation by gravity, EBs with similar sizes were collected.

2.3. Establishment of induction system

The chosen EBs were continuously cultured in EB medium for 5–7 days. Then they were seeded into new petri dishes in EB medium supplemented with 200 ng/ml of stem cell factor (SCF, Perprotech), 10 ng/ml of interleukin (IL)-3 (Perprotech), and 10 ng/ml of IL-7 (Perprotech) as control. Previous reports demonstrate that TP5, in concentrations ranging from 10^{-7} M to 10^{-3} M, can induce the differentiation of prothymocytes to thymocytes (Goldstein et al., 1979), that Tα-1 from 1 to 10 μg/ml can enhance thymopoiesis of CD34+ stem cells *in vitro* (Knutsen et al., 1999) and that 50 μg/ml of thymopeptides is an optimum concentration for thymopoiesis of mouse ES cells (Zhou et al., 2003). We used 10 μg/ml (10^{-5} M) of TP5 (Zhonghe Pharmaceuticals Inc., China), 10 μg/ml of Tα-1 (Zadaxin, SciClone Pharmaceuticals, Inc.), or 50 μg/ml of thymopeptides (Saisheng Pharmaceuticals, Inc., China) in three different induction systems, respectively. On the 8th, 15th and 22nd days, the induced EBs were collected and dissociated into single cells by incubation (2–5 min, 37 °C) in 0.25% trypsin–EDTA (Sigma) and mechanical shearing. Individual cells were then counted using a hemocytometer and analyzed by flow cytometry.

2.4. Flow cytometry analysis

After dissociation, we removed dead cells by density gradient centrifugation, fixed the cells using 4% paraformaldehyde, and then washed and resuspended the test cell population at 10^6 cells/100 μl in phosphate buffered saline (PBS). The cells were incubated for 20 min at 4 °C with mouse CD16/CD32 monoclonal antibody (Ebioscience) at 1 μg/1 × 10^6 cells to block non-specific binding. The cells were then incubated for 25 min at 4 °C with 1 μg/1 × 10^6 cells Cy-5 anti-mouse CD3 (PharMingen), FITC anti-mouse CD4 (PharMingen), PE anti-mouse CD8 (PharMingen), FITC anti-mouse TCRγδ (Ebioscience), and PE anti-mouse TCRαβ (Ebioscience), and then washed twice with staining buffer. After the final wash, flow cytometric analysis was performed on a FACS-A (BD Biosciences).