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Study for the Production of Immunodeficiency Animals for Xenotransplantation

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ABSTRACT

Transgenes in HSV-TK gene driven by the lck promoter was tested for the expression in immune cells (Jurkat cells) to apply xenotransplantation of human cells into transgenic animals for the potential use of the proliferation or differentiation of human stem cells in the large animal such as an pig. Also, lck-GFP gene was used for transfection experiment into Jurkat cell to confirm the proper regulation of lck promoter for transgene expression in the T cells. Transfection of lck-GFP gene into Jurkat cells induced GFP expression in transfected cells. The expression of lck-TK and lck-GFP genes was confirmed by RT-PCR using RNAs extracted from Jurkat cells. When Jurkat cells transfected with TK and GFP genes were selected against G418 or gancyclovir treatments, Jurkat cells transfected with TK gene were not proliferated in G418 and gancyclovir medium while intact cells or cells transfected with GFP gene could grow in gancyclovir medium. However, Jurkat cells transfected with TK or GFP gene were proliferated in G418 medium probably due to Neo^r gene in the vector. Gancyclovir treatment destroyed Jurkat cells expressing TK gene indicating that T-cells expressing TK gene can be selectively eliminated by TK gene expression driven by lck promoter.

(Key words : HSV-TK gene, lck promoter, Jurkat cells, Gancyclovir treatment, xenotransplantation)

Ⅰ. INTRODUCTION

Animals that have a human immune system would be extremely valuable for biomedical research. Also, the development and regeneration of human tissues or cells in animals can be explored for the repair of damaged tissues or genetic defects. Human-animal transplantation models are fascinating to explore human cell proliferation and differentiation in vivo. Sever combined immunodeficiency (SCID) mice that lack functional T and B cells have an inability express an effective cellular and humoral immune repose to foreign antigen (Bosma,

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et al., 1983; Dorshkind et al., 1984). Moiser et al. (1988) and McCune et al. (1988) showed that SCID mice accepted the xenotransplantation of mature lymphocytes, human hematopoietic stem cells, and lymphjoid tissues. Recently, human stem cells and bone marrows tried to transplant into the tissues of animals for the induction of differentiation as a therapeutic purpose (Azizi et al., 1998; Bjornson, et al., 1999). However, mice or rats are not appropriate for the real therapeutic applications due to the organ size as well as immune differences compared to human. Immunodeficient large animals would be required for the further practical applications.

To ablate cell lineage in animals specifically, toxin genes such as diphtheria toxin A fragment or ricin A can be expressed using tissue-specific promoters or enhancers (Arase et al., 1999; Bartell, et al. 2000). The TK gene can be used for the conditional depletion of specific cell population. Herpes simplex virus thymidine kinase (HSV-TK) gene has no detrimental effect on mammalian cells. However TK gene product inhibit DNA synthesis with nucleoside analogs such as acyclovir or gancyclovir leading cell death. Addition of these nucleotide analogs will kill TK gene-expressing cells among dividing cell population.

In this study we constructed transgene in that HSV-TK gene expression was driven by the lck promoter for the efficient control of immune system in animals and tested the elimination of immune cells expressing TK gene for the potential use of the proliferation or differentiation of human stem cells in immunodeficient large animals such as pigs produced by TK gene.

Ⅱ. MATERIALS ANA METHODS

1. Gene Construction

The TK gene was obtained from PKO select TK vector (obtained from Lexicon Corp.) by PCR, using PKOTKF primer 5'-CTAGCGGATCCGTAGAAGCGCGTATGGCTTCG-3' and PKODTR 5'-TAGCAGGATCCCCAGTGCCAAGCTTCTGATGG-3' and cloned into the Not I-BamHI site of pBluescript II KS(+), which contain the mouse lck proximal promoter and polyadenylation signal. The resulting plasmid was digested with Hind III, Not I and Bam HI, yielding a 2.2-kb DNA fragment consisting of the 3.2-kb lck proximal promoter followed by TK gene (1700bp) and SV 40 polyadenylation signal (Fig. 1). GFP gene derived from CMV-EGFP vector was also cloned into pBluescript II KS(+), which contain the mouse lck proximal promoter and polyadenylation signal. The linearized DNA fragment was purified by agarose gel electrophoresis followed by extraction using DEAE membrane (Schleicher & Schuell corp.).

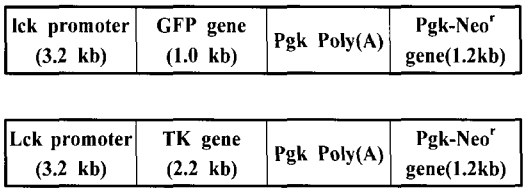

Fig. 1. Schematic representation of transgene construct.

2. Transfection into Jurkat Cells

Jurkat cells (human leukemic T cell line) were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 medium (RPMI; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), at 37℃ in a humidified atmosphere of 5% CO_2. DNA carrying the mouse proximal lck promoter and the TK gene or GFP gene was transfected into Jarkat cells by GenePORTER^{TM}(Gene Therapy Systems corp.). For stable transfection, cells were seeded in 100-mm cultured dishes at a density of 5 $\times 10^5$ cells per plate. After incubation for 24 h, the

cells were transfected with 20 μg of DNA. Forty -eight hours after transfection, the transfected cells were selected with 200ug/ml of G418 (Gibco-BRL) for 10 days and later tested the resistance against 3mM of Gancyclovir. Total cellular RNA from Jurkat cell was extracted with Trizol (Life Technologies Inc.).

3. PCR and RT-PCR

PCR was performed according to the manufacturers guidelines (Takara Corp.) using 0.5 μl of Taq DNA polymerase (Takara Corp.) in a 50- μl reaction containing 1.5mM $MgCl_2$, 0.2 mM dNTP, 0.8 mM of each primer, and template DNA. PCR conditions included an initial 10 minutes at 95℃ followed by 30cycles each of 95℃ for 1 minute, 60℃ for 1 minute, 72℃ for 1 minute, and a final 8-minute extension period.

Total cellular RNA was extracted with Trizol (Life Technologies Inc.). cDNA was produced using the reverse transcriptase superscript II (Life Technologies) and a poly-dT oligo, according to manufacturers instructions. Specific detection of GFP and TK transgenes was performed with forward primer inside genes and oligo (dT) primer. RT-PCR were performed as follows: a mix of 0.5 μmol of each primer; 2 mM $MgCl_2$; 0.2mM dNTP; 1.25 U Gibco Taq polymerase II (Life Technologies) was applied to a denaturation step of 5 min at 95℃ followed by 35 cycles consisting each of 30 s at 94℃, 30 s at 60℃ and 1 min at 72℃, terminated with an elongation step of 10 min at 72 ℃. PCR and RT-PCR products were identified with 2% agarose gel electrophoresis.

Ⅲ. RESULTS

In this experiment, transgenes for the expression in immune cells were constructed and tested with trasfection into Jurkat cells (human leukemic T cell line). To induce transgene expression in immune cells, lck promoter sequences were used in this experiment. The transgene was consisting of the 3.2-kb lck proximal promoter followed by TK gene (1700bp) and SV 40 polyadenylation signal (Fig. 1). Also, GFP gene was ligated with lck promoter sequence as a control. The lck-GFP gene was used for transfection experiment into Jurkat cell that is a human leukemic T cell line to confirm whether lck promoter drives correctly transgene expression in the T cells. Following transfection of lck-GFP gene into Jurkat cells, GFP expression was observed in transfected cells whereas no expression was detected in intact cells (Fig. 2). Efficiency of transient transfection in Jurkat cells was about 30 % in this experiment.

Fig. 2. Transfection experiments using lck-GFP construct in Jurkat cells. A: Control Jurkat cells, B: Jurkat Cells transfected with lck-GFP gene.

To investigate whether cells transfected with TK gene can be destroyed selectively with gancyclovir treatment, the lck-TK DNA was transfected into Jurkat cells. The expression of lck-TK and lck-GFP genes was check by RT-PCR using RNAs extracted from Jurkat cells following transfection (Fig. 3). RT -PCR of RNA from Jurkat cells by using Poly(T) and internal primers in TK and GFP genes was performed. A 556 bp band of TK transcript in Jurkat cells was detected while a 686 bp band of GFP RNA was amplified in RT-PCR analysis (Fig. 3). No band was detected in RT-PCR anaylsis with RNA of intact Jurkat cells.

Jurkat cells transfected with TK and GFP genes

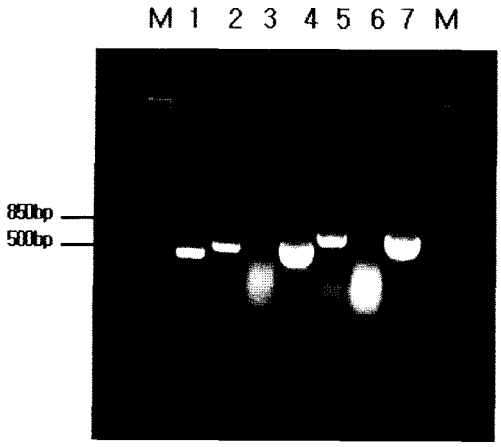

Fig. 3. RT-PCR analysis of TK and GFP genes in Jurkat cells. M : DNA size marker, Lane 1: RT-PCR control RNA, Lane 2: Positive TK cDNA, Lane 3: TK negative control, Lane 4: RNA of Jurkat cells transfected with lck-TK gene, Lane 5: Positive GFP cDNA, Lane 7: GFP negative control, Lane 7: RNA of Jurkat cells transfected with lck-GFP gene.

were selected against G418 or gancyclovir treatments. Intact Jurkat cells could not survive in G418 medium, but could survive in gancyclovir medium. However, Jurkat cells transfected with TK or GFP gene were proliferated in G418 medium probably due to Neo^r gene in the vector. Most Jurkat cells transfected with TK gene were not proliferated in G418 and gancyclovir medium (only 0.14% survival) while cells transfected with GFP gene could grow in G418 and gancyclovir medium (Table 1). Intact cells could not survive in G418 or G418 and gancyclovir medium. Gancyclovir treatment could affect the survivals of Jurkat cells expressing TK gene while it did not affect proliferation of GFP -expressing cells suggesting that Jurkat cells expressing TK gene were selectively destroyed by TK gene expression.

Ⅳ. DISCUSSION

In this study we constructed HSV-TK transgenes driven by lck promoter for the efficient control of immune system in animals especially and tested the expression in cells to evaluate the potential use for ablation of immune cells in animals for transplantation of human cells for the proliferation or differentiation of human stem cells. Early studies have shown that the lck promoter regulates the expression of transgenes at high level in developing thymocytes (Garvin et al., 1990). The lck gene is expressed throughout T-cell development and it may play a role in signal transduction through T cell receptor (Perlmutter et al., 1993). In our experiment, the lck promoter has successfully directed transcription of transgene in Jurkat cells (Fig. 3). Even though amount of transcripts was not quantified, RNA transcribed by lck promoter in this experiment was supposed to be abundant in cells by estimation with band intensity.

TK gene can be successfully used for depleting

Table 1. Cell survivals following G418 and gancyclovir treatment

Cells	No. cells ($\times 10^3$)	GFP-expressing cells (%)	No. $G418^r$ colonies (%)	No. $G418^r$+$Ganc^r$ colonies
Intact	200	0	0	0
Cells with lck-GFP*	200	30	NR[a]	NR
Cells with lck-TK*	200	0	NR	27 (0.14 %)

* Cells were selected against G418 medium for 10 days.

[a] non-readable due to too many colonies.

the specific cell population. Gancyclovir killed specifically cells with TK gene in this experiments (Table 1). In previous transgenic study (light chain promoter-TK gene induced incomplete depletion of T-cell population probably due to promoter strength (Hayamn et al., 1989). The lck promoter may drive more efficient expression of TK gene in the transgenic animals. Therefore, lck promoter and TK gene seems to be a reliable method for generating transgenic animals lacking immune cell population in an inducible manner.

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