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Co-expression of sCD40LIg and CTLA4Ig mediated by adenovirus prolonged mouse skin allograft survival*

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Abstract: Objective: To investigate the role of simultaneous blockade of CD40/CD40L and B7/CD28 pathways in the immune tolerance via co-expression of sCD40LIg and CTLA4Ig mediated by replication-defective adenovirus. Methods: Ad-sCD40LIg-IRES$_2$-CTLA4Ig, replication-defective adenovirus co-expressing sCD40LIg and CTLA4Ig, was constructed and identified. The co-expression of sCD40LIg and CTLA4Ig was evaluated with confocal laser scanning microscope and Western blotting. Skin transplantations of C57BL/6 to BALB/c mice were performed. PBS, Ad-Shuttle-CMV and Ad-sCD40LIg-IRES$_2$-CTLA4Ig were administered. Skin graft survival was monitored and the mRNA expression of both genes was evaluated in the skin allografts. Results: Ad-sCD40LIg-IRES$_2$-CTLA4Ig was constructed successfully and identified. The co-expression of sCD40LIg and CTLA4Ig was identified with confocal laser scanning microscopy and Western blotting. Compared to the skin graft mean survival time (MST) of non-treated group ((5.75±0.71) d) or Ad-Shuttle-CMV-treated group ((5.50±0.53) d), the skin graft MST was dramatically prolonged in the Ad-sCD40LIg-IRES$_2$-CTLA4Ig-treated group ((16.38±1.19) d, P<0.001). The mRNA expression of both genes was detected. Conclusion: Ad-sCD40LIg-IRES$_2$-CTLA4Ig, a replication-defective adenovirus carrying genes encoding sCD40LIg and CTLA4Ig, was constructed. Simultaneous blockade of CD40/CD40L and B7/CD28 costimulatory pathway mediated by replication-defective adenovirus significantly prolonged skin allograft survival in mice.

Key words: Immune tolerance, CTLA4Ig, sCD40LIg, Replication-defective recombinant adenovirus, Skin graft
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INTRODUCTION

Costimulatory signals play a vital role in T cell activation. B7/CD28 and CD40/CD40L have been well-established as important costimulatory pathways, with the blockade of either resulting in suppression or anergy of T cells associated with prolongation of graft survival (Akalin *et al*., 1996; Bolling *et al*., 1996; Kita *et al*., 1999; Reddy *et al*., 2001; Laumonier *et al*., 2003; Thiel *et al*., 2005). Cytotoxic T lymphocyte associated antigen-4 (CTLA4), a ligand for B7, delivers negative signals to antigen presenting cells (APCs) to down-regulate proinflammatory responses, and competitively inhibits the binding of B7 and CD28 (Li *et al*., 2005). CTLA4Ig is a soluble recombinant fusion protein consisting of the extracellular domain of CTLA4 and the human IgG Fc region (Gao *et al*., 1999). A study indicated that CTLA4Ig alone or in combination with low-dose cyclosporine could not reverse acute rejection of renal allografts in rats (Perico *et al*., 1996). However, the combination of CTLA4Ig with T cell depletion (Rehman *et al*., 1996; Tu *et al*., 1996) or FTY720 (Ohba *et al*., 2000; Kita *et al*., 2002), or anti-LFA-1 monoclonal antibody (Corbascio *et al*., 2002) prolonged allograft survival. Signals from the CD40/CD40L costimulatory pathway also play an important role in acute rejection of organ grafts such as kidneys and hearts (Kawai *et al*., 2004; Pearson *et al*., 2002; Guillot *et al*., 2002).

To study the effect of blockade of both B7/CD28

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and CD40/CD40L pathways on allograft rejection and immune tolerance, we constructed replication-defective recombinant adenovirus to co-express sCD40LIg (a soluble recombinant fusion protein consisting of the extracellular domain of CD40L and the human IgG Fc region) and CTLA4Ig. The co-expression and role of sCD40LIg and CTLA4Ig in mouse skin allograft survival were observed in the present study.

MATERIALS AND METHODS

Construction of Ad-sCD40LIg-IRES$_2$-CTLA4Ig

Total RNA from peripheral blood lymphocytes was extracted using Trizol (Invitrogen, San Diego, CA, USA) to synthesize cDNA encoding extracellular domain of CD40L. Briefly, 2.5 μg of RNA were heated at 85 °C for 3 min and then reverse-transcribed into cDNA in a 25-μl solution containing 200 units of Superscript II RNase H-RT (Invitrogen), 50 ng random hexamers (Invitrogen), 160 μmol/L dNTP, and 10 mmol/L dithiothreitol. The reaction consisted of 10 min at 25 °C, 60 min at 42 °C, and 5 min at 99 °C. Polymerization reactions were performed in a 50-μl reaction volume containing 1 ng of cDNA, 5 units of AmpliTaq Gold DNA polymerase (PerkinElmer, USA), 1.5 mmol/L $MgCl_2$, 160 μmol/L dNTPs, and 10^{-5} nmol oligonucleotide primers. The oligonucleotide primers were as follows: Forward: 5′-TTT AGA TCT ACC ATG GGT CTA CTG CTC ACA CA-3′, Reverse: 5′-ACA GGT ACC AGT TTG AGT ACG CC-3′. All above-mentioned primers were from TaKaRa (BioTech, Liaoning, Dalian, China). The thermal cycle profile used a 5-min denaturing step at 94 °C followed by 30 cycles (1 min of denaturation at 94 °C, 45 s of annealing at 53 °C, and 1 min of extension at 72 °C) and an extension step of 10 min at 72 °C. The products were then separated by agarose gel electrophoresis. Secretory CD40L (sCD4L) cDNA was produced by ligation of the signal peptide and extracellular domain of CD40L. Ligation of sCD40L encoding and human IgG Fc was performed to produce sCD40LIg and sCD40LIg cDNA was separated by agarose gel electrophoresis. Then, sCD40LIg cDNA encoding sCD40LIg was inserted into the plasmid pAdShuttle-IRES$_2$-CTLA4Ig (internal ribosome entry site, IRES), which was a gift from Dr. HE Wei-feng, to produce the pAd-sCD40LIg-IRES$_2$-CTLA4Ig. Homologous recombination of the linearized pAd-sCD40LIg-IRES$_2$-CTLA4Ig and pAdEasy-1 vector (Stratagene, La Jolla, CA, USA) was performed in electrocompetent *E. coli* BJ5183 cells (Stratagene) by electroporation (2500 V, 200 Ohms, 25 μFD). The resultant plasmids were linearized with *Pac*I and then transfected into the adenovirus packaging cell line AD-293 using Dosper liposome (Clontech, Palo Alto, CA, USA) to observe the cytopathic effect (CPE). AD-293 cells are human embryonic kidney cells, in which E1a proteins are provided in trans, allowing the production of infectious virus particles when cells are transfected with E1-deleted adenovirus vectors such as the pAdEasy-1 vector. CPE is characterized by cells rounding up and detaching from the plate, with the nucleus occupying a major part of the cell due to the high level of virus production.

Western blotting

Whole-cell extracts obtained from Ad-sCD40LIg-IRES$_2$-CTLA4Ig-infected and Ad-Shuttle-CMV-infected 293 cells were fractionated by 8% SDS-PAGE and transferred to cellulose nitrate membrane (Ad-Shuttle-CMV is a gift from Dr. HE Wei-feng). After blocking, the membranes were incubated at 4 °C overnight in Tris-buffered saline (TBS: 50 mmol/L Tris-HCl, 150 mmol/L NaCl) containing a 1:1000 dilution of rabbit-anti-human CD40L antibody (Santa Cruz, California, CA, USA) and rabbit-anti-human CTLA4 antibody (Santa Cruz) and then incubated for 1 h at room temperature in TBS containing a 1:2000 anti-rabbit IgG antibody conjugated horseradish peroxidase (Santa Cruz). Immunoreactive bands were visualized by incubation with LumiGLO (Cell Signaling Tech, Beverly, MA, USA) and exposure to light-sensitive film.

Confocal laser scanning microscopy

As the kidney is one of the most important organs associated with transplantation in clinical therapy and the adenovirus constructed in the present study may be applied to renal transplantation, Human kidney-2 cells (HK-2 cells), which are immortalized human proximal tubular cells, were transfected (ATCC, Rockville, MA, USA) with Ad-sCD40LIg-IRES$_2$-CTLA4Ig in Dulbecco's modified Eagle's

medium (DMEM, Gibco) with 10% fetal bovine serum (Hyclone, Logan, UT, USA) supplemented with 100 IU/ml penicillin (Gibco, Grand Island, NY, USA) and 100 μg/ml streptomycin (Gibco). HK-2 cells transfected by Ad-Shuttle-CMV were used as the vehicle control. The third day, HK-2 cells were fixed with 4% formalin for 10 min, labelled with phycoerythrin (PE) labelled goat-anti-human CD40L and fluorescent isothiocyanate (FITC) labelled goat-anti-human CTLA4Ig antibody (Invitrogen), then mounted with 60% glycerin. The co-expression of sCD40LIg and CTLA4Ig was surveyed with confocal laser scanning microscopy (Leica, TCS 4 D, Wetzlar, Germany) under conditions of 488 nm wavelength for FITC and 568 nm for PE.

Skin grafting

Inbred C57BL/6 and BALB/c mice (Chinese Academy of Medical Sciences) weighing 20~25 g were used as donors and recipients, respectively. All animals were housed under special pathogen-free conditions. There were three groups (eight mice in each group): Group 1, non-treated; Group 2, receiving Ad-Shuttle-CMV; Group 3, Ad-sCD40LIg-IRES$_2$-CTLA4Ig. Skin grafting was performed using a method described previously (Hashimoto *et al*., 2002) with modification. Briefly, donors and recipients were anesthetized with intraperitoneal barbanylum (10 g/L). Full thickness donor skin (10 mm×20 mm) was harvested from the dorsal skin of C57BL/6 mice and immersed immediately into ice-cold PBS (Group 1) or 5×10^9 pfu adenovirus in 5 ml of DMEM with 10% fetal bovine serum (FBS) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (Groups 2 and 3). Four hours later, skin was transplanted into BALB/c mice with graft beds (20 mm×20 mm). Grafts were covered by bandages, which were removed on the postoperative 5th day. The condition of the skin allografts was evaluated from day 5 to rejection. Grafts were considered rejected when 80% or more of the graft was altered in color and consistency.

RT-PCR analysis in the skin allografts

Total RNA was extracted from frozen skin allografts at 1st day, 3rd day, 5th day and 8th day after skin grafting and RT-PCR was performed to assess the sCD40LIg and CTLA4Ig mRNA expression according to the method described above. RNA from the skin of untransfected BALB/c mice was used as a control. The oligonucleotide primers were as follows: sCD40LIg: forward: 5′-TTT AGA TCT ACC ATG GGT CTA CTG CTC ACA CA-3′, reverse: 5′-CTA GAA GCA TCC TCG AGC GAC CG-3′; CTLA4: forward: 5′-AAC ATA TGC AGT GGC CAG CCT GCT GTG-3′, reverse: 5′-GCG GAT CCT TAG TCA GAA TCT GGG CAC GGT TCT GG-3′. All above-mentioned primers were from TaKaRa (Bio-Tech, Liaoning, Dalian, China). The RT-PCR products were then separated by agarose gel electrophoresis.

Statistical analysis

Data were expressed as the mean±*SE*. Skin graft survival was evaluated by Kaplan-Meier analysis and log-rank tests. *P*-values less than 0.05 were considered significant (SPSS statistical software, Version 10, USA).

RESULTS

Identification of Ad-sCD40LIg-IRES$_2$-CTLA4Ig

The RT-PCR amplification product of CD40L of 750 bp and the ligation product sCD40LIg of the sCD40L and IgG Fc were separated by agarose gel electrophoresis. There was no band in the controls without mRNA or ligation product (Fig.1). The sCD40LIg product of 60 kDa and CTLA4Ig product of 50 kDa were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig.2) in the Western blotting analysis. The construction of Ad-sCD40LIg-IRES$_2$-CTLA4Ig was confirmed via identification with agarose gel electrophoresis and Western blotting analysis. The CPE was observed in the 7th day after the 293 cells were transfected with Ad-sCD40LIg-IRES$_2$-CTLA4Ig. Compared to the untreated 293 cells, the 293 cells transfected by replication-defective adenovirus became larger in the nucleus, more round in shape, and detached from the plate (Fig.3).

Co-expression of sCD40LIg and CTLA4Ig

The co-expression of the sCD40LIg and CTLA4Ig was examined in HK-2 cells by detecting the green and red fluorescence. The expression of sCD40LIg and CTLA4Ig was mainly in the cell

membrane and cytoplasm. The expression locations of the two proteins were similar (Fig.4). There was no expression of the sCD40LIg or CTLA4Ig in the HK-2 cells transfected by Ad-Shuttle-CMV (data not shown).

Skin allograft survival and mRNA expression of both genes

Compared to the skin graft MST of Group 1 or Group 2 ((5.75±0.71) and (5.50±0.53) d, respectively), simultaneous blockade of both costimulatory signals by Ad-sCD40LIg-IRES$_2$-CTLA4Ig dramatically prolonged the skin allograft MST up to (16.38±1.19) d (P<0.001) (Fig.5). Analysis of sCD40LIg and CTLA4Ig mRNA in the skin allograft showed that adenovirus-mediated gene expression was optimal in the 3rd~5th day after grafting. However, the level of CTLA4Ig gene expression decreased 8 d after transplantation and expression of sCD40LIg was not detected after day 5 (Fig.6).

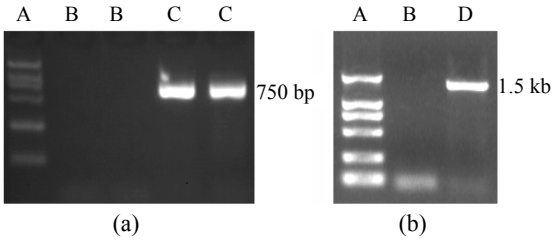

Fig.1 (a) The RT-PCR amplification products of CD40L of 750 bp, and (b) the ligation product sCD40LIg of the sCD40L and IgG Fc
A: DL2000 marker; B: Control; C: CD40L; D: sCD40LIg

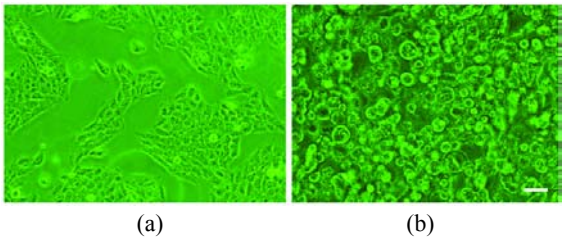

Fig.3 The CPE of the 293 cells transfected by adenovirus. Compared to the untreated 293 cells (a), the 293 cells transfected by Ad-sCD40LIg-IRES$_2$-CTLA4Ig (b) became larger in the nucleus, more round in shape, and detached from the plate (200×). Scale bar=20 μm

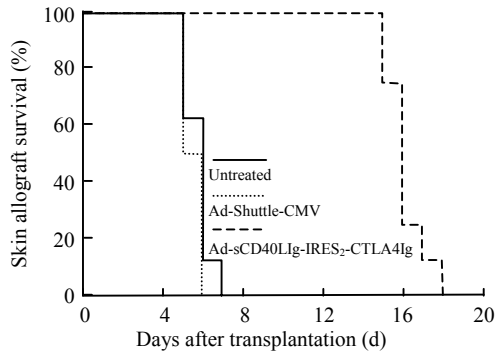

Fig.5 Kaplan-Meier curve of skin allograft survival in mice. Skin graft MST was prolonged by Ad-sCD40LIg-IRES$_2$-CTLA4Ig to (16.38±1.19) d as compared to control, P<0.001

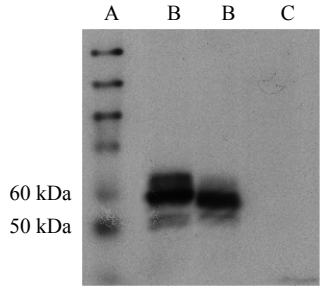

Fig.2 Western blotting showing expression of sCD40LIg (60 kDa, upper bands in Lanes 2 and 3) and CTLA4Ig (50 kDa, lower bands in Lanes 2 and 3). No band was observed in the vehicle control (Lane 4)
A: Protein marker; B: Ad-sCD40LIg-IRES$_2$-CTLA4Ig; C: Vehicle control

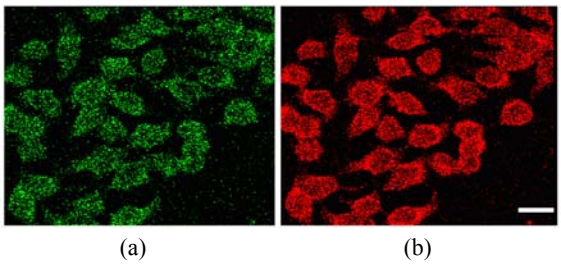

Fig.4 The co-expression of sCD40LIg and CTLA4Ig in HK-2 cells. Co-expression of sCD40LIg (a) and CTLA4Ig (b) was detected with confocal laser scanning microscope (400×). Scale bar=20 μm

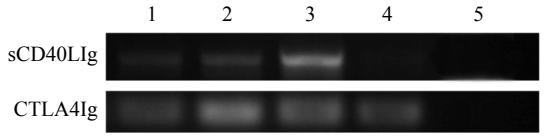

Fig.6 The mRNA expression of sCD40LIg and CTLA4Ig in the skin allograft. The sCD40LIg (upper panel) and CTLA4Ig (lower panel) mRNA expression was examined on the 1st, 3rd, 5th, 8th day after grafting. The mRNA expression also was examined in the skin of the untransfected BALB/c mouse as control
Lane 1: 1st day; Lane 2: 3rd day; Lane 3: 5th day; Lane 4: 8th day; Lane 5: Untransfected

DISCUSSION

The co-expression of two proteins coded by different genes via IRES (internal ribosome entry site) was identified in our study. When the IRES is located between two different genes, the first gene initiates its translation depending on catabolite gene activator protein (CAP), whereas the translation initiation of the second gene is IRES-dependent (Molla *et al*., 1992; Mountford and Smith, 1995). In some cases, the co-expression of two (Gurtu *et al*., 1996) or three genes (Li and Zhang, 2004) was simultaneously successful. In our study, the sCD40LIg and CTLA4Ig proteins were expressed simultaneously, but a different study (Yu *et al*., 2003) indicated that when each transgene was under the independent control of one internal promoter, the co-expression of the two separate genes is more efficient than that mediated by IRES. Therefore, promotion of the co-expression efficiency of two genes ligated by IRES is a problem to be addressed in future studies.

Increasing recent years evidence showed that replication-defective adenovirus without E1a and E3 domains may serve as a useful vector for gene transduction. The advantages of this vector may include the following: the procedure and techniques for construction of this viral vector are straightforward; the vector can carry long exogenous DNA fragments; infect more cell types at higher infectious rate. In addition, there is less risk of inducing alteration of the host DNA since the infection cannot interfere with the host genome (Kanaya *et al*., 2003). However, the application of the gene expression mediated by replication-defective adenovirus is hindered by the short expression period. The susceptibility of different cell types to adenoviral infection is often decided by the availability of the coxsackievirus and adenovirus receptor (CAR) (Walters *et al*., 1999), as well as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, cellular receptors for adenovirus internalization (Chen *et al*., 2002). Adenovirus vectors can efficiently infect a wide range of cell types with the exception of some celltypes with low expression level of CAR, such as T cells (Chen *et al*., 2002), and human dendritic cells (DC) (Tillman *et al*., 1999) and murine DC (Okada *et al*., 2003). Further experiments will be necessary to confirm the distribution of the CAR and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in the skin allograft.

In addition to receptors, the immune response also plays a crucial role in the gene expression mediated by adenovirus. Specific immune responses directed against viral proteins and some transgene products cause the complete elimination of the transduced cells within a few weeks after vector administration (Harvey *et al*., 1999). The immunities induced by adenovirus-mediated gene expression are both cellular and humoral. Cellular immunity results in the elimination of transduced cells or in depletion of vector, and humoral immunity prevents successful readministration of vector. Control of adenoviral antigen specific cellular immune responses will be critical to successful gene therapy if long-term transgene expression is required (Lochmüller *et al*., 1996; Guibinga *et al*., 1998; Jiang *et al*., 2001). The macrophages in the liver and spleen constitute the first barrier of defense against blood-borne pathogens (Worgall *et al*., 1997). A study showed that depletion of macrophages from spleen and liver decreases hepatic inflammation, significantly prolongs transgene expression, and delays the onset of humoral immune responses after systemic administration of an adenovirus vector (Kuzmin *et al*., 2001). In our study, the non-vascular administration of vector may avoid the phagocytosis by macrophages mainly located in the liver and the spleen. Therefore, the gene expression period should be prolonged to benefit the allograft survival. However, our study showed that the mRNA expression of sCD40LIg gene just lasted for about 5 d and the mRNA expression of CTLA4Ig gene got weaker 5 d later, which may be the result of the low efficiency of infection due to the non-vascular administration way of vector under the low temperature condition.

During antigen presentation, one of the most important costimulatory pathways is the interaction of CD40L on activated T lymphocytes and CD40 on macrophages, dendritic cells (DCs), and B cells. Interfering with CD40/CD40L binding prevents the activation and maturation of macrophages and DCs (Mackey *et al*., 1998), and prevents isotype switching and generation of memory B cells (Cerutti *et al*., 1998). CTLA4, another important costimulatory molecule, inhibits T-cell activation by downregulating the interaction between CD28 on T cells and B7 on antigen presenting cells. CTLA4Ig results in systemic immunosuppression by downregulating CD28-

dependent T-cell activation and proliferation, and IL-2 production (Walunas *et al*., 1996; Krummel and Allison, 1996). The potential benefit of the blockade of the B7/CD28 and CD40/CD40L interactions includes nontoxic, transient immune tolerance specific to those adenoviral antigens required for successful gene transfer. Many studies revealed that the blockade of CD40 signaling caused the elimination of activated B cells (Sakata *et al*., 2000; Kuss *et al*., 1999). A study has reported that antiviral cytolytic T lymphocyte (CTL) responses against recombinant adenovirus were strongly reduced in CD40L-deficient mice or in mice treated with anti-CD40L antibodies (Whitmire *et al*., 2000; Andreasen *et al*., 2000). Macrophage depletion in combination with temporary blockade of CD40/CD40L completely stabilized transgene expression and inhibited the production of neutralizing anti-adenovirus antibodies, permitted successful vector readministration via reducing the pool of proinflammatory cytokines and the immediate inflammatory responses induced by adenovirus vectors. This in turn prevented the rapid destruction of highly transduced cells, thereby permitting higher initial levels of transgene production (Kuzmin *et al*., 2001). Another report showed that a high-capacity adenovirus expressing CTLA4Ig prolonged transgene expression in skeletal muscle and showed low levels of infiltrating $CD8^+$ and $CD4^+$ T cells. In contrast, a control vector without CTLA4Ig administration to skeletal muscle resulted in the accumulations of both $CD4^+$ and $CD8^+$ T cells 30 d posttreatment (Jiang *et al*., 2002). Guo *et al*.(2003) demonstrated that AdCTLA4Ig combined with anti-ICOS antibody potently induced a stable immune tolerance after heart allografting in rat, which is mediated by the induction of $CD4^+CD25^+$ regulatory T cells. Blockade of the CD40/CD40L and B7/CD28 costimulatory pathways by anti-CD40L antibody and soluble CTLA4Ig protein, respectively, has been used to abolish adenovirus-specific B-cell functions and severely compromise T-cell responses, prolong transgene expression, and allow efficient readministration in liver, lung, brain, and muscle (Chirmule *et al*., 2000; Guerette *et al*., 1996; Guibinga *et al*., 1998; Ideguchi *et al*., 1999; Jooss *et al*., 1998; Kay *et al*., 1995; 1997; Wilson *et al*., 1998; Yang *et al*., 1996). A bicistronic vector expressing both CTLA4Ig and CD40Ig has been observed to provide better immune suppression than vectors expressing either protein alone (Jiang *et al*., 2001). In the present study, CTLA4Ig and sCD40LIg were simultaneously expressed via replication-defective with IRES to block the B7/CD28 and CD40/CD40L pathways. We can speculate that the adenovirus-mediated CTL and immune responses against viral proteins or transgene products are reduced in this study. In turn, the expression of the CTLA4Ig and sCD40LIg can prolong the corresponding gene expression mediated by adenovirus. One interpretation of this difference between the theoretical period and the short time (about 8 d) of the mRNA expression is that the non-vascular administration of the adenovirus vector affected the infection efficacy.

Additional blockade of CD40/CD40L facilitates the ability of CTLA4Ig-modified dendritic cells to induce immune tolerance (Sun *et al*., 2003). When the B7/CD28 and CD40/CD40L pathways were blocked simultaneously with other techniques, the survival of grafts was significantly prolonged (Saito *et al*., 1998; Safley *et al*., 2005; Yamashita *et al*., 2003; Benda *et al*., 2002). Consistent with that finding, our results showed that simultaneous blockade of both pathways via co-expression of sCD40LIg and CTLA4Ig mediated by replication-defective adenovirus dramatically prolonged the skin graft survival in mice. We think that the prolongation of the skin allograft in mice is due to the immune suppression by sCD40LIg and CTLA4Ig. However, we did not evaluate the effect of the adenovirus on the normal tissue or organ function. The safety of the replication-defective adenovirus will be investigated in future.

CONCLUSION

In the present study, we successfully constructed adenovirus-carrying genes encoding both sCD40LIg and CTLA4Ig. This provides an important tool for using adenovirus for gene therapy in vivo. Compared to the vehicle control, our study indicated the simultaneous blockade of both costimulatory signals significantly prolonged the graft survival. It suggests that simultaneous blockade of the CD40/CD40L and B7/CD28 costimulatory pathways via co-expression of sCD40LIg and CTLA4Ig mediated by replication-defective adenovirus is an acceptable method to induce immune tolerance.

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