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Original Article

Human interleukin-10 gene inhibits acute rejection by triggering apoptosis in allograft vascular transplantation

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Abstract: The aim this study is to explore effect of IL-10 on apoptosis of VSMCs in allograft arterial transplantation rats, and to investigate mechanism. SD rats were divied into three groups, including control group (CN, with physiological saline), blank vector group (BV, with blank adenovirus) and combined gene group (CG, with adenovirus carried IL-10 gene). The isolated donor vascular was transfected with the adenovirus carried hIL-10 gene for 30 minutes by immersing method. Forty-five days post transplantation, the grafts were harvested. The allografts pathologioc changes were observed and the size of vascular intima and middle layer of allografts were measured. The expression of hIL-10 was detected by RT-PCR, ELISA and immunohistochemistry, respectively. The repression of Fas/Fasl in artery allografts was also examined by immunohistochemistry method. The results indicated that 45 days after transplantation, the intimal and middle hyperplasia ratio in CG group was significantly lower than that in CN and BV group ($P < 0.05$). The transgene expression of human interleukin-10 was significantly enhanced in CG group compared to CN and BV group by ELISA, RT-PCR and immunohistochemistry ($P < 0.05$). The expression of Fas/FasL was higher in CG group compared with the other groups ($P < 0.05$). The level of apoptotic smooth muscle cells were significantly increased in CG group compared to CN and BV group ($P < 0.05$). In conclusion, adenovirus mediated IL-10 expression could up-regulate Fas/FasL expression, induce smooth muscle cell apoptosis and alleviate angiosclerosis process. The IL-10 gene transfer to allograft artery could inhibit acute rejection reaction of allograft vascular transplantation.

Keywords: Human interleukin-10, gene transfer therapy, apoptosis, allograft arterial transplantation

Introduction

The previous study indicates that the smooth muscle cells (VSMCs) hyperplasia is the main pathological basis of Allograft vascular disease [1, 2]. The proliferation and migration of MSMCs have pivotal roles in the progression of atherosclerosis and the development of restenosis after vascular interventions [3]. VSMCs can be divided into two types, contractile and synthetic/proliferative [4]. The synthetic VSMCs contribute to the progression of atherosclerosis and the formation of neointimal hyperplasia after vascular injury [5]. Furthermore, the VSMCs apoptosis is also very important for the Allograft vascular disease, which has not been fully discussed in the previous reports, and has been become the most promising fields for the present transplantation immunology.

Inflammatory response, both acute and chronic, is mediated by cytokines, such as interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF-α), and mediators, such as the cysteinyl leukotrienes and isoprostanes [6]. Among these factors, IL-10 is the outstanding one as the immunosuppressive cytokine. The proliferation of the VSMCs could be caused by growth factors, such as the basic fibroblast growth factor (bFGF), the platelet-derived growth factor (PDGF), the epidermal growth factor (EGF) and the insulin-like growth factor-1 (IGF-1) [7]. The PDGF, EGF, IGF-1 and bFGF can activate the mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K) signaling pathways and lead to proliferation of VSMCs [8, 9]. Therefore, the IL-10 can inhibit the bFGF caused proliferation of the VSMCs [10].

The purpose of this study is to explore the effect of IL-10 on the apoptosis of the VSMCs in allograft arterial transplantation rats, and to investigate the mechanism in further step.

Table 1. Primer sequences for the hIL-10mRNA RT-PCR

Primer		Sequence	Length (bp)
Hil-10	Up-stream	5'-ACCTGGGTTGCCAAGCCTT-3'	155
		5'-ATCGATGACAGCGCCGTAG-3'	
β-actin	Down-stream	5'-GAGCTATGAGCTGCCTGACG-3'	410
		5'-AGCACTTGCGGTCCACGATG-3'	

Materials and methods

Materials

The health SD rats, weight 300-350 g, were provided by Shanghai Experimental Animal Center of Chinese Academy of Sciences. The adenovirus vector carried with IL-10 gene, AdenoVec-hIL-10, was purchased from Invitrogen company (Invitrogen, CA, USA). The antibodies anti- rat IL-10, anti- rat IL-1, anti- rat IFN-γ, SABC immunohistochemistry kit and DAB staining kit were purchased from Boshide Biology Company (Boshide, Wuhan, China). hIL-10 ELISA detection kit was purchased from Jingmei Biology (Jingmei, Shenzhen, China). hIL-10 PCR amplification kits was purchased from MBI company (MBI Fermentas, CA, USA). Fluorescence imaging microscopy (PM10SP) was purchased from Olympus (Olympus, Japan). Gel image analysis system (Emager2200) was purchased from Alpha company (Alpha, MA, USA). Ultraviolet spectrophotometer was purchased from Beckman Company (Beckman, CA, USA).

Trial grouping

Twenty-nine SD rats were included in this study, which were divided into 3 groups randomly, including control group (CN), blank vector group (BV) and combined gene group (CG). For the CN group, the donor vascular was immersed in the physiological saline with the heparin for 30 min at 10°C. For the BV group, the donor vascular was immersed in the transfection solution with the blank adenovirus vector for 30 min at 10°C. For the CG group, the donor vascular was immersed in the tranfection solution with the combined adenovirus vector with the hIL-10 gene for 30 min at 10°C.

Establishment of animal model

The donor SD rats were anaesthetized, and cut a median incision at the neck. The arteria carotis communis was dissociated, and cut a length of 1.5 cm to be as the donor vascular. The Allograft vascular transplantation model was established by using the carotid artery fiber surgery anastomosis transplantation technology [11]. Three out of the 39 rats died for the excessive anaesthesia.

Gene transfection

Transplanting the vascular of CG group into the neck of the recipient rats. Forty-five days post transplantation, the arteria carotis communis was isolated and dissociated, and the heparin (200 U/g) was injected intraperitoneally. The isolated arteria carotis communis was cut for the length of 1 cm, which was washed slightly for the following process. The cut arteria carotis communis was cleaved into several minor sections for tissue fixation, embedding, slicing and HE staining. The specific process of the HE staining performed according to Xe et al.'s report [12]. The changes of vascular intima, middle layer and vascular adventitia, the changes of vascular vessel and macrophage infiltration were observed under optical microscope. The imaging systems were used to calculate the results above.

RT-PCR

The RT-PCR method was used to detect the transcription of the hIL-10 mRNA in transplanted vascular. The specific primers for hIL-10 mRNA were listed in **Table 1**. In parallel, we selected the individual β-actin as the internal control, which sequence has also been indicated in **Table 1**. The total RNA was isolated using RNAsimple Total RNA Kit according to the manufacturer's protocol (TIANGEN, Beijing, China). The RNA purity and concentration were examined on the wavelength (A256nm/A280nm) more than 1.8. Reverse transcription was performed using SuperScriptTM III First-Strand Synthesis System (Invitrogen, CA, USA) as the manufacturer's protocol. The procedure and condition of the RT-PCR reaction for IL-10 were performed as the followings: predenaturation for 5 min at 94°C, denaturation for 30 s at 94°C, annealing for 40 s at 57°C, extending for 50 s at 72°C, for 30 cycles, and finally extending for 10 min. The procedure and condition of the RT-PCR reaction for β-actin were performed as the followings: pre-

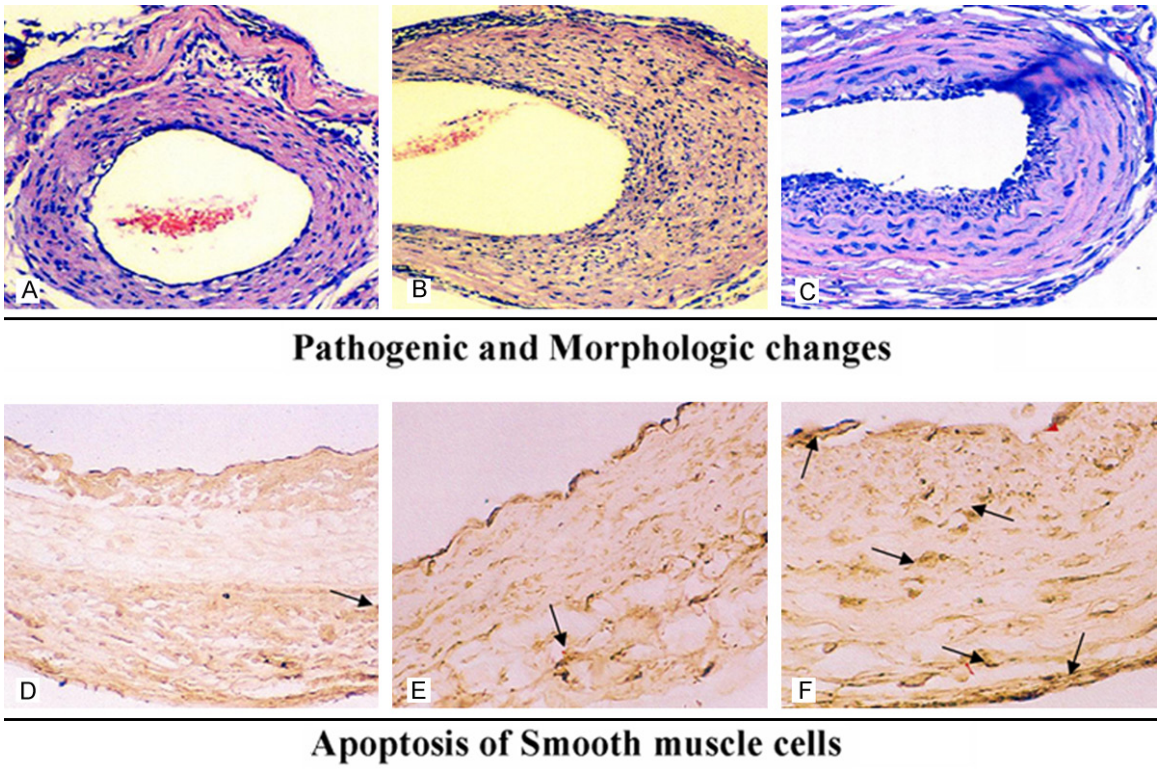

Figure 1. Morphologic changes (HE staining, 200×) and apoptosis (TUNEL assay, 200×) of the smooth muscle cells in every group. A: Pathogenic and morphologic changes of CN group; B: Pathogenic and morphologic changes of BV group; C: Pathogenic and morphologic changes of CG group; D: Apoptosis in CN group; E: Apoptosis in BV group; F: Apoptosis in CG group. The arrows in D to F represent the stained positive apoptotic cells.

denaturation for 5 min at 94°C, denaturation for 20 s at 94°C, annealing for 20 s at 55°C, extending for 75 s at 72°C, for 25 cycles, and finally extending for 10 min. Quantitative RT-PCR were performed using SYBR Premix ExTaq II (TaKaRa, Dalian, China), and detected with the ABI7500 Real-time PCR system instrument (Applied Biosystems, Foster city, USA). After electrophoresed on 1% agarose gel, the gel images of each PCR product were digitally captured with a CCD camera and analyzed with the gel image analysis system (Emager 2200).

ELISA

The ELISA method was employed to detect the expression of IL-10 in transplanted vascular. The ELISA analysis was completed according to the instruction of the ELISA kit (Jingmei, Shenzhen, China).

Immunohistochemistry (SABC method)

The expression of IL-10 and Fas/Fasl were detected by using the immunohistochemistry method. Firstly, the vascular samples performed the antigen repair process. The samples were examined by the immunohistochemistry SABC method. The yellowish-brown nucleus represents the positive cells under the optic microscope. The positive rate of the immunohistochemistry was observed in the total 5 visual fields.

Tunel assay

Potential DNA fragment was examined by the TUNEL apoptosis detection kit (Chemicon, USA). Briefly, the samples were fixed with 4% paraformaldehyde in 0.1 M NaH_2PO_4, pH7.4, and endogenous peroxidase was inactivated by 3% H_2O_2. Cells were incubated with a solution containing biotin-dUTP and the terminal deoxynucleotidyl transferase (TdT) for 60 min. After end-horseradish peroxidase, stained with diaminobenzidine, and counterstained with ethyl green to detect biotin-labeled nuclei. Apoptosis bodies were stained as brown. Cell nuclei were counted under the light microscope. Apoptosis index (AI) was calculated as the percentage of apoptosis cells. At least three

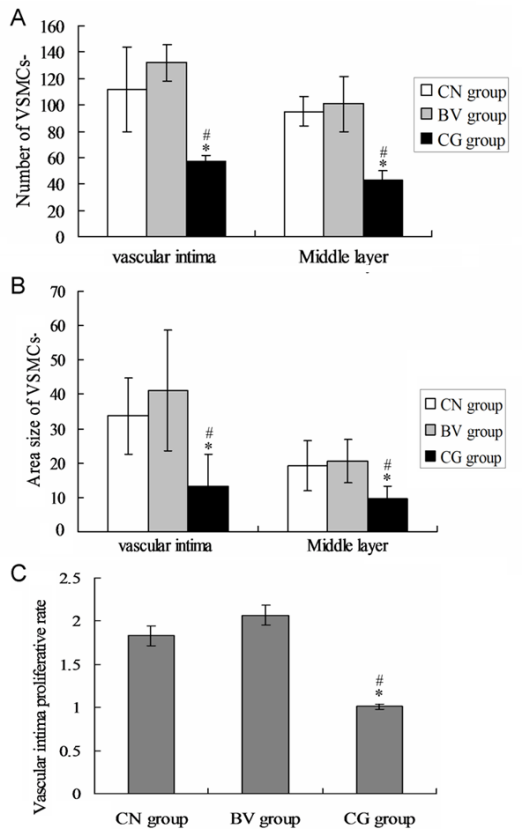

Figure 2. Changes of VSMCs number and area size in vascular intima and middle layer. A: Number of VSMCs in intima and middle layer of smooth muscle cells; B: Area size of VSMCs in intima and middle layer of smooth muscle cells; C: The vascular intima proliferative rate of the intima. $^{*}P < 0.05$, $^{\#}P < 0.05$ represent the index in CG group compared to the CN and BV group, respectively.

independent observers counted the positive-staining nuclei in ten fields (200×). The detailed process of TUNEL assay was performed due to Wang et al's methods [13].

Statistical analysis

All data were analyzed by using SPSS 10.0 software, and presented as the mean ± SD. Statistical analysis was performed employing the t test. Probabilities of less than 0.05 were considered to be statistically significant.

Results

Morphologic changes of transplanted vessels

Forty-five days post transplantation in the CN group, the thickness of the vascular intima was increased diffusely, the vessel was decreased, and the smooth muscle cells were significantly increased (**Figure 1A**). The thickness of vascular intima was decreased, and the smooth muscle cells degenerated obviously. There were also a few mononuclear cell infiltration in the vascular adventitia. The pathogenic and morphologic changes in BV were similar to the CN group (**Figure 1B**). In the CG group, the smooth muscle cells indicated significant changes 45 days post transplantation. The vascular intima indicated an obvious hyperplasia, and the vessel was increased compared to the CN group (**Figure 1C**). The smooth muscle cells and the collagen fibrils were decreased significantly. No obvious mononuclear macrophage infiltration was observed in the vascular intima. There were no changes of smooth muscle cells in middle layer, and the Flexible fibre layer was intact.

IL-10 gene triggers changes of VSMCs number and area size of vascular intima and middle layer

From the **Figure 2**, we found that the number of VSMCs in the intima and middle layer of CG group were decreased significantly compared to the CN and BV group (**Figure 2A**, both $P < 0.05$). The vascular intima size and middle layer size in CG group were decreased significantly compared to CN and BV group (**Figure 2B**, both $P < 0.05$). Meanwhile, we also examined the vascular proliferative rate in all of the three groups. The results showed that the intima proliferative rate was significantly decreased in the CG group compared to the CN and BV group (**Figure 2C**, both $P < 0.05$).

Expression of hIL-10

By the PCR amplification fcific band was found in the CN and BV group. The western blot results also indicated that the expression of IL-10 in the CG group was higher significantly compared to the CN and BV group (**Figure 3B**, $P < 0.001$).

Furthermore, the expression of hIL-10 was also detected by the immunohistochemistry. Only a few expression of hIL-10 was discovered in the CN and BV group (**Figure 4A**, **4B**). However, there was an intense hIL-10 expression in the CG group, and which mainly distributed in the cytoplasm of VSMCs with the chocolate brown (**Figure 4C**).

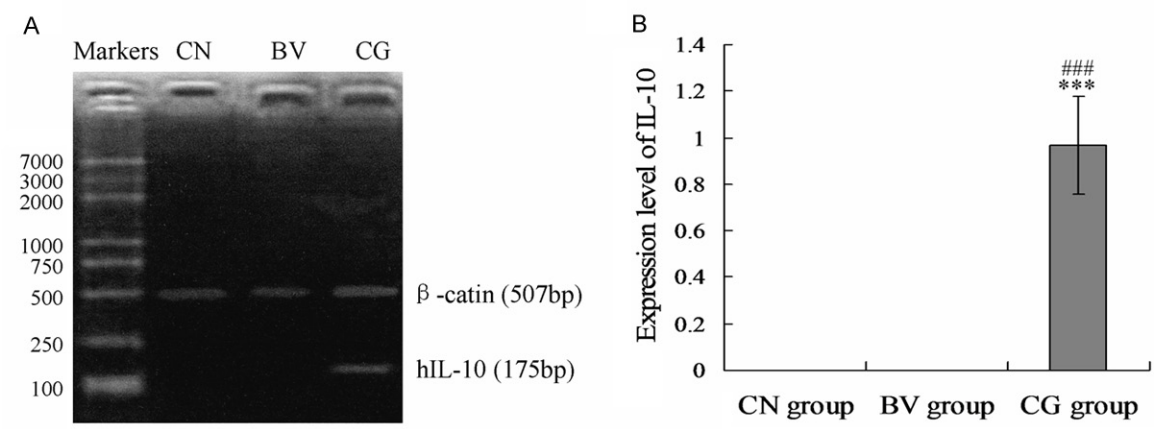

Figure 3. hIL-10 expression in VSMCs. A: RT-PCR detection for the hIL-10 mRNA expression; B: ELISA assay detection for the hIL-10 protein expression. $^{***}P < 0.001$, $^{###}P < 0.001$ represent the index in CG group compared to the CN and BV group, respectively.

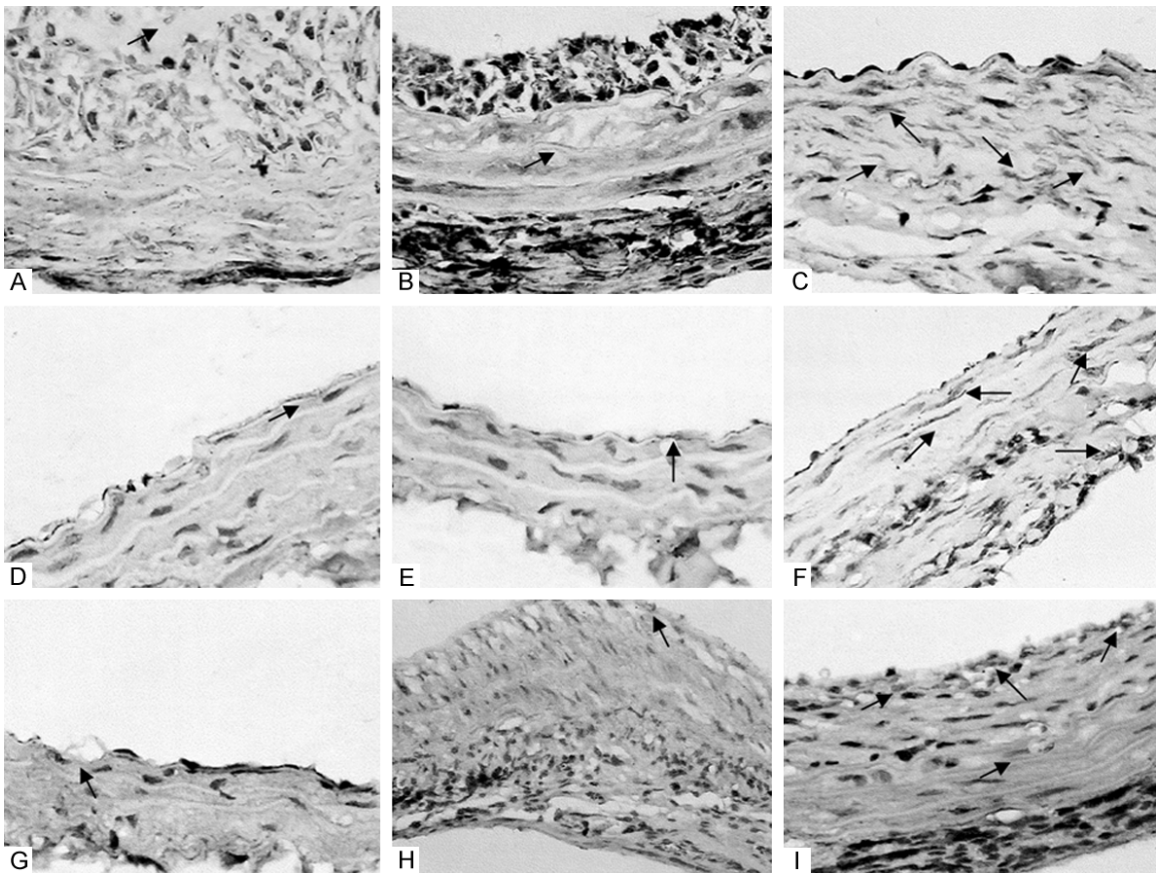

Figure 4. IL-10, Fas and FasL expressing detection by using the immunohistochemistry assay. A to C represent the IL-10 expression in CN, BV and CG group, respectively; D to F represent the Fas expression in CN, BV and CG group, respectively; G to I represent the FasL expression in CN, BV and CG group, respectively. The arrows represent the positively stained smooth muscle cells.

IL-10 induces VSMCs apoptosis

The positive staining signals mainly distributed in nucleus of the intima VSMCs cells. The apoptotic VSMCs cells were stained with the chocolate brown and the nucleus were significantly condensed. The apoptotic cells in the CG group (**Figure 1D**) were increased significantly com-

Table 2. Apoptosis index and Fas/FasL level in transplanted vascular tissues

Groups	AI	Fas	FasL
CN group	21.3 ± 5.6	74 ± 12	57 ± 11
BV group	19.4 ± 3.2	69 ± 15	61 ± 21
CG group	53.7 ± 11.2*,#	164 ± 32*,#	142 ± 23*,#

*$P < 0.05$ represents the values in CG group compared to the values in CN group; #$P < 0.05$ represents the values in CG group compared to the values in BV group.

pared to the CN and BV group (**Figure 1E**, **1F**). BV group indicated the equal amounts of positive staining compared to the CN group. Moreover, by calculating the stained cells, the apoptosis index was significantly enhanced in CG group compared to CN and BV group (**Table 2**, both $P < 0.05$).

Fas/FasL expression in VSMCs

Fas/FasL expression is correlated closely with the apoptosis. The Immunohischemistry results indicated that the Fas positive stained tissues in CG group were significantly higher compared to the CN and BV group (**Figure 4D-F**), and indicated a statistical analysis significance (**Table 2**, both $P < 0.05$). Also, the expression of the FasL in the CG group were higher significant compared to the CN and BV group (**Figure 4G-I**; **Table 2**, both $P < 0.05$).

Discussion

The chronic rejection is the extensively accepted viewpoint in the allograft vascular transplantation caused angiosclerosis [14], but the specific mechanism has been fully investigated. The characteristics of the angiosclerosis in the allograft vascular transplantation include the vessel wall increased, vessel becoming narrow, vascular occlusion and function lose of graft, which were all dependent on the the VSMCs migration and proliferation [15].

Recent years, the main strategy for the chronic rejection is characterized by targeting the above histological changes and the related factors of these changes. Using the genetic engineering technology to modify the gene of donor could resolve the immunological rejection response in the allograft organ transplantation [16, 17]. There are many merits of the adenovirus vector, including fast transfection, strong adsorptive power to smooth muscle, higher infective rate for resting stage cells, which enhances about 20 times compared to the most novel transfection method with the new liposome plasmid complex [18]. The infective efficiency of the gene products is very higher, which possesses the most extensive application potential in the field of organ transplantation and gene therapy.

The previous reports indicate that there are many similarity between the immunologic suppression effects of IL-10 and immunologic suppression status in the late stage of organ transplantation [19]. Some scientists have been transfected the IL-10 gene into organs to observe the protective effects of IL-10, and received some promising results [20, 21]. However, there also no related reports to investigate the protective effects of IL-10 gene for allograft vascular transplantation caused angiosclerosis.

This study established the model of allograft vascular transplantation induced angiosclerosis. The vascular were immersed in the solutions with the adenovirus vector carried the IL-10 gene for therapy. The results indicated that there extensive and exogenous expression of IL-10 at both mRNA and protein levels 45 days post transplantation. However, no obvious signals could observe in the CN and BV group. The immunohistochemistry results showed that the IL-10 mainly expressed in vascular intima smooth muscle cells and some of the inflammatory cells, which strongly confirmed that the gene transfection method of IL-10 is very effective. Actually, our experiment has also proved that the IL-10 was highly expressed, and inhibited the proliferation of smooth muscle cells, and alleviated the pathogenic injury of transplanted vascular. Thus, we though this method could achieve satisfying therapeutic effects.

Sata et al. [22] has been transfected the adenovirus mediated Fas/FasL gene into rats transplanted arteria carotis. The results indicated that Fas/FasL inhibits the intimal hyperplasia by inducing the apoptosis of smooth muscle cells. Our results also indicated that

comparing to the control group, the apoptotic rate in the CG group significantly enhanced, and the expression of Fas with FasL was significantly up-regulated. Therefore, we speculated that the Fas/FasL may participate in the protective effects of IL-10 gene transfection for the angiosclerosis. The results also illustrated that the expression of IL-10 could enhance the smooth muscle cells apoptosis, and further alleviate the angiosclerosis process by up-regulating the expression of Fas/FasL.

In conclusion, adenovirus mediated IL-10 expression could up-regulate the Fas/FasL expression, and induce smooth muscle cell apoptosis and alleviate the angiosclerosis process. The IL-10 gene transfer to allograft artery could inhibit acute rejection reaction of allograft vascular transplantation.

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Disclosure of conflict of interest

None.

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