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Research Paper

Histomorphological observation in arterial remodeling following New Zealand rabbit auto-extremity artery transplantation

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Abstract

Objective: To investigate the histomorphological change in auto-extremity artery following transplantation. **Methods:** 50 New Zealand rabbits were randomly divided into 5 groups(postoperative 1 d, 3 d, 7 d, 14 d, 56 d, $n = 10$). Femoral artery was harvested and end-to-side anastomosed with carotid in order to build the auto-extremity arterial graft animal model. On the postoperative 1st, 3rd, 7th, 14th and 56th days, grafts for morphometric analysis under the Image analysis system were obtained; and electron microscope was scanned to observe endothelial cells. In addition, Immunostaining of sections were performed with the mouse monoclonal antibody of the α-smooth muscle isoform of actin and proliferating cell nuclear antigen antibody. **Results:** Overall patency rate for all conduits was 86%.The intimal hyperplasia was first observed in the 7th day group, and continued to increase in the 56th day group(183.21 ± 111.74) μm, $P < 0.01$. Additionally, the luminal narrowed(32.43 ± 18.28)% in the 56th day group. Smooth muscle cells were the mainly hyperplastic components. The most active proliferation of cells was detected in the 14th day group, where the extracellular matrix gradually deposited in the intima. **Conclusion:** Moderate intimal hyperplasia occurred in arterial conduits and vascular structure experienced constrictive remodeling after auto-transplantation.

Key words: extremity artery; auto-transplantation; artery remodeling; rabbit

INTRODUCTION

With the number of coronary artery bypass grafting (CABG) surgery and CABG redo surgery increasing annually, the concept of total arterial coronary revascularization had been so widely accepted that the radial artery(RA) was introduced as a second order arterial graft next to the internal thoracic artery(ITA)[1-3]. A large number of studies have shown that the post-operation patency rate of the RA graft is at least as good as the saphenous vein grafting(SVG) in the short term, while middle and long-term post-operation revealed large differences[1-5]. Alterations in RA conduit have not been thoroughly investigated. In this experimental study, an animal model of the auto-extremity artery graft is established in order to observe the vessel remodeling of the arterial conduit after transplantation.

MATERIALS AND METHODS

Creation of arterial graft animal model

50 New Zealand rabbits of both sexes, weight range 2.3-2.5 kg, were randomly divided in to 5 groups (postoperative 1 d, 3 d, 7 d, 14 d, 56 d, $n = 10$). All animals received pentobarbital solution diluted 30 mg/kg through an ear vein. To prepare for the operation, cervical part and left iliac region were unhaired using an 8% NaS solution. 5 cm femoral artery was harvested and stored in the preservative fluid(mix normal saline, liquemine, dolicaine, cardoverine). Mdian incision at the ante-cervicum was performed to separate bluntly 6 cm of right carotid. After 1.5 mg/kg heparinizing, the free carotid was clamped at both sides. Two 3 mm long

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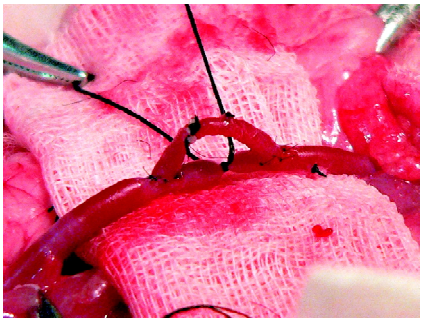
incisions were performed on the carotid. Femoral artery and carotid, were anastomosed interrupt end-to-side by 10-0 polypropylene. The carotid was unblocked if stomas didn't bleed obviously, then ligated between two stomas in order to ensure blood only flow through the graft(***Fig 1***). The animals received one dose of heparin 10 mg/kg(hypodermic injection) and antibiotics for 3 days after the operation.



The carotid was ligated between the two stomas in order that blood flowed through the graft completely.

Fig 1 Femoral artery was anastomosed with carotid

Histopathology and morphology

Normal femoral artery and all grafts of each group were harvested and fixed in 4% formaldehyde solution. Multiple transverse slices(5 μm) of arterial conduit were processed in paraffin wax. All sections were stained with hematoxylin-eosin and Verhoeff Van Gieson's elastin. Morphometic measurement was obtained by color image analysis system(Motic DMBA400). The distribution changes of the collagen fibril and elastic fiber in conduit were measured with the software(Motic Image Advance3.2) at 4 different places of each slice. The width of intima(WI), width of media(WM), hyperplasy intimal area(HIA), medial area(MA), internal elastic lamina circumference(IELC), external elastic lamina circumference(EELC) were each measured 4 times and averaged. The internal elastic lamina area(IELA, IELA=IELC^2/4π), external elastic lamina area(EELA, EELA=EELC^2/4π), percentage of luminal narrowing (PLN, PLN=(HIA/IEL IELA)× 100%), intimal thickness index(ITI, ITI=HIA/MA), intima-to-media ratio (IMR, IMR=at maximal intimal thickness/ at maximal intima thickness) were calculated respectively.

Electron microscopy protocal

Vessel tissues were fixed in 2% glutaraldehyde for 24 h(pH 7.4, 4℃). After being stained with 1% osmic acid, the tisue pieces were frozen and dried under vacuum before being sputtered. Collected specimens were then observed under the scanning electron microscopy and photographs were taken.

Immunohistochemistry

Vessel tissues were estimated by immunohistochemical staining of proliferating cell nuclear antigen(PCNA) antibody. The smooth muscle cell(SMC) was evaluated by performing Envision immunostaining using the mouse monoclonal antibody(α-smooth muscle isoform of actin).

statistical analysis

All experimental values were expressed as mean ± standard deviation and SPSS 12.0 software package was used for data processing. One-Way ANOVA was used for multiple group comparisons and LSD test was used for two group comparisons. The level of significant was set at $P < 0.05$.

RESULTS

There was no cases of death during and after operation. In the 1st day, 3rd day, 7th day, 14th day and 56th day groups, the number of closure were 1, 2, 0, 2 and 2, respectively. Overall patency rate for all conduits was 86%. Gross examination of the closure of conduits revealed thrombus inside grafts of the 1st, 3rd day group and began organzing in the 14th day group. In 56th day group, organized thrombus became fiber bundles so that it's difficult to distinguish the structure of the lumen.

Histopathology and morphology

Light microscopy examination of slices showed moderate intimal hyperplasia occurring in the 7th day group. In the 14th day group, intimal hyperplasia became obvious and a number of irregular SMCs were observed. Extra cellular matrix accumulation was found at the same time. In the 56th day group, endomembrane hypertrophied and the extracellular matrix continued to accumulate.

Morphomtric measure revealed WI was significantly greater in the 56th day group than that in the other groups (all $P < 0.01$; ***Fig 2***).

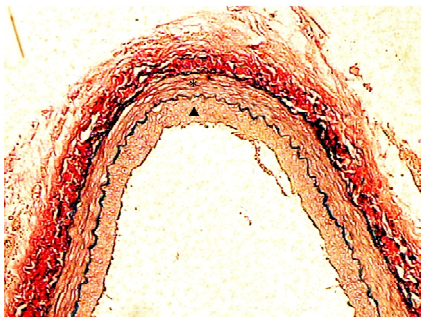


Fig 2 On the 56th day after operation, slice of conduit showed mild intimal thickening(arrow) and multiple elastic lamina within media(star)(Verhoeff van Gieson's elastin, original magnification,× 50)

The HIA was also significantly greater in the 56th day group than that in the other groups with the exception of the 14th day group(***Tab 1***). The PLN, ITI, IMR were significantly greater in the 56th day group than those in the other groups(***Tab 2***).

Tab 1 **Comparative morphology of every group** ($\mu m, \bar{x} \pm s$)

	1d(n = 9)	3d(n = 8)	7d(n = 10)	14 d(n = 8)	56d(n = 8)
WI	4.10 ± 0.75	3.73 ± 0.60	7.90 ± 3.32	64.14 ± 57.87	183.21 ± 111.74
WM	72.73 ± 33.74	87.90 ± 47.86	70.01 ± 27.70	100.11 ± 61.06	73.99 ± 17.34
HIA	0	0	8 067.68 ± 9 919. 17	283 376.99 ± 238 746.95	546288.14 ± 320090.66$^{\triangle\triangle}$
MA	311 448.03 ± 71 928.44	325 450.66 ± 181 192.76	265 792.02 ± 88 247.43	508 278.98 ± 378 802.42	336440.18 ± 146901.13
IELA	2 500 096.51 ± 1 159 908.10	1 395 095.80 ± 1 711 844.74	1 711 844.74 ± 787 012.77	2 773 092.32 ± 2 460 551.57	2379598.07 ± 1808266.64
EELA	2 932 416.32 ± 1 368 051.68	1 775 908.17 ± 865 506.20	2 032 127.81 ± 823 801.75	3 308 309.83 ± 2 395 791.05	2785565.21 ± 1975815.61

Compared with 1 d, 3 d, 7 d, 14 d,$^{\triangle}$ $P < 0.01$; Compared with 1 d, 3 d, 7 d, $^{\triangle\triangle}$ $P < 0.01$.

Tab 2 **Severity indices of every group** ($\bar{x} \pm s$)

	1 d(n = 9)	3 d(n = 8)	7d(n = 10)	14 d(n = 8)	56 d(n = 8)
PLN(%)	0	0	0.34 ± 0.37	10.52 ± 5.60	32.43 ± 18.28*
ITI	0	0	0.02 ± 0.03	0.51 ± 0.30	1.72 ± 1.24*
IMR	0.10 ± 0.05	0.07 ± 0.03	0.26 ± 0.12	0.99 ± 0.69	5.01 ± 3.44*

Compared with 1 d, 3 d, 7 d, 14 d, $^{*}P < 0.01$.

Electron microscopy protocal

In the 1st and 3rd day groups, endothelial cell(EC) and subendothelial layer loss were observed under electron microscopy. In the 7th day group, regenerating ECs were fusiform and without particular arragement. However, endothelial fiber connected with anagenetic ECs, and the EC's long axis became oriented with the direction of the blood flow. In the 14th day group, ECs presented lamellar patches and fiber among ECs increased(***Fig 3A***). In the 56th day group, the quantity of EC increased visibly with the accumulated fiber connecting to the collagens around ECs, however the volume of EC was larger than the normal, and an increase of cellular gaps was found (***Fig 3B***).

Immunohistochemistry

Cells with PCNA expression were stained tan and were subsequently found in the adventitia, the media and the intima at various moments. PCNA positive cells in the media first appeared in the 1st day group and increased greatly up to the 14th day group(***Fig 4A***). Such cells could still be detected in the 56th day group, but their quantity visibly diminished when compared with the 14th day group. Alternation of PCNA positive cells in intima was similar to the ones in the media, except that they initially appeared in the 7th day group. PCNA positive cells were found in the adventitia only in the 14th day group.

Cells positive for α-smooth muscle isoform of actin began to appear in the intima in the 7th day group. The positive cells expression in hyperplastic endomembrane were strongest in the 14th day group(***Fig 4B***). There were still a few positive cells in hyperplastic endomembrane in 56th day group, but their quantity clearly reduced compared with the 14th day group.

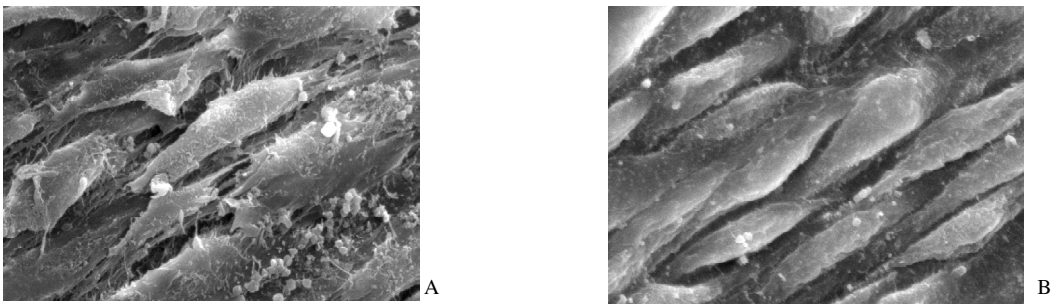
DISCUSSION

The patency of graft directly influences the outcome of the operation and the patient's living quality. In CABG, arterial grafting is more suitable than the venous graft, because the artery is more closed to its physiological condition. The patency rate in RA graft was less than it in ITA graft[1-3]. In order to improve treatment outcome, RA graft's long-term patency rate has been investigated by elementary and clinical scientists.

The femoral artery belongs to extremity artery as well as RA, and it would spasm without nerve control. The caliber proportion between rabbit femoral artery and carotid artery is close to that of human's RA and coronary artery. So the arterial graft animal model closely simulates the situation of RA graft after CABG.

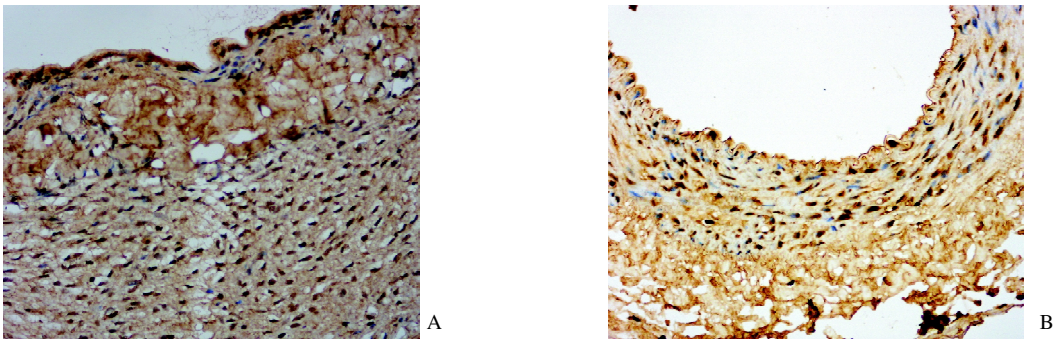
The concept of arterial remodeling is not new, but its mechanisms are unkown. Arterial remodeling is recognized as an important determinant in most vascular pathology, in which narrowing of the artery is the predominant feature. The mode of remodeling includes expansive and constrictive remodeling[6-7].

Changes in the artery after auto-transplantation can be divided into three stages, in which different composition of the vessel repaired and proliferated. The principal character of the first stage was EC recovery. Reactive initmal hyperplasia took place at the same time of traumatic inflammation. The regenerating EC appeared and increased in number. Its arrangement became more sequential under electron microscope. The principal character of the second stage was SMC



A:a mount of fiber connected with collagens around ECs on the 14th day after operation. B:the volume of EC was larger and the cellular gap increased on the 56th day after operation.

Fig 3 Electron microscopy(original magnification,× 2000)



A:PCNA positive cells in media increased in number greatly on the 14th day after operation(immunohistochemical staining of PCNA, original magnification,× 200); B: α-actin positive cells in hyperplastic endomembrane on the 14th day after operation.

Fig 4 Immunohistochemical stain(immunohistochemical staining of α-actin, original magnification, × 100)

behavior. In PCNA positive cells expressed at earlier stage of EC recovering(1,3 days after operation), proliferation and apoptosis in the media remained in balance. At the intermediate stage(7 days after operation), SMCs in the media proliferated significantly with some cells migrating to the endomembrane. 14 days post operation, SMCs achieved the maximum number. Masses of cells expressed in both the endomembrane and the media. Endomembrane continuously thickened into the 56th day after operation while SMCs in both the endomembrane and the tunica media gradually decreased. The principal character in the third stage was extracellular matrix which was mainly made up of collagens. There was no conspicuous extracellular matrix deposition until 14 days after the operation. The collagens continued to increase into the 56th day after operation, while the PCNA positive cells decreased.

In short term of post-operation, conduits displayed constrictive remodeling; the lumina reduced 32.43% on average by intimal hyperplasia. The comstrictive remodeling resulted from multiple factors:①Dysfunction of endothelium: Endothelium undertakes multitude physiological functions. Besides elementary barrier function, it also has responsibility of accepting signal stimulation, in addition to synthesizing and secreting bioactive compound to regulate many bio-behavior, such as blood coagulation, fibrolysis, angiotasis, SMC behavior and so on[7-9]. Some indirect evidences imply that dysfunctional endothelium is one of initiators of constrictive remodeling. Langille et al[10] found that completed endothelium play an important part in vascular remodeling. NO, a principal mediator of vascoregulation secreted by endocheliocyte, play a significant role in positive remodeling[6,7,11-12]. ECs regeneration requires considerable time, especially for the recovery of function if vascellum is impaired by ischemical reperfusion, operation, inflammation and hemodynamic changes. In the 56th day after operation, regenerated ECs had abnormal morphology and cellular gap, which implied a dysfunctional endothelium. ②Haemodynamic change: New vascular bed had also promoted the structure changes of the conduit while inflammatory reaction was taking place. The blood flow, blood pressure and shear stress of the femoral artery are different from those of the carotid so that hemodynamic changed remarkably after femoral artery transplantion. Because of end-to-side anastomosis and bending pon-vascellum, laminar flow is likely to become turbulent flow, thereby causing variations of shear stress when blood flowed through conduit[11]. Both higher and lower shear stress could stimulate endothelial change[13]. ③ Apoptosis and hyperplasy of SMC:SMC in media of conduit proliferated actively and migrated to endomembrane, thereby breaking the balance of apoptosis and hyperplasy at

middle and late stages of endomembrane repair similar to the changes after balloon angioplasty[13]. However, the balance of apoptosis and hyperplasia in SMCs remained the stable at the earlier stage. We speculate this is due to the initial restriction on potential compensatory expansion. ④ The changes of extracellular matrix: After impairment, SMC secreted extracellular matrix as stimulated by growth factors and cyto kinase[14]. The deposition of extracellular matrix in the intima of conduit significantly increased at the later stage. Breakdown and build up of extracellular matrix continued throughout the process of arterial remodeling. The loss of equilibrium of breakdown and build up, perhaps the main reason for constrictive remodeling[15].

Clinical research observed the radial enlargement of conduit arteries in media and long term of postoperation[1,11,17]. And in this experimental study, the IELA had no statistical difference in any group, which explained why the arterial wall didn't dilate or retract obviously in early days of post-operation. The possible reason was that the observing time was only 8 weeks, in which the function of the vessel didn't recover completely. The intimal hyperplasia maybe a widespread phenomenon in the vessel after impairent, and the intimal hyperplasia can existed in the stenotic and closure vessels, even in some un-stenotic vessels[18]. In the middle and later stage of postoperation, more researche is needed on auto-extremity artery transplantation to observe whether the intimal hyperplasia will continue or not and to reveal whether it will decrease obviously by MSC gradually turn into the constringent type[19].

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