Study on mechanism of c-Myc in restenosis after coronary artery bypass grafting

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Abstract. – OBJECTIVE: To study the actions of c-Myc gene fragments in restenosis after coronary artery bypass grafting (CABG).

MATERIALS AND METHODS: A total of 25 healthy pure breeds New Zealand white rabbits were randomly divided into 5 groups according to weight, 5 in each group. The external jugular vein is placed at ipsilateral common carotid artery and sampling at 6h, 2d, 7d, 14d and 28d. The expression of the c-Myc positive cell population was observed in different time using immunohistochemistry and morphological analysis. The thickness and ratio of luminal intima and media were measured by the computer image analytical method.

RESULTS: The luminal intima and media thickness at day 7 is significantly thickening (p<0.01) from 6h while it has not changed (p>0.05) at day 14 and 28 compared to day 7. C-Myc proteins are gradually increased from 6h to day 7, reached a peak (p<0.01) at day 7; started declining from day 14-28. The difference has statistical significance (p<0.01) compared to day 7.

CONCLUSIONS: C-Myc positive cell population has reached a peak after transplantation, which is identical with the peak of fast intimal proliferation. It indicates that c-Myc protein expression is closely associated to intimal proliferation. It can act as an indicator for intimal proliferation after vascular injuries in the early stage of reactions.

Key Words: C-Myc genes, Coronary artery bypass surgery (CABG), Restenosis, Intimal proliferation.

Introduction

The major failure cause of coronary artery bypass grafting (CABG) is restenosis of implants. Its key link is hyperplasia of vascular smooth muscle cells (VSMC). The mechanism of VSMC hyperplasia is not clear. At present, it is believed that multiple vasoactive substances may have participated in occurrence and development of restenosis together. C-Myc is a transcription factor which has been studied a lot in recent years. A large number of studies suggest that c-Myc is a response gene in the early stage. It has participated in the cellular generation, proliferation, differentiation and apoptosis. It is a joint pathway for actions of cell growth factors, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGD) and transforming growth factor (TGF). Using antisense c-Myc gene to suppress the c-Myc gene expression may become a potential target for the treatment of vascular restenosis. However, massive trials were limited to arteries and in vitro animals in the past, and few studied on c-Myc expression in vivo implants.

Materials and Methods

Experimental Materials

A total of 25 healthy pure breed New Zealand male and female rabbits were provided by National Institute for the Control of Pharmaceutical and Biological Products, Chinese Academy of Medical Sciences (CAMS). The weight was between 2.5 kg and 3.0 kg in conventional feeding. They were randomly taken and divided into 5 groups based on the weight, 5 in each group. Mouse Streptavidin-Peroxidase (SP) kits and 3’3-diaminobenzidine (DAB) kits were purchased from Zymed Company (San Francisco, CA, USA). Mouse-anti-rat c-Myc antibodies (1:50) were purchased from Santa Cruz Company (Santa Cruz, CA, USA). Table operation microscopes and microsurgical operating instruments and medical non-invasive stitches were sponsored by Shanghai Medical Apparatus Factory. Pathological image capture analysis workstation is developed by Beijing University of Aeronautics and Astronautics.

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Modeling
Vein graft preparation: Giving ketamine (30 mg/kg) induction and 2% pentobarbital (30 mg/kg) anesthetic maintenance, the rabbit was fixed on the operation table for neck skin preparation and disinfection. A median incision was taken in the neck. The right external jugular vein was freed about 1.5 cm. The branch was ligated, and 2 cm of the right external jugular vein (EJV) was cut out. The lumen was washed by heparin and natural saline (NS) and kept in heparin and NS solution. The right common carotid artery (CCA) was freed about 1.5 cm along the side of the trachea. Each branch was ligatured. A non-invasive vascular clamp was placed at distal and proximal end respectively at a distance of 1 cm. The middle artery was removed.

EJV-CCA bypass transplantation: Both ends of vein segment were cut and reversed. End-to-end anastomosis for two ends was respectively taken place with lacerated ends of CCA. Interrupted exostrophy suture was conducted using 10-0 non-invasive sutures. The lumen and stomas were frequently washed by heparin and NS during anastomosis in case of thrombosis. The vascular clamp was removed after finishing anastomosis to check whether there was blood leaking. If there is blood leaking, additional sutures can be given. Whether the vein graft was unobstructed, it was checked by a conventional method. Hemostasis by compression for stomas exudation was conducted by gauzes. The subcutaneous tissue and skin were stitched layer by layer. The rabbit was given routine penicillin im for 3 days after the operation in order to prevent infection and it was fed by common fodder.

Vein Graft Sampling
Sampling was taken place at 6h, 2d, 7d, 14d and 28d after the operation. Anesthesia and operative incision were same to before. Free the EJV graft and ligature vessels at distal and proximal ends of stomas by 8-0 surgery sutures. The vascular tissue between two ligatures was cut off. After washing by heparin and NS, these cutting tissues were immediately placed in neutral 4% paraformaldehyde for fixation. Conventional dehydration, paraffin embedding, section, HE staining and elastic fibers staining were taken place within 4 hours for morphologic observation.

Immunohistochemical Staining
Immunohistochemical staining is carried out according to the specification of SP kit about c-Myc expression. The main steps include conventional dewaxing in water, blocking endogenous hydrogen peroxide enzyme activities, serum blocking antibodies, adding in c-Myc monoclonal antibodies (1:100) of primary antibodies, incubating overnight in a moist box under 4°C, phosphate buffered saline (PBS) washing 5 minutes for 3 times, adding in horseradish peroxide enzyme markers of second antibodies, incubating for 30 minutes in the moist box under 37°C, washing, developing, counterstaining, dewatering, neutral gum sealing and observing. The positive staining was determined based on endonuclear deep brown staining of the smooth muscles. The percentage of positive cells accounting for total cells was observed and counted under a 400× light microscope. The negative control was carried out by PBS replacing primary antibodies.

Measurement of Vein Graft’s Intima and Media Thickness
The thickness and ratio of intima and media of vein graft were measured by BUA Pathological Image Acquisition and Analysis Workstation in order to evaluate the intimal hyperplasia levels of the vein graft.

Statistical Analysis
Input and analysis were carried out using SPSS19.0 statistical software (SPSS Inc., Chicago, IL, USA). The quantitative data were expressed by mean±standard deviation. Repeated measurement data analysis of variance was used, and the pairwise comparison was tested by LSD or Bonferroni method. \( p<0.05 \) means the difference has statistical significance.

Results
Comparisons of Luminal Intima and Media Thickness and Ratio
No animal deaths were happened during the experimental process. The finished stoma was unobstructed. There were 2 cases of vein graft blocking found within 7-10 days after the operation. A small amount of intimal bleeding was reported at 6h. Inflammatory cells were adhered to the endothelium after 2 days. Smooth muscle cells were staggered with extracellular matrix between them. Luminal intima and media thickness and ratio were increased rapidly between 6h and day 7 but not significant from day 7 to day 28 (Table I).

Comparison of c-Myc Positive Rate
C-Myc protein expression was not tested in normal vascular smooth muscle cells. A small
amount of c-Myc positive cells were turned up at stomas 6 hours after transplantation. The expression was significantly increased from day 2 to day 7 and decreased from day 14 to day 28. The difference has statistical significance (F=35.627, \( p < 0.001 \)) (Figures 1 and 2).

**Discussion**

The patency rate after CABG is a key to decide the success or failure of the operation. The great saphenous vein is most used for a graft currently. However, its blocking rate one year after the operation is 15-30%, ascending year by year, and up to 50% at year 10. The main reason of intimal hyperplasia of vein grafts is media smooth muscle cells (SMC) moving to the intima with massive proliferation and extracellular matrix secretion and deposition. The endothelial cells can suppress or promote intimal hyperplasia factors via secretion function, as well as adjust the migration and proliferation of SMC. VMSC proliferation is converted from contract form to synthetic form to produce a large number of extracellular matrix (ECM), including collagen I, III, fibronectin and hyaluronan stacking in intercellular spaces resulting in vascular wall thickening and luminal stenosis. Meanwhile, vascular remodeling plays an important role in graft restenosis mechanism. Simultaneously, neointima can be deemed as an atherosclerotic “soil” to combine with the atherosclerosis-induced lipoprotein resulting in the degenerative changes of vein grafts and affecting the life and long-term effects of vein grafts. This study shows that the thickness and ratio of luminal intima and media have increased rapidly between 6h and day 7, not significantly between day 7 and 28. It indicates that day 7 is a peak of vascular remodeling.

Burns et al. found that the intake of tritium labeled-thymine in VSMC of the proximal vascular wall was more than the media after artery injuries using tritium labeled-thymine soaking into the vascular wall. Kato and Dang identified the VSMC by α-actin antibodies. Mouse anti-c-Myc antibody’s dual immunohistochemistry found that c-Myc oncogenic protein expression was significant in actively proliferative cells. This study suggests that no c-Myc protein expression is found in normal VSMC. After 6 hours after transplantation, a few c-Myc positive cells are turned up at stomas. The expression is significantly increased at day 2-7 and decreased at day 14-28. The difference has statistical significance. It indicates that c-Myc expression is closely associated to intimal proliferation in transplanted veins. The stronger the c-Myc protein expression, the more significant the intimal proliferation will be. This result is same to the outcome of balloon catheter damage model obtained by Zeymer et al. C-Myc belongs to intra-nuclear protide. Its coding products are specific binding intra-nuclear DNA. C-Myc is a cell messenger with “fast reactions in early stage”. Its expression is elevated due to sti-

<table>
<thead>
<tr>
<th>Intima Thickness</th>
<th>Media Thickness</th>
<th>Intima/Media Thickness</th>
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<tr>
<td>6h 5.5±0.3</td>
<td>26.3±4.7</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>2d 27.0±4.2</td>
<td>40.7±5.9</td>
<td>0.32±0.06</td>
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<tr>
<td>7d 51.3±6.6</td>
<td>48.9±5.3</td>
<td>1.17±0.08</td>
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<tr>
<td>14d 53.2±6.2</td>
<td>51.2±5.2</td>
<td>1.03±0.07</td>
</tr>
<tr>
<td>28d 55.4±6.3</td>
<td>52.8±5.0</td>
<td>1.12±0.06</td>
</tr>
<tr>
<td>F 24.527</td>
<td>16.924</td>
<td>10.326</td>
</tr>
<tr>
<td>p &lt;0.001</td>
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Figure 1. Comparison of c-Myc positive rate. C-Myc expression was found to significantly increase from day 2 to day 7 and decrease from day 14 to day 28.
mulation of mitosis sources before entering a proliferation state from stationary state, namely cells enter into the phase $G_1$ from phase $G_0$. Under actions of multiple growth stimulating factors, c-Myc is strongly induced in first 2 hours of phase $G_1$ and kept a high expression during the entire cell cycle of continuous proliferative cells. C-Myc protein is deemed as having an important meaning in promoting cells entering into phase S to finishing DNA synthesis. When other fast early genes have not induced expressions, activated c-Myc can make cells enter into phase S ahead of time. PGDF, FGF and TGF can accelerate c-Myc gene transcription and induce cell cycle and proliferation. Cell proliferation inhibitor-heparin can mediate c-Myc inductive pathway via suppressing PKC and then suppress c-Myc expression and cell growth\textsuperscript{17}.

Studies in recent years have shown that c-Myc protein expression is increased, closely associated to VSMC proliferation and migration in some cardiovascular disease such as atherosclerosis, hypertension, restenosis after blood vessel molding operation\textsuperscript{18,19}. As a transcriptional activation protein, it can activate promoters with E-box sequence genes, including cell cycle’s regulatory genes. c-Myc protein can directly interact with gene products that play key roles in regulating the cell cycle. Its mechanism\textsuperscript{19} is likely to be deemed as the carboxy-terminal binding specific DNA sequence and opening genes that are related to cell proliferation. Amino-terminal binding inhibitor genes will relieve its suppressor cell proliferation; induce serine-threonine protein kinase P34; and produce cell migration. A large number of trials indicate that c-Myc antisense oligonucleotide can significantly suppress c-Myc protein expression and effectively reduce VSMC proliferation and migration rate\textsuperscript{20}.

Conclusions

c-Myc protein expression is closely associated with intimal proliferation. It can be an indicator for intimal proliferation in early reactions after vascular injuries.

Conflicts of interest
The authors declare no conflicts of interest.

References


