Research of glycosylated granulocyte colony-stimulating factor on mobilizing cell regeneration of bone marrow stem cells and repairing injured myocardium

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ABSTRACT

Objectives: To compare the differences between glycosylated and non-glycosylated granulocyte colony-stimulating factor (G-CSF) on mobilizing cell regeneration of bone marrow stem cells and repairing injured myocardium.

Methods: In the acute myocardial ischemia model, 40 Sprague-Dawley rats (8 weeks old, weight 200-250 g) were successfully established with isoproteronol (ISO) and randomly and evenly divided into 4 groups: Group G (injected with glycosylated G-CSF), Group N (nonglycosylated G-CSF), Group C ([control] normal saline for 7 days), and Group M (model). At day 5, all rats were labeled with bromodeoxyuridine (BrdU). At day 28, the proportion of CD34+ cells, myocardial BrdU immunohistochemical stain, myocardial capillary density, myocardial fibrosis and cardiac function was detected among the 3 groups. The study was carried out at the Central Laboratory, Tongji Hospital of Tongji University, Shanghai, China between 1 July 2009 and 31 July 2009.

Results: In Group G, the proportion of CD34+ cells and cardiac function was significantly higher than groups N and C, and BrdU positive cells in myocardium were higher than those other groups. Diaminobenzidine stained positive cells in striated muscle tissue possessed more cardiocyte-like structure, higher myocardial capillary density, and less myocardial fibrosis in group G compared with other 2 groups.

Conclusions: Glycosytated G-CSF might possess stronger capability to mobilize cell regeneration of bone marrow stem cells and repair injured myocardium.
The cardiocytes of adult mammals lacked proliferation and differentiation potentiality, and damaged and necrotic cardiocytes were inevitably replaced with fibrous connective tissues, which resulted in contractile and diastolic functional disturbance, and then congestive heart failure, and eventually death. Although traditional treatments including drug treatment and interventional therapy and surgery could improve the symptoms of myocardial ischemia and heart failure to unblock the obstructed blood vessels, they could not replace necrotic cardiocytes. Stem cells possessed reproductive activity and multidirectional differentiation potentiality, and thus they could fundamentally increase the number of functional cardiocytes to improve cardiac function. Recently, stem cells open up a new way to treat ischemic heart disease. Stem cells mobilization reagents mobilized bone marrow stem cells to increase the amount of peripheral stem cells, stem cells homing; and horizontal differentiation promoted injured myocardium, which was named as autografting. Theoretically, glycosylated granulocyte colony-stimulating factor (G-CSF) had better mobilizing effects than traditional non-glycosylated G-CSF. In this study, rat acute myocardial ischemia model was established, and the differences between glycosylated and non-glycosylated G-CSF on mobilizing cell regeneration of bone marrow stem cells and repairing injured myocardium were compared, in order to promote widespread application of glycosylated G-CSF on ischemic heart diseases.

Methods. Forty clean inbred male Sprague-Dawley (SD) rats, 8 weeks old, weight 200-250 g, were provided by the Shanghai Laboratory Animal Center of Chinese Academy of Sciences. The study was carried out at the Central Laboratory, Tongji Hospital of Tongji University, Shanghai, China between 1 July 2009 and 31 July 2009. The main drugs and reagents included Isoproterenol (Hefong Shanghai Pharmaceutical Co., Ltd, Shanghai, China), Lenograstim (Zhongwai Pharmaceutical Consulting Co., Ltd, Shanghai, China), Teerjin (Tebao Xiamen Bio-engineering Co., Ltd, Shanghai, China), CD34+ monoclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA), bromodeoxyuridine (BrdU) reagents (Shanghai Jiemei Gene Technology Pharmaceutical Co., Ltd, Shanghai, China), BrdU monoclonal antibody (Sinopharm Group Chemical Reagent Co., Ltd, Beijing, China), rabbit anti-rat antibody and fluorescent secondary antibody (Sigma Company, Rockford, IL, USA), flow cytometer (Becton, Dickinson and Company, New Jersey USA); immunohistochemistry Kit (Bor-Cheng Technology Co., Ltd, Taiwan); ordinary and inverted microscopes (Olympus Optical Co., Ltd Tokyo, Japan), and Vivid 7 echocardiography (General Electric Company, Louisville, USA). Isoproterenol (ISO) is a strong β-adrenergic stimulants, it can increase myocardial oxygen consumption through accelerating heart rate, increasing cardiac contractility and other sectors, resulting in cardiac overload, myocardial microcirculation, and form a myocardial infarction-like change if used continuously.

Forty SD rats were intraperitoneally injected with ISO (5 mg/kg/d) after 3 days. They were randomly divided into 4 groups: glycosylation group (Group G), non-glycosylation group (Group N), control group (Group C) and model group (Group M); 10 rats in each group. The myocardium tissues of rats in group M were observed immediately after modeling and diffused white punctiform ischemic focus were found in endocardium, indicating successful modeling was performed in all 4 groups. One hour after modeling, rats in group G were subcutaneously injected with 10 μg/kg glycosylated rhG-CSF (Lenograstim) diluted with normal saline, once a day at the same time for 7 days, while non-glycosylated rhG-CSF (Teerjin) in group N and normal saline (NS) in group C. After administration with mobilizing agents or NS on day 5, all rats in 3 groups were intraperitoneally injected with 50 mg/kg 5-bromo-2’-deoxyuridine (BrdU) twice with an interval of 2 hours. After administration with mobilizing agents or NS on day 5, 1 ml blood was immediately drawn from the tail vein of the rats in 3 groups. Then, peripheral mononuclear cells were separated with ficoll stratified fluid method, and CD34+ monoclonal antibody was added to determine the proportion of CD34+ cells in all mononuclear cells with flow cytometer. Ejection fraction (EF) values was determined with echocardiography on day 28, Vivid 7 echocardiography with probe frequency of 7.5 MHz was performed in all rats to determine left ventricular long-axis 2-dimensional images, M-mode ultrasonography of left ventricular papillary muscles and EF. Measured value was the mean of 5 cardiac cycles. After cardiac functions examination, rats were anesthetized with pentobarbital and sacrificed by intravenously injecting with 15% potassium chloride to ensure cardiac arrest in diastole. After perfusion with 2% paraformaldehyde, hearts were taken out following with fixation, dehydration, embedding with embedding medium and frozen sections. Then, capillary density was observed and counted after hematoxylin and eosin staining (the number of capillaries in diameter <10 μm in a visual field under 400 x optical microscope). And myocardial fibrosis was observed by Masson staining. Sections were

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incubated with rabbit anti-mouse BrdU antibody or fluorescent secondary antibody to observe fluorescent positive cells. Diaminobenzidine (DAB) staining was performed with BrdU monoclonal antibody through ABC method (avidin-biotin-peroxidase) to observe stained positive cells. This study was approved by the Biomedical Ethics Committee of Tongji University, and all work was conducted in accordance with the Declaration of Helsinki (1964).10

The data was expressed as mean ± standard deviation and group comparison was performed by t-test. All statistical analyses were performed with SPSS version 14.0 statistical software, and p<0.05 was considered significantly different.

**Results.** All 30 SD rats survived throughout the experiment. After subcutaneous administration of mobilizing agents or NS, the proportion of peripheral CD34+ cells in group G was higher than group N and group C, with a significant difference (p<0.05) (Table 1). The echocardiography on day 28 showed higher EF values in Group G compared to Group N and Group C (p<0.05) (Table 1). Under high power field, the myocardial capillary density in group G was higher than group N and group C, with significant difference between any 2 groups (p<0.05) (Table 1).

**Myocardial fibrosis.** More ordered arrangement of cardiocytes were found in group G and N while fewer collagen fibers in group C, especially in group G.

**Table 1 -** Main data of the study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Proportion of peripheral CD34+ cells</th>
<th>Ejection fraction value</th>
<th>Myocardial capillary density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group G</td>
<td>4.350 ± 0.7487*</td>
<td>74.700 ± 3.0569*</td>
<td>20.200 ± 3.6148/HP</td>
</tr>
<tr>
<td>Group N</td>
<td>2.500 ± 5.1424*</td>
<td>71.000 ± 3.0551*</td>
<td>16.100 ± 3.3483/HP</td>
</tr>
<tr>
<td>Group C</td>
<td>0.330 ± 0.2111</td>
<td>65.900 ± 4.0675*</td>
<td>8.000 ± 2.0000/HP</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD. *data are in percentage, HP - high power objective

Group G - glycosylation group, Group N - non-glycosylation group, Group C - control group

**Figure 1 -** The fibrosis change showing the blue collagen fibers (arrow) (pink cardiac muscle fibers, interstitial substance, and red blood cells) (Masson ×200). Group G - glycosylation group, Group N - non-glycosylation group, Group C - control group

**Figure 2 -** The bromodeoxyuridine (BrdU) immunofluorescent staining fluorescent positive cells (arrow). Group G - glycosylation group, Group N - non-glycosylation group, Group C - control group
irregular arrangement of cardiac muscle fibers was found in group C and presented large lamellar or island-like shape separated with criss-cross collagen fibers (Figure 1). After myocardial BrdU immunofluorescence staining, relatively intense and uniformly distributed fluorescent positive cells were found in group G and group N, especially in group G, while only scattered fluorescent positive cells in group C (Figure 2). Striped muscle BrdU-DAB staining revealed: regular arrangement of stained positive cells were found in group G and group N, with obvious directionality and clear transverse striation and sarcomere structure of cardiocytes; there were also connections between positive cells and host cardiocytes, especially in group G. While, scattered stained positive cells were found in some sections of group C (Figure 3).

**Discussion.** Ischemic heart disease (IHD), which is caused by coronary artery stenosis or blockage, is one of high-fatality diseases in the world. Recently, basic experiments and initial clinical researches reveal good prospects of stem cells in IHD treatment. Due to many advantages including simple, adjustable, high cell viability and without toxic side effects, autografting becomes one of research hotspots in this field, and meanwhile the ethical and immunological problems of transplantation in vitro can avoided. Autografting mainly includes the following 3 steps:11-14 1) stem cell mobilizing reagents stimulate bone marrow stem cell proliferation and then release into peripheral blood; 2) whether or not bone marrow stem cells correctly get to myocardium because of "the role of homing" after being mobilized; 3) bone marrow stem cells differentiates into cells possessing similar function with cardiocytes (cardiocyte-like cells). Glycosylated granulocyte colony-stimulating factor, a stem cell mobilizing reagent playing role in late progenitor cells, can increase the number of colony forming unit-granulocyte-monocyte (CFU-GM) for up to 100 times. At present, it is one of the most widely used mobilizing agents in clinic and in experiments. Recombination human granulocyte colony-stimulating factor (rhG-CSF) included 4 kinds of products: filgrastim (Neupogen), nartograstim (Ro25-8315), lenograstim (Granuocyte) and SD/01 (filgrastim sustained duration). The former 2 were clinically applied in the United States while the last 2 in Europe.15 Of them, lenograstim is the only glycosytated G-CSF. In experiments, in vitro glycosylation can enhance the stability of G-CSF and decrease the degradation against serum enzymes when pH and temperature changes. In fact, the affinity between lenograstim and receptors is 3 times than filgrastim, while the concentration stimulating neutrophil cloning maturation is 1/16 of filgrastim. Related bone marrow culture research revealed that the intensity of lenograstim in stimulating neutrophil colony forming is greater than filgrastim and G-CSF used in experiments (non-glycosylated G-CSF), indicating that the intensity advantages of lenograstim in experiments in vitro derived from glycosylation. Therefore, acute myocardial ischemic model was established, and then 3 steps how stem cells repaired damaged myocardium were verified to compare the curative effects between glycosylated and non-glycosylated G-CSF. Due to no definite morphological characteristics, all stem cells show like lymphocyte-like mononuclear cells. Thus, most studies recognize CD34+ cells as stem cells, and CD34+ cells are preferred cells of stem cell biological activity and clinical application.16 CD34+ cells might...
be the common genealogical ancestor of hematopoietic stem cells, bone marrow mesenchymal stem cells, and bone marrow hemangioblasts. Therefore, we indirectly judged stem cells mobilization effect through further determining the amount of peripheral CD34+ cells. It was reported that the peak value of bone marrow stem cells stimulated by G-CSF appeared on day 5 after mobilization. Therefore, we drew peripheral blood on day 5 after mobilization, and it was found that the proportion of CD34+ cells in mononuclear cells in G group was significantly higher compared to the other 2 groups. It also shows that glycosylated G-CSF can promote bone marrow stem cells proliferate and release into peripheral blood compared with non-glycosylated G-CSF. BrdU, a analogue of DNA thymidine precursor, can replace thymine by competitive insertion into single stranded DNA nucleotide sequences of S-phase cells. The competitive insertion intensity of BrdU into thymidine is closely related with cell proliferation. The higher proportion of cells was inserted, the more vigorous cell proliferation. In this experiment, myocardium sections and immunofluorescence processing revealed that, in group G, more bone marrow stem cells were received to myocardium and participated into the second step of damaged myocardium repair. The striated muscle is the effective muscle group of myocardium. Therefore, DAB staining with BrdU monoclonal antibody was performed in striated muscle sections of myocardium. Under high power field, obvious couplings between stained positive cells and original cardiocytes were found in group G and N. Then, they participated in myocardial contractile activity and differentiated into cardiocyte-like cells, especially in group G. Myocardial capillary density and myocardial fibrosis determination also revealed that damaged myocardium was better repaired in group G. Besides, echocardiography prompted group G had an advantage in improving cardiac function, which had great reference value in clinical application, thus, we assumed that the main reason was the effective regeneration of myocardial cells.

The above 3 steps confirmed in different degrees that glycosylated G-CSF possessed higher ability to mobilize bone marrow stem cells and repair damaged myocardium compared with non-glycosylated G-CSF. Under the same conditions, we extrapolated that glycosylation played a very important role. Therefore, we believe that glycosylated G-CSF possessed great potential in treating IHD. The effect of glycosylated G-CSF is more significant than non-glycosylated G-CSF, at the same dose and the same environment, and can further improve the cardiac function. It will be able to be more widely applied in the future. Of course, there are also many unresolved ambiguities. For example, the existence of myocomma was not verified by electron microscope in the aspect of cardiocyte function; the survival time of “cardiomyocyte-like” cells after horizontal differentiation was not discussed; slight phagocytosis of original cardiocytes could not be excluded by BrdU labeling (a small amount of cell division was found in cardiocytes) and so on. The above-mentioned questions need to be resolved by further studies.

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References

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