Hyperglycemia has no effect on development of restenosis after percutaneous transluminal angioplasty (PTA) in a diabetic rabbit model

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Abstract

It is well known that hyperglycemia is a trigger of atherosclerosis in patients with diabetes mellitus. However, the role of hyperglycemia in restenosis remains unclear. In this study, we investigated the effects of hyperglycemia on restenosis. Stenosis was evaluated in two sets of diabetic rabbit models: i) diabetic restenosis versus nondiabetic restenosis and ii) diabetic atherosclerosis versus nondiabetic atherosclerosis. Our results indicated that there was no difference in rates of stenosis between the diabetic and the nondiabetic groups in restenosis rabbit models. However, the incidence of stenosis was significantly higher in the diabetic atherosclerosis group compared with the nondiabetic atherosclerosis group. Similarly, the intima–media thickness and cell proliferation rate were significantly increased in the diabetic atherosclerosis group compared with the nondiabetic atherosclerosis group, but there was no difference between the diabetic restenosis and the nondiabetic restenosis groups. Our results indicate that hyperglycemia is an independent risk factor for atherosclerosis, but it has no evident effect on restenosis. These findings indicate that the processes of atherosclerosis and restenosis may involve different pathological mechanisms.

Key Words
- hyperglycemia
- restenosis
- percutaneous transluminal angioplasty
- atherosclerosis

Introduction

Peripheral vascular disease (PVD), one of the major clinical manifestations of atherosclerosis, is highly prevalent in diabetic patients, and may cause intermittent claudication and critical limb ischemia (Bosevski 2012, Delbin & Trask 2014). Revascularization is often recommended for patients who are resistant to conservative therapies.

Although surgical bypass grafting is the gold standard, acceptable midterm secondary patency and limb salvage rates have led us to consider percutaneous transluminal angioplasty (PTA) as the first-line treatment for patients with lower extremity ischemia (Abularrage et al. 2010). Successful revascularization of the lower limbs by PTA
can improve patients’ quality of life and functional status and decrease future atherothrombotic events (Giugliano et al. 2013). However, the application of PTA to PVD is largely limited due to the high restenosis rate (Jones et al. 2013).

Intimal hyperplasia is a major process of restenosis after PTA. In the diabetic state, hyperglycemia, which can enhance intimal hyperplasia by induction of platelet hypersensitivity, dyslipidemia, and dysregulation of expression of chemotactic factors (Barbieri et al. 2013, Zeadin et al. 2013), is potentially related to the process of restenosis. However, conflicting results have been reported with respect to the role of hyperglycemia in the pathogenesis of restenosis (Aronson et al. 1996, Park et al. 2001, Mazeika et al. 2003, Carter 2004, Lindsay et al. 2007, Lavi et al. 2008, Saxon et al. 2008).

To assess the effects of hyperglycemia on restenosis after PTA, we developed models of restenosis in New Zealand white rabbits (Chatterjee et al. 2014). Diabetes was induced by injection of alloxan. Stenosis rate, intima–media thickness, cell proliferation rate, and their relationship with glucose levels were analyzed. Rabbit models of atherosclerosis were also established. The effects of hyperglycemia on models of restenosis and atherosclerosis were studied.

Materials and methods

Animals and treatments

Three-to-four-months-old male New Zealand white rabbits were obtained from the Animal Center of Shandong Agriculture Science Academy, China. All rabbits were allowed free access to food and water during the whole study. The rabbits were allowed to acclimatize for at least 7 days, and then were fed with a high-cholesterol diet (1% cholesterol) thereafter. All animal care and experimental procedures were in accordance with the guide for the care and use of laboratory animals published by the Chinese National Institutes of Health, and the protocol was approved by the ethical committee of Shandong University Medical School. All surgical procedures were performed under anesthesia with sodium pentobarbital.

All rabbits were randomly divided into four groups: the diabetic restenosis group, nondiabetic restenosis group, diabetic atherosclerosis group, and nondiabetic atherosclerosis group. After 1 week of a high-cholesterol diet, 24 rabbits received injections of alloxan (80 mg/kg) via their ear veins to induce type 1 diabetes. As blood glucose levels should become stable within a week or so (Haddock et al. 1991, Hao et al. 2013), blood glucose concentrations were measured 1 week after injection of alloxan. Sixteen rabbits with fasting plasma glucose levels of over 300 mg/dl were considered to be diabetic, and divided into two groups: the diabetic atherosclerosis group ($n=8$) and the diabetic restenosis group ($n=8$). Another 16 age-matched rabbits which received injections of saline into their ear veins instead of alloxan were used as nondiabetic controls, and assigned to the nondiabetic atherosclerosis group ($n=8$) and the nondiabetic restenosis group ($n=8$). No exogenous insulin and hypoglycemic agents were given to these animals during the whole study period.

Surgical procedures for production of models of restenosis and atherosclerosis

One week after the injection of alloxan (80 mg/kg) or saline mentioned above, we started to develop models of restenosis and atherosclerosis in both diabetic and nondiabetic rabbits.

Restenosis models  

Rabbits were anesthetized with phenobarbital. An arteriotomy in the right saphenous artery was made with standard surgical techniques (Fig. 1A). A 2.5-mm, wire-guided balloon catheter was inserted into the iliac artery (the length was about 15 cm) through the incision (Fig. 1B). The injury consisted of three gentle tractions of the balloon catheter at 8, 10, and 12 atm to ensure complete endothelial denudation. At the end of the procedure, the balloon catheter was withdrawn, the vessel was ligated, and the surgical site was closed by a simple continuous intradermal pattern with a 4-0 polydioxanone suture. After surgery, the rabbits received i.m. injections of 800 000 units of penicillin daily for 3 days. In order to induce restenotic plaque, PTA was performed if the iliac artery developed severe atherosclerotic damage (about 4 weeks after the first surgical procedure), which was confirmed by ultrasound examination (Visualsonics, Toronto, ON, Canada) (Fig. 1E). After anesthesia, a midsagittal incision was made in the dissected distal ends of the right femoral artery (Fig. 1C). A 2.5-mm, wire-guided balloon catheter was inserted into the narrow iliac artery and the balloon was inflated to 12 atm three times for 5 s each time at the site of stenosis (Fig. 1D). After the dilatation, we removed the catheter and ligated the femoral artery. The rabbits received an i.m. injection of penicillin at the dose of 800 000 units daily for 3 days. Another color Doppler ultrasonogram was performed to demonstrate the patency of the stenosed iliac arteries after PTA (Fig. 1F).
Atherosclerosis models
On the same day, as the rabbits in restenosis groups underwent PTA surgery, balloon-induced injuries of the right iliac arteries (with no plaques, Fig. 1G and H) were performed on rabbits in nondiabetic and diabetic atherosclerosis groups to develop models of atherosclerosis.

Tissue harvesting and histological processing
For assessment of neointimal hyperplasia, the animals were killed under anesthesia on day 28 after the last surgical procedure. Blood samples were collected from the ear veins and HbA1c levels were evaluated using a commercial diagnostic kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The injured iliac arteries were cut into 3 mm pieces. Five segments were fixed in 10% neutral buffered formalin solution, embedded in paraffin, and processed for histopathological analysis. The sections (5 μm) were stained with hematoxylin and eosin (HE) for general appearance, Masson’s trichrome for collagen, and elastic van-Gieson dye for elastin and observed by microscopy.

Histomorphometric analysis was performed as described previously (Ali et al. 2007). Total vessel area and the area of lumen, media, and neointima were measured using Image Pro-Plus Software (Image Pro-Plus 6.0, Media Cybernetics, Silver Spring, MD, USA).

Intimal growth was estimated from the ratio of the intimal area to the area bounded by the internal elastic lamina (luminal cross-sectional area narrowing) and the ratio of intimal to medial areas.

Immunohistochemical analysis
Tissue sections (4 μm) deparaffinized in xylene and rehydrated through graded alcohol washes were incubated with 1% H2O2 in methanol for 10 min to block endogenous peroxidase activity. The sections were then incubated overnight in a humid chamber at 4°C with primary antibody against: proliferating cell nuclear antigen (PCNA; Merck, Millipore, Darmstadt, Germany) and α-smooth muscle actin (Abcam, Inc., Cambridge, MA, USA). After washing with 1× PBS, sections were incubated with biotinylated anti-mouse secondary antibody (Zhongshan, Beijing, China) for 30 min. Diaminobenzidinetetrahydrochloride was used to visualize the staining. The samples were counterstained with hematoxylin before addition of coverslips. The primary antibodies were omitted for negative control sections.

Figure 1
(A) After isolated, the distal saphenous artery was ligated. A small arteriotomy (Art) was made in the proximal saphenous artery. (B) A balloon catheter (BC) was advanced into the femoral artery and iliac artery (after topical application of lidocaine). Then three gentle tractions of the BC at 8, 10, and 12 atm were made to ensure complete endothelial denudation. (C) Four weeks after the first surgery, a midsagittal incision was made in the dissected distal ends of the femoral artery. (D) A BC was inserted into the narrowed iliac artery and inflated at 12 atm three times for 5 s each at the site of stenosis. (E) Four weeks after Balloon-induced endothelial injuries, severe iliac artery stenosis were formed in models of restenosis. (F) Results of color Doppler ultrasonography demonstrated the patency of the stenosed iliac arteries after PTA in models of restenosis. (G and H) Results of color Doppler ultrasonography demonstrated blood flow through normal arteries in models of atherosclerosis when severe iliac artery stenosis were formed in models of restenosis.
PCNA-positive cells were counted in ten randomly selected 200× high-power fields under a microscope. The PCNA index was calculated according to the following formula: number of PCNA-positive cells/total cell count × 100%.

**Statistical analyses**

All data are presented as mean ± s.d. Comparisons were made using one-way ANOVA with SPSS Software (version 20.0, SPSS China). A value of \( P<0.05 \) was considered statistically significant.

**Results**

**Animals**

The experiment was carried out using 40 New Zealand white rabbits. Baseline nonfasting blood glucose levels were normal in all animals (Table 1). Of the 24 alloxan-treated rabbits, 16 developed hyperglycemia; eight were excluded due to inadequate glucose levels. After all surgical procedures, three animals (two in the diabetic atherosclerosis group and one in the diabetic restenosis group) died due to severe hyperglycemia. Three rabbits (two in the nondiabetic atherosclerosis group and one in the nondiabetic restenosis group) died during ultrasound examination and three (one in the nondiabetic restenosis group and two in the diabetic atherosclerosis group) died of thrombosis. Before harvesting of tissue, six rabbits remained in the nondiabetic atherosclerosis group, four in the diabetic atherosclerosis group, six in the nondiabetic restenosis group, and seven in the diabetic restenosis group. Rabbits in the diabetic groups had a slight weight gain, while the levels of HbA1c were notably higher than those of rabbits in the nondiabetic groups (Table 1). The blood glucose levels of rabbits in each group are given in Table 1.

**Effects of hyperglycemia on rate of stenosis and intima/media areas of the atherosclerotic and restenotic plaques**

The samples were collected from the iliac arteries of rabbits undergoing different treatments. A progressive increase in intimal cross-sectional area of the iliac arteries was detected in all four groups (Fig. 2A).

The rate of stenosis in the diabetic restenosis group (92.33 ± 5.46%) was not significantly different from the nondiabetic restenosis (89.17 ± 7.14%) group (\( P>0.05 \)). Whereas the diabetic atherosclerosis group showed a higher stenosis rate (72.37 ± 24.55%) than the nondiabetic atherosclerosis group (35.54 ± 40.97%) \( (P<0.05) \) (Fig. 2B). The calculated intima/media ratio results were consistent with stenosis rates (Fig. 2C). The intima/media ratio in the diabetic restenosis group was 2.39 ± 0.43 compared with 2.13 ± 0.88 in the nondiabetic restenosis group \( (P>0.05) \), and 1.55 ± 0.47 in the diabetic atherosclerosis group compared with 0.37 ± 0.43 in the nondiabetic atherosclerosis group \( (P<0.05) \).

**Effects of hyperglycemia on cell proliferation in the atherosclerotic and restenotic plaques**

A large number of PCNA-positive cells were detected in the intima and media, and the cell arrangement was extremely disordered. The PCNA index in the diabetic atherosclerosis group was significantly higher than that in the nondiabetic atherosclerosis group \( (P<0.05) \). In contrast, in both the diabetic and nondiabetic restenosis groups, cell proliferation was both markedly increased in the restenotic plaques and no significant difference was found between these two groups (Fig. 3).

**Smooth muscle cells content changes**

As revealed by immunohistochemical staining for \( \alpha \)-smooth muscle actin (a marker for smooth muscle cells (SMCs)), the number of SMCs was higher in restenotic plaques than in

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of animals. Blood glucose levels were measured 1 week after injection of alloxan</th>
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</thead>
<tbody>
<tr>
<td><strong>New Zealand white rabbits</strong></td>
<td><strong>NDA (n=6)</strong></td>
</tr>
<tr>
<td><strong>Weight (g)</strong></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>2160.4 ± 216.6</td>
</tr>
<tr>
<td>At death</td>
<td>3143.0 ± 426.4</td>
</tr>
<tr>
<td><strong>Glucose (mg/dl)</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>104.76 ± 12.24</td>
</tr>
<tr>
<td><strong>HbA1c (ABS/10 g)</strong></td>
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<td></td>
<td>26.24 ± 3.32</td>
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</tbody>
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\*\( P<0.05 \) versus control group in each diabetic model. NDA, nondiabetic atherosclerosis rabbits; DA, diabetic atherosclerosis rabbits; NDR, nondiabetic restenosis rabbits; DR, diabetic restenosis rabbits; HbA1c, glycosylated hemoglobin; ABS, absorbance.
atherosclerotic plaques. In samples from both diabetic and nondiabetic models, large number of SMCs were observed in the intima of restenotic plaques, whereas fewer SMCs were found in the atherosclerotic plaques (Fig. 4).

**Discussion**

Restenosis after PTA in diabetic patients with PVD is an unsolved clinical issue. Factors contributing to restenosis remain poorly defined. In this study, we investigated the effects of hyperglycemia on restenosis in two sets of diabetic rabbit models: restenosis and atherosclerosis models. We found that i) build-up of atherosclerotic plaques was much faster in diabetic rabbits than in nondiabetic rabbits; ii) formation of restenotic plaques in diabetic restenosis rabbits was not different from that in nondiabetic restenosis rabbits and iii) numbers of PCNA-positive cells in diabetic atherosclerotic plaques were higher than those in nondiabetic atherosclerotic plaques; whereas in diabetic restenotic and nondiabetic restenotic plaques, numbers of PCNA-positive cells were not significantly different. These findings indicate that hyperglycemia is a risk factor for atherosclerosis but there was no evident effect on the process of restenosis.

Hyperglycemia, a hallmark of diabetes, has been well accepted as a major risk factor for atherosclerosis (Barbieri et al. 2013, Nagareddy et al. 2013). In addition, it might potentiate the response to arterial injury and be involved in multiple steps in the process of restenosis. In this study, we confirmed that hyperglycemia is closely correlated with atherosclerosis; however, it had no obvious effect on restenosis.

Restenosis is a secondary injury to atherosclerotic vessels. Restenotic lesions cause structural changes in the arteries. The mechanisms underlying the formation of restenotic plaques may be different from those for the primary atherosclerotic plaques. Atherosclerosis is an inflammatory process, during which endothelial cells, monocytes, and T lymphocytes interact with each other. Hyperglycemia can accelerate inflammatory processes via the activation of multiple signaling pathways, and finally leads to the development of atherosclerosis. Whereas inflammation does not appear to be essential in the development of restenosis, results from previous studies indicated that there are almost no white blood cells (WBCs) in restenotic plaques (Roque et al. 2002, Edlin et al. 2009). Therefore, hyperglycemia, which accelerates the process of atherosclerosis mainly by promoting
inflammatory responses, may have little effect on the development of restenosis.

So what accounts for rapid formation of restenotic plaques? Unlike atherosclerotic plaques in which WBCs and inflammation are major mediators (Roque et al. 2002, Edlin et al. 2009, Barbieri et al. 2013, Nagareddy et al. 2013), restenotic plaques is typically hypercellular with foci of vascular SMCs and extracellular matrix (ECM) (Zargham 2008, Muthiah et al. 2014) (Fig. 4). In the acute phase of restenotic plaques, arterial injury evokes loss of the contractile phenotype in the tunica media, leading to migration of SMCs from the media toward the intima. Then the presence of these SMCs result in the intimal thickening of the restenosis via the excessive synthesis of ECM and cell proliferation (Zargham 2008). Thus, the migration of vascular SMCs from the media to the intima may be the principal step in the development of restenosis (Marx et al. 2011), and restenotic lesion-activated specific signaling pathways leading to the migration of SMCs may play essential roles in this process.

In addition, we found a strong trend toward a higher stenosis rate and cell proliferation activity in the non-diabetic restenosis group compared with the diabetic atherosclerosis group, indicating that migration of SMCs to arterial plaques may play a key role in the pathogenesis of restenosis.

In this study, we used alloxan to establish models of type 1 diabetes and observed only the effects of hyperglycemia on restenosis. The influence of hyperinsulinemia and insulin resistance on restenosis was not investigated. Insulin has several biological activities, which may be related to the process of restenosis. Therefore, future studies are needed with models of type 2 diabetes. In addition, due to the complex surgeries and the long observation time, there were only four animals alive in the diabetic atherosclerosis group, which may weaken the statistical power to some degree.
In summary, we demonstrated that hyperglycemia plays a critical role in the development of atherosclerosis, but has little effect on restenosis after PTA, indicative of distinct mechanisms for these processes. Further studies are needed to explore the mechanisms underlying the formation of restenosis in both animal models and human.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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