A new rat model of diabetic macrovascular complication

Céline Bouvet a, Wouter Peeters b,1, Simon Moreau a,1, Denis deBlois c, Pierre Moreau a,⁎

a University of Montreal, Montreal, Québec, Canada
b Department of Nephrology, University Medical Center, Utrecht, The Netherlands
c Department of Pharmacology, Faculty of Medicine, University of Montreal, Québec, Canada

Received 10 May 2006; received in revised form 16 October 2006; accepted 1 November 2006
Available online 3 November 2006
Time for primary review 21 days

Abstract

Objectives: Age-related medial calcification (elastocalcinosis) of large arteries is accelerated in diabetes and appears mainly in distal arteries. The aim was to devise a rat model of elastocalcinosis in association with diabetes to examine the hypothesis that diabetes accelerates vascular calcification experimentally.

Methods: Male Wistar rats received a high fat diet during 2 months followed by a low dose of streptozotocin to induce diabetes (D). Elastocalcinosis was facilitated by 3 weeks of treatment with warfarin and vitamin K (WVK). We started WVK treatment 1 week (D4WVK) and 4 weeks (D7WVK) after the injection of streptozotocin and in age-matched healthy rats. Measurements of hemodynamic and metabolic parameters, aortic and femoral calcium content, and immunohistochemistry for alkaline phosphatase, osteopontin, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β were performed.

Results: Three weeks of WVK treatment alone did not increase the calcium content in the aorta and femoral arteries. However, in the D7WVK group, femoral calcification, but not aortic calcium content, increased significantly as compared to the WVK group. This response was not observed in the D4WVK group. In femoral arteries, strong immunostaining for alkaline phosphatase and osteopontin was observed in the D7WVK group. TNF-α and TGF-β expressions were mainly localized in the adventitia of arteries from diabetic rats.

Conclusion: We have established a model of accelerated elastocalcinosis in diabetes related to its duration and localized in distal arteries. The modification of local protein expression is also in accordance with clinical data, suggesting that this model could be useful to investigate mechanisms related to this important clinical macrovascular complication of diabetes.

© 2006 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Diabetes; Elastocalcinosis; Arteriosclerosis; Alkaline phosphatase; Osteopontin

1. Introduction

The prevalence of diabetes is estimated to be 2.8% of the global population, gradually reaching epidemic proportions [1]. Patients with type 2 diabetes present a 2–3 fold increased risk of cardiovascular diseases as compared to the general population [2]. Diabetes induces several complications, including vascular alterations that represent the principal cause of morbidity and mortality. One of the macrovascular complications associated with diabetes is an increase of vascular stiffness, as evidenced by reduced arterial compliance [3], and enhanced pulse wave velocity (PWV), pulse pressure (PP) and systolic blood pressure (SBP) [4,5]. In fact, the elevation of PP occurs approximately twenty years earlier in diabetic patients as compared to non-diabetics [6].

Medial elastocalcinosis (MEC or medial arterial calcification) differs from intimal calcification associated with advanced atherosclerotic lesions. It is characterized by a deposition of calcium-containing minerals on a fragmented...
the severity of diabetes [10] and, at least in early stages, it moreover, the extent of MEC is related to the duration and the severity of diabetes [10] and, at least in early stages, it appears to be mainly located in lower limbs [11]. Although vascular calcification was previously believed to be a passive phenomenon, there is mounting evidence suggesting that it is a regulated process involving the expression of mineralization-regulating proteins in the vascular wall [12–14]. For example, osteopontin (OPN), a noncollagenous protein binding calcium and hydroxyapatite in bone and teeth, is present in calcified atherosclerotic lesions, as well as in MEC [15]. Furthermore, Shanahan et al. demonstrated that alkaline phosphatase expression, an enzyme responsible for hydroxypatite formation, was markedly elevated in intimal and medial forms of calcification [16]. In addition, osteopontin, type I collagen, bone sialoprotein, osteonectin (SPARC), osteocalcin and alkaline phosphatase were reported to be upregulated and strongly associated with medial calcification in diabetes [17,18]. Matrix Gla protein (MGP) is a noncollagenous protein preventing the deposition of calcium minerals in the vascular wall [19,20]. Its activity depends on the γ-carboxylation of glutamic acid residues, a vitamin K-dependent process. Warfarin, which inhibits the recycling of vitamin K, has been shown to lead to MEC by inhibiting MGP activation [20]. Phylloquinone (vitamin K1) administration allows the carboxylation of MGP in the arterial wall [21]. The relevance of the warfarin-vitamin K1 model (WVK) to diabetic patients comes from the observation that MGP is reduced in arteries from these patients [18]. However, this model does not exhibit the metabolic abnormalities found in diabetes. The aim of the present study was to evaluate the effect of combining experimental diabetes with WVK administration in order to examine the hypothesis that diabetes accelerates vascular calcification.

2. Methods

2.1. Treatments

Male Wistar rats (3–4 weeks old) were obtained from Charles River Breeding Laboratories (St-Constant, Qc, Canada). They were fed a high fat diet containing 45 kcal % fat (soybean oil and lard that included 0.95 mg cholesterol by g of lard), 35 kcal % carbohydrates and 20 kcal % protein (Research diets, D12451) ad libitum during 8 weeks, followed by a single low dose of streptozotocin (STZ, 30 mg/kg intra-peritoneally). Rats were then allowed to develop diabetes for 4 (D4) and 7 (D7) weeks. Fig. 1 depicts the treatment protocols. Half of these groups were treated with the calcification regimen for the last 3 weeks. Thus, warfarin (20 mg kg$^{-1}$ day$^{-1}$ in drinking water) and vitamin K (phylloquinone, 15 mg kg$^{-1}$ day$^{-1}$ sub-cutaneous injection) were administered, starting one (D4WK) or 4 weeks (D7WK) after STZ injection. Dosages were adjusted every second day. At the end of treatments, rats were 16 (D4, D4WK) and 19 (D7, D7WK) weeks old. Controls consisted of age-matched untreated rats (because the values for each parameter were not statistically different, they were pooled for presentation clarity). In addition, 13 week-old rats were treated for 3 weeks with a WVK treatment (WVK group).

The Animal Care and Use Committee of Université de Montréal approved all animal experiments that comply with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Hemodynamic parameters

Twelve hours before sacrifice, food was removed. Animals were anesthetized with pentobarbital (65 mg/kg) during 8 weeks, followed by a single low dose of streptozotocin (STZ, 30 mg/kg intra-peritoneally). Rats were then allowed to develop diabetes for 4 (D4) and 7 (D7) weeks. Ctrl: control; WVK: 3 week warfarin/vitamin K treatment; D4: rats studied 4 weeks after the administration of STZ; D4WK: D4 rats also treated with WVK for the last 3 weeks; D7: rats studied 7 weeks after the administration of STZ to induce diabetes; D7WK: D7 rats also treated with WVK for the last 3 weeks. *: P<0.05 vs respective control without WVK; †: P<0.05 vs WVK group. ANOVA followed by Bonferroni’s correction for multiple comparisons.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Weight (g)</th>
<th>Glucose (mmol/L)</th>
<th>Insulin (ng/mL)</th>
<th>Cholesterol (mmol/L)</th>
<th>Triglycerides (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>18</td>
<td>476±9</td>
<td>4.45±0.26</td>
<td>1.35±0.10</td>
<td>2.88±0.21</td>
<td>0.38±0.02</td>
</tr>
<tr>
<td>WVK</td>
<td>14</td>
<td>470±7</td>
<td>5.14±0.40</td>
<td>1.60±0.17</td>
<td>2.39±0.21</td>
<td>0.41±0.07</td>
</tr>
<tr>
<td>D4</td>
<td>10</td>
<td>521±14</td>
<td>7.44±0.88</td>
<td>0.66±0.19</td>
<td>1.62±0.24</td>
<td>0.16±0.06</td>
</tr>
<tr>
<td>D4WK</td>
<td>12</td>
<td>495±26</td>
<td>11.40±1.81 †</td>
<td>0.84±0.27 †</td>
<td>1.89±0.30</td>
<td>0.34±0.03</td>
</tr>
<tr>
<td>D7</td>
<td>9</td>
<td>463±29</td>
<td>19.90±1.62</td>
<td>0.10±0.02</td>
<td>2.43±0.64</td>
<td>0.49±0.03</td>
</tr>
<tr>
<td>D7WK</td>
<td>9</td>
<td>440±11</td>
<td>17.25±1.49 †</td>
<td>0.24±0.04 †</td>
<td>0.94±0.11 †</td>
<td>0.23±0.03*</td>
</tr>
</tbody>
</table>

Fig. 1. Schematic representation of chronic treatments in rats. Open bars represent a normal diet, gray bars a high fat diet and diagonals represent the warfarin/vitamin K treatment. The vertical dashed lines represent the injection of streptozotocin.
and short catheters (polyethylene-50, approx. 10 cm, Folio-plast SA, Sarcelles, France) were inserted into the distal abdominal aorta through the left femoral artery and into the aortic arch through the left carotid. Catheters were connected to a pressure transducer to allow the measurement of systolic (SBP) and diastolic blood pressure (DBP) at each location. Mean arterial blood pressure (MBP), carotid and femoral pulse pressures (PP) were calculated from these parameters. Pulse wave velocity (PWV) was measured by the foot-to-foot method, as previously described [22]. The femoral catheter was used to collect arterial blood. Finally, the aorta and the femoral arteries were harvested. Portions were frozen at −80 °C for calcium amount. A section of femoral arteries was fixed in 4% cacodylate-buffered paraformaldehyde and embedded in paraffin blocks for immunohistochemistry.

2.3. Metabolic parameters

Before sacrifice, glycemia was quantified on a sample of arterial blood with a glucometer (AccuSoft Advantage, Roche, Laval, Qc, Canada). A 1 mL blood sample was collected in a tube containing EDTA. Plasma was extracted by a 10 min centrifugation at 3000 rpm. Insulinemia was measured with a radioimmunoassay kit (Rat insulin RIA kit, Linco Research, St-Charles, MO, USA) and cholesterol and triglyceride amounts with a colorimetric kit (Cholesterol and Triglycerides Infinity, ThermoElectron corporation, Melbourne, Australia).

2.4. Vascular composition

To measure calcium content, portions of aorta and femoral arteries were dried at 55 °C and calcium was extracted with 10% formic acid (30 μL/mg of dry tissue) overnight at 4 °C. The colorimetric quantification was achieved through a reaction with o-cresolphtalein (Teco Diagnostics, Anaheim, CA, USA).

For immunohistochemistry, sections of paraffin-embedded femoral arteries (7 μm thickness) were mounted on glass slides and deparaffinized with xylene, followed by hydration in graded concentrations of ethanol. Each tissue section was incubated overnight at room temperature with primary antibodies. In order to assess the phenotypic changes, antibodies against either tissue non-specific alkaline phosphatase (TNAP, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or osteopontin (OPN, courtesy of Dr Larry W. Fisher, National Institutes of Health, MD, USA) were used. Elastin degradation was evaluated with an antibody against TGF-β (Santa Cruz Biotechnology Inc. Santa Cruz, Ca, USA) and inflammation was estimated with an antibody against tumor necrosis factor-alpha (TNF-α, US biological, Swampscott, MA, USA). After washing, primary antibodies were incubated with appropriate secondary antibody conjugated to biotin.

![Fig. 2. Effect of 3 weeks of warfarin/vitamin K (WVK) treatment on aortic (A) and femoral calcium content (μg/mg of dry tissue). Open circles: control and WVK groups; Closed circles: D4 and D4WVK groups; Closed squares: D7 and D7WVK. *: P<0.05 vs without WVK; †: P<0.05 vs WVK group (open circle). Fig. 1 represents the treatment protocols. The same X-axis scale was used to allow visual comparisons between the two arteries.](https://academic.oup.com/cardiovascres/article-abstract/73/3/504/367858)

![Fig. 3. Von Kossa staining for calcification in femoral arteries from WVK (A) and D7WVK (B). L: lumen; M: media; A: adventitia. Arrows point to calcification nuclei.](https://academic.oup.com/cardiovascres/article-abstract/73/3/504/367858)
Biotin was detected with a Vectastain ABC-AP kit (Vector Laboratories Inc, Burlingame, CA, USA) and color development was achieved with Fast Red TR/Naphthol AS-MX phosphate alkaline phosphatase substrate with 1 mM levamisole. Adjacent slices were stained with the von Kossa protocol to localize calcium deposition. Slices from the tibia of untreated rats were used as positive controls for phenotypic changes and TGF-β, while the spleen of rats treated with LPS served as a positive control of inflammation. For negative controls, the primary antibody was omitted.

2.5. Drugs and statistical analysis

All drugs were purchased from Sigma Chemical Co. (Oakville, On, Canada) unless specified otherwise. Values are expressed as mean ± SEM. An ANOVA followed by Bonferroni’s correction was used to compare the groups. Since our aim was to determine the effect of diabetes on calcification, a priori-selected comparisons were: WVK group compared to respective control (without WVK group) and diabetes+WVK compared to WVK alone. Since 5 comparisons were selected, \( P \) had to be smaller than 0.01 \( (0.05/5) \) in individual comparisons to reach significance.

3. Results

Two months of high fat diet associated with an injection of a low dose of STZ induced a significant increase of glycemia, which progressed from 4 to 7 weeks after STZ injection (Table 1). Although insulinemia was mildly reduced after 4 weeks of diabetes, rats that were studied after 7 weeks of diabetes exhibited a marked reduction of plasma insulin levels. In general, the WVK treatment did not modify these metabolic responses (Table 1). Cholesterol was not elevated in rats receiving the high fat diet (diabetic groups). However, WVK treatment modified the cholesterol profile in an inconsistent manner: cholesterol was reduced by WVK in non-diabetic rats, but elevated in 4-week diabetic rats. Triglycerides were elevated only in rats having diabetes for 7 weeks and this was abrogated by WVK treatment (Table 1).

Three weeks of WVK treatment resulted in a small but not significant elevation of calcium content in the aorta that reached significance in the D7WVK group (Fig. 2A). Furthermore, femoral elastocalcinosis was not elevated by WVK alone, but it was significantly increased in rats that had diabetes for 4 weeks before starting the WVK treatment (D7WVK group, Fig. 2B). In contrast, one week of diabetes prior to WVK treatment (D4WVK group) did not produce calcification. Von Kossa staining revealed that femoral calcification in the D7WVK group was localized in the media, with a dotted distribution (Fig. 3). These calcification spots appeared adjacent to elastin filaments.

Immunohistochemistry of TNAP revealed that this enzyme was not detectable in femoral sections from control rats (Fig. 4). Seven weeks of diabetes (D7 group) or
3 weeks of WVK (WVK group) produced a slight but consistent staining for alkaline phosphatase. Femoral arteries from rats of the D7WVK group revealed a marked staining throughout the arterial wall. Osteopontin staining demonstrated a similar pattern: mild staining in WVK or diabetic rats, but more intense staining in the D7WVK group (Fig. 5). Interestingly, OPN staining appeared more intense in the adventitia of femoral arteries during diabetes. TGF-β was not detected in control rats but it was strongly expressed in the adventitia of WVK, D7 and D7WVK rats. An occasional and weak coloration was detected in the intima or in the media of D7 and D7WVK rats (data not shown). Finally, TNF-α was not expressed in control and WVK rats, but slightly expressed in D7 and D7WVK rats (data not shown).

Central and peripheral hemodynamic parameters were not modified by any treatment (data not shown). Pulse wave velocity, an index of aortic stiffness, was not elevated at this early time-point following WVK treatment.

4. Discussion

To our knowledge, the experiments presented in this study represent the first experimental evidence that diabetes accelerates vascular calcification. In addition, the present study suggests that the model has important similarities with the human pathological condition.

4.1. Metabolic parameters

Our first objective was to select a model of diabetes that shares similarities with the human condition of type 2 diabetes, since vascular calcification normally occurs later in life. Type 2 diabetes is associated with a reduction of insulin sensitivity and a gradual impairment of pancreatic β cell function. By the time fasting hyperglycemia appears and diabetes is diagnosed, insulin resistance and reduction of β cell function have already occurred [23]. The insulin resistance phase is characterized by a normal glycemia and compensatory hyperinsulinemia. In our model, the administration of the high fat diet induced an insulin resistance state (elevated insulinemia and HOMA, data not shown), confirming the results obtained by other groups working with rats [24] and mini swine [25]. The administration of STZ aimed at slightly reducing the β cell function to obtain hyperglycemia, as shown by Zhang et al [24]. It has been demonstrated that a 30 mg/kg dose of STZ has minimal effect on pancreatic function [26] and was used to trigger diabetes in our study. After 4 weeks of diabetes, insulinemia decreased slightly below control values and rats were clearly hypoinsulinemic at 7 weeks. The transition to hypoinsulinemia occurred very rapidly and the relevance of this accelerated β cell dysfunction to the human condition, where this transition is much slower, is not known. More importantly, the duration of hyperinsulinemia was, if any,
very limited in time. Thus our model focused exclusively on the effect of hyperglycemia rather than on the combined effect of hyperglycemia and hyperinsulinemia. In any case, our model recapitulates the sequence of events observed in type 2 diabetes in a very short time frame, although, 7 weeks after STZ administration, diabetes could also be considered of type 1. This is at variance with the study of Zhang, which showed sustained normoinsulinemia [24]. The dose of STZ used in the current study may have been slightly elevated. As suggested by Srinivasan et al., there is a narrow dose window between obtaining hyperglycemia on high fat diet-fed while maintaining hyperinsulinemia (above 25 mg/kg of STZ) and overt insulin deficiency (above 35 mg/kg) [27]. Nonetheless, the elevation of fasting glycemia supports the notion that this model is relevant for the study of diabetes effects on vascular calcification, although it may not be fully relevant to study the physiopathology of diabetes. Along these lines, our rats did not show increased triglycerides and cholesterol levels, contrary to human type 2 diabetic patients and other animal models [24,27]. This could be explained by a weight loss after the injection of STZ [28]. This apparent normal lipid metabolism should not influence our results since medial calcification is not associated with lipid deposition.

4.2. Vascular calcification and hemodynamic parameters

In order to induce MEC, rats were treated with warfarin and vitamin K. We previously demonstrated that 4 weeks of WVK treatment induced a 10-fold increase in aortic calcium content, which was associated with an elevation of PWV [22]. However, 2 weeks of WVK treatment was not associated with aortic calcification (unpublished observation). In order to study the acceleration of MEC, rats were treated during 3 weeks, when minimal calcification was expected. Indeed, WVK induced only a 2-fold increase in aortic calcification. Diabetes did not amplify or accelerate the development of MEC in the aorta. The lack of modification of hemodynamic parameters, including normal aortic PWV, is consistent with this modest aortic calcification. Prolongation of the WVK treatment beyond these 3 weeks leads to elevation of PWV (data not shown), as seen in patients with type 1 and type 2 diabetes [6,29].

Clinical reports on diabetic patients have revealed that MEC is most commonly observed in lower limbs and is associated with a 5.5-fold rate of amputations [10]. Indeed, a five-year follow-up study of patients with newly diagnosed type 2 diabetes demonstrated a higher incidence of lower limbs vascular calcification [11]. Moreover, Edmonds et al. observed that diabetic patients with neuropathy present MEC more often in feet [30]. For these reasons, MEC was also evaluated in femoral arteries from our treatment groups, although it is important to note that femoral circulations in human and in rats are different, with greater pressures in man due to the upright position. As in the aorta, 3 weeks of WVK treatment did not produce significant calcification of femoral arteries. However, when the same treatment was initiated after 4 weeks of diabetes, significant medial calcification was observed in femoral arteries. Thus, diabetes seems to accelerate the development of MEC. This was not the case after one week of diabetes, suggesting that the development of MEC is related to the duration of diabetes. This is in line with clinical observations in Pima Indians, showing an effect of diabetes duration on the incidence of macrovascular complications [10]. Interestingly, calcification occurred in the media closer to the lumen of the arteries, while in the WVK model, calcification normally occurs closer to the adventitia [31]. This suggests that distinct mechanisms adding to the MGP inhibition could be involved in diabetes.

4.3. Expression of osteogenic proteins, TGF-β and TNF-α

Arterial calcification in general and MEC in particular have been associated with the expression of bone-related proteins, suggesting that cells are not passive bystanders. In diabetes, the expression of alkaline phosphatase has been shown to be elevated in Mönckeberg’s sclerosis, a form of medial calcification [18]. Tissue non-specific alkaline phosphatases are important for the mineralization process. Indeed, for hydroxyapatite generation, Ca2+ and P_i are required and TNAP generates inorganic phosphate from pyrophosphate [32]. As in human diabetic arteries, TNAP was expressed in diabetic rats presenting vascular calcification and in femoral arteries of rats treated with WVK even if calcium accumulation was not yet significant. This suggests that the enzyme is probably involved in the initiation of the calcification process, which is coherent with the role of alkaline phosphatase in calcification [33]. A similar pattern was observed with OPN, another protein invariably expressed in areas of vascular calcification [34], and more specifically in calcified diabetic arteries [18,35]. Interestingly, the increase of OPN expression was more marked in the adventitia. Zhang et al. showed that streptozotocin-induced diabetes caused an important inflammatory response in the adventitia [24]. Moreover, inflammation stimulates bone-related protein expression [36]. We have previously reported that staining for ED-1, a macrophage marker, was negative in the WVK model, partially ruling out cellular infiltration and overt inflammation [15]. However, since diabetes is associated with inflammation, TNF-α expression was assessed to determine its potential impact on calcification. In contrast to arteries from non-diabetic rats (control and WVK), TNF-α staining was present in the arterial wall from diabetic animals and remained mainly unaffected by the addition of the WVK treatment. In addition to inflammation, TGF-β, through Smads signaling molecules, is also a stimulus for bone-related protein expression [37]. Since high glucose conditions enhance TGF-β signaling [38], we tested its local expression. Similarly to TNF-α expression, TGF-β was detected in the adventitia of diabetic groups or rats (D7 and D7WVK), but also in WVK-treated rats. The weak expression of TGF-β in the media of femoral arteries of rats treated with WVK is in line with elastin degradation.
during calcification [39], but our observation time-point may be too early to observe significant shedding. Taken together, our results seem to highlight a role of the adventitia in medial calcification, especially in diabetes. This conclusion is in line with a recent report by Shao et al [40]. TNF-α and TGF-β expressions could explain why OPN and TNAP were also observed in femoral arteries of diabetic rats without calcification. In general, the observations made in this experimental model in terms of phenotypic changes in femoral arteries mimic the changes observed in vessels of patients with medial calcification.

In conclusion, we have devised a new rat model of accelerated elastocalcinosis induced by diabetes that shares the following similarities with the human condition: 1) calcification is present earlier in the diabetic rats than in normal rats treated with WVK, 2) it occurs first in a distal localization (femoral arteries) and 3) it is positively associated with the duration of diabetes. Finally, calcified vessels present phenotypic changes that are consistent with what can be found in human arteries from diabetic patients. This new model will offer new possibilities to elucidate and understand mechanisms implicated in diabetes-accelerated elastocalcinosis.

Acknowledgments

The authors acknowledge the skilful technical assistance of Louise Ida Grondin. The Canadian Institutes for Health Research (CIHR) funded the study. CB receives a student-fellowship from the Fonds de la recherche en santé du Québec.

References


