Cardiovascular disease has been recognized as the most common complication of type II diabetes mellitus (DM). Although hyperinsulinemia, insulin resistance and the abnormal lipoprotein profile have important roles in macrovascular disease development, considerable controversy exists regarding the process of arterial wall damage. Nevertheless, most studies have examined aortic damage in an animal model of type I DM and in diagnosed DM patients, as it has been difficult to investigate serial changes in aortic wall morphology and function at the prediabetic stage in an animal model or in humans. Otsuka Long-Evans Tokushima Fatty (OLETF) rats, as an animal model of type II DM, were characterized by late onset of hyperglycemia (after 20 weeks of age) and diagnosable DM by oral glucose tolerance test (OGTT) from 25 weeks of age, reduction in insulin level from 8 weeks of age, and insulin resistance of the peripheral tissues from 12 weeks of age, and inheritance by males and contribution of sex hormones to the onset of DM. In addition, recent advances in high resolution intravascular ultrasound mean that intraluminal size can be measured in conjunction with direct determination of pressure, measurements that enable evaluation of vessel distensibility in vivo. 

In the present study, we investigated the serial changes in aortic wall stiffness using intravascular ultrasound, histopathology and metabolic disorders, such as hyperglycemia, hyperinsulinemia and aortic wall collagen accumulation, during the process of DM development in OLETF rats.

**Key Words:** Arteries; Collagen; Remodeling; Type II diabetes mellitus; Ultrasound

**Methods**

Thirty, male OLETF rats, established as models of spontaneous long-term hyperglycemia with diabetic complications, were used as the experimental subjects. Thirty male Long-Evans Tokushima Otsuka (LETO) rats, which were developed from the same colony by selective mating but do not develop DM, were used as control animals. All animals were maintained at the Kagawa Medical University animal experiment center from 5 weeks of age. They were kept in a pathogen-free facility under controlled temperature (23±2°C) and humidity (55±5%) with a 12-h artificial light and dark cycle, and given free access to standard laboratory rat chow (MF, Oriental Yeast Corp, Tokyo, Japan) and tap water. All procedures were in accordance with institutional guidelines for animal research.

**Experimental Protocol**

At the ages of 15 (n=10 per group) and 30 (n=10 per group) weeks, OLETF and LETO rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal injection). A heparin-mixed, saline-filled catheter (P-50) was canulated from the left carotid artery to the thoracic aorta to measure arterial blood pressure. Intravascular ultrasound images and aortic pressure were recorded and the stiffness parameter was calculated. The aortic walls were excised at 5, 15 and 30 weeks for histopathology and the measurement of hydroxyproline. At 10 weeks, blood glucose (mg/dl) and insulin concentrations (ng/ml) of the OLETF rats (2h; 168±30 and 0.82±0.15) were significantly high (nonDM: 118±15; p=0.02 and 0.16±0.64; p=0.003). At the prediabetic stage (15 weeks), in the OLETF rats (2.5±0.9) was larger than in nonDM rats (1.4±0.4; p=0.0006), and the collagen (hydroxyproline) content/dry weight (mg/g) of the aortic wall was significantly higher in OLETF (33.5±3.1) than in nonDM rats (28.7±3.5; p<.05). Histopathological examination showed that from 15 weeks of age the medial wall thickness increased gradually. In the prediabetic stage, collagen accumulation may contribute to impairment of aortic wall stiffness in the OLETF rats, which would accelerate the aging process in the aortic wall. (Jpn Circ J 1999; 63: 988–993)
Calculation of Aortic Wall Stiffness

Intravascular ultrasound images, as replayed, were input into a Clear View system (Cardiovascular imaging system, Boston Scientific Corp) after completion of the protocol. For each intravascular echogram, we identified the endo-thelium–blood border and traced this surface to measure lumen diameter. For each recording, measurements of short-axis diameter were performed on the frames identified as the largest or smallest areas by 2 observers. All significant difference in aortic wall morphology was observed between the 2 groups. The cyclic variation of aortic dimension was analyzed for evaluation of the wall stiffness. P, artifact of probe.

Table 1 Hemodynamics During Intravascular Ultrasound

<table>
<thead>
<tr>
<th></th>
<th>15 weeks</th>
<th>30 weeks</th>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OLETF</td>
<td>104±14</td>
<td>117±5</td>
</tr>
<tr>
<td>LETO</td>
<td>101±7</td>
<td>119±21</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OLETF</td>
<td>79±17</td>
<td>99±5</td>
</tr>
<tr>
<td>LETO</td>
<td>90±2</td>
<td>102±23</td>
</tr>
<tr>
<td>Pulse pressure (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OLETF</td>
<td>25±15*</td>
<td>18±2</td>
</tr>
<tr>
<td>LETO</td>
<td>10±8</td>
<td>17±8</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OLETF</td>
<td>343±57</td>
<td>271±46*</td>
</tr>
<tr>
<td>LETO</td>
<td>338±53</td>
<td>361±17</td>
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</table>

Systolic lumen area (mm²) | OLETF | LETO |
<table>
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<tbody>
<tr>
<td>4.3±1.1</td>
<td>4.1±0.8</td>
<td></td>
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<tr>
<td>Diastolic lumen area (mm²)</td>
<td>5.4±1.1</td>
<td>5.4±1.0</td>
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</table>

Data are presented as mean±SD; n=10 for all groups. *p<0.05 vs age-matched LETO rats.

Histopathological Examination

Specimen Preparation The thoracic aorta was excised at 5, 15 and 30 weeks of age for histopathology using hematoxylin-eosin staining (n=10 per group), and later at 15 and 30 weeks of age for intravascular ultrasound examination (n=10 per group). The excised aortic wall was divided into 2 parts, which were used for histopathological examinations and for measuring collagen content. The specimens for histopathology were fixed in formalin solution, embedded in paraffin and cut into 4-μm thick sections.

Morphometric Analysis Histomorphometric measurements were performed on the images using a Macintosh computer (Apple Corp), into which lit microscopic images stained by hematoxylin-eosin were input directly via a 3-CCD video camera (Victor, Tokyo, Japan). Medial wall thickness was measured as the distance between the intimal and adventitial leading edges on the images at ×100 magnification, and vessel and lumen areas were measured on the images at ×20 magnification. Vessel and lumen areas were measured by tracing the adventitia-media borders and intimal surfaces, respectively. Medial wall area was calculated as vessel area – (lumen area), and the ratio of medial to vessel area was calculated as (medial area)×100 / (vessel area). The nuclear numbers of smooth muscle cells per visual field were measured on the images at ×400 magnification. Measurements of medial wall thickness and the nuclear number of the smooth muscle cells were performed at 3 different sites on each specimen and the average values were used for analysis.

Collagen Content

Hydroxyproline concentration in the aortic wall was measured as an indicator of collagen accumulation. Each specimen was dried and hydrolyzed by adding 6 mol/L HCl at 110°C for 24h. Hydrolysates were transferred to flasks and dried in a desiccator overnight to remove hydrochloric acid by evaporation. After dissolving in 2 ml of 0.02 mol/L

Fig 1. Intravascular ultrasound cross-sectional images of the systolic lumen area of aorta in OLETF and LETO rats. Systolic lumen area was identified as the largest lumen area and diastolic lumen area was the smallest lumen area during the cardiac cycle. No significant difference in aortic wall morphology was observed between the 2 groups. The cyclic variation of aortic dimension was analyzed for evaluation of the wall stiffness. P, artifact of probe.
HCl, solutions in 50 μl aliquots were applied to the amino acid analyzer (Amino Acid Analyzer Model 835; Hitachi) to determine the hydroxyproline concentration. Because hydroxyproline is incorporated only into collagen and assuming that collagen contains 14% hydroxyproline, the total collagen content (mg/g dry weight) can be calculated.\textsuperscript{12,13}

**Statistics**

All values are expressed as mean ± standard deviation. Statistical analysis was performed using Statview software (Power PC version, Abacus Concepts Inc). Comparisons of values were done between the age-matched OLETF and LETO rats using the Mann-Whitney U test. Statistical comparisons of serial changes were performed by one-way analysis of variance (ANOVA). A probability (p) value of <0.05 was considered statistically significant.

**Results**

**Stiffness of Aortic Wall**

The hemodynamics during intravascular ultrasound are shown in Table 1. At 15 weeks of age, the stiffness parameter $\delta$ in OLETF rats (2.5±0.9) was significantly higher than that in LETO rats (1.4±0.4, p=0.0006). No significant difference between OLETF (2.1±0.6) and LETO (1.9±0.9) rats was observed at 30 weeks of age. The change in stiffness parameter $\delta$ from 15 to 30 weeks of age was significant in LETO rats (p=0.05), but was not significant in OLETF rats.
**Histopathological Examination**

At 5 weeks of age, neither medial wall thickness nor medial wall area were significantly different between the 2 groups. At 15 and 30 weeks of age, the medial wall thickness and medial wall area of the OLETF rats gradually increased and was significantly greater than that of LETO rats. Medial wall area to vessel area ratios in OLETF rats increased gradually and were significantly larger than that in LETO rats at 15 weeks. Nuclear number/area in both groups gradually decreased with aging and that in the OLETF rats tended to be smaller at 15 and 30 weeks. No atherosclerotic plaque was observed from 15 to 30 weeks of age (Figs 2, 3).

**Collagen Content**

At 5 weeks of age, no significant difference in collagen content was observed between OLETF and LETO rats. At 15 weeks of age, the collagen content was significantly higher in the OLETF rats compared with the LETO rats. At 30 weeks of age, there was no significant difference between the 2 groups. In OLETF rats, the collagen content markedly increased from 5 to 15 weeks of age with no changes from 15 to 30 weeks of age. In LETO rats, the collagen content tended to increase gradually, but was not significantly changed from 5 to 30 weeks of age (Fig 4).

**Oral Glucose Tolerance Test**

Hyperglycemia at baseline was not observed in either group at 10 and 20 weeks of age. However, the baseline insulin levels of OLETF rats were significantly higher at 10 weeks of age. After oral glucose loading, the blood glucose levels and the plasma insulin levels in OLETF rats were significantly higher than those in LETO rats. These increases were more prominent at 20 weeks of age, which means that the OLETF rats had been hyperinsulinemic and insulin-resistant from 10 weeks of age. At 30 weeks of age, the high glucose levels of OLETF rats were prolonged 2 h after loading, and the insulin levels at both baseline and at 2 h after loading were significantly higher than those of LETO rats (Fig 5).

**Body Weight and Metabolic Features**

The body weights of OLETF rats were significantly
Interobserver and Intraobserver Variability

The comparison of lumen area measurements on intravascular ultrasound images obtained by the 2 independent observers demonstrated a high correlation, as did the 2 separate measurements by the single observer. The mean difference between observer 1 and observer 2 was 0.01 mm² with a 95% confidence interval of –0.07 to 0.09 mm², and that between measurement 1 and measurement 2 was 0 mm² with a 95% confidence interval of –0.07 to 0.07 mm².

Table 2  General Characteristics of OLETF and LETO rats

<table>
<thead>
<tr>
<th></th>
<th>5 weeks</th>
<th>15 weeks</th>
<th>30 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OLETF</td>
<td>87±5</td>
<td>457±54*</td>
<td>576±65*</td>
</tr>
<tr>
<td>LETO</td>
<td>85±3</td>
<td>378±30</td>
<td>447±30</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OLETF</td>
<td>40±16</td>
<td>37±37</td>
<td>65±8*</td>
</tr>
<tr>
<td>LETO</td>
<td>20±8</td>
<td>20±15</td>
<td>16±8</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OLETF</td>
<td>134±6</td>
<td>93±13</td>
<td>132±11*</td>
</tr>
<tr>
<td>LETO</td>
<td>136±6</td>
<td>92±9</td>
<td>86±8</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD; n=10 for all groups. *p<0.05 vs age-matched LETO rats.

greater than those of LETO rats from 15 weeks of age. Total cholesterol and triglyceride were not significantly different between the 2 groups at 15 weeks of age, but at 30 weeks of age, both the total cholesterol and triglyceride of the OLETF rats were significantly higher than those of the LETO rats. However, the value of total cholesterol in OLETF rats at 30 weeks of age was not significantly different from that in LETO rats at 5 weeks, which may be within normal range (Table 2).

Interobserver and Intraobserver Variability

The comparison of lumen area measurements on intravascular ultrasound images obtained by the 2 independent observers demonstrated a high correlation, as did the 2 separate measurements by the single observer. The mean difference between observer 1 and observer 2 was 0.01 mm² with a 95% confidence interval of –0.07 to 0.09 mm², and that between measurement 1 and measurement 2 was 0 mm² with a 95% confidence interval of –0.07 to 0.07 mm².

Discussion

In patients with DM, complications of atherosclerosis and an alteration in arterial wall stiffness have been previously demonstrated. In the present study, OLETF rats were used as a model of type II DM, and an alteration in wall stiffness could be demonstrated even at the prediabetic stage. To our knowledge, this is the first study that provides serial data on dynamic aortic wall elastic properties from the prediabetic stage in an animal model developing type II DM, along with data on glucose and lipid metabolism, aortic wall collagen content and histopathology.

Stages of DM in OLETF Rats

Based on data from the OGTT, from 10 to 20 weeks the OLETF rats showed postprandial hyperglycemia and relatively higher plasma insulin levels both at baseline and at 2 h after loading compared with age-matched LETO rats. At 30 weeks of age, the high glucose levels, at 2 h, of the OLETF rats were prolonged, which indicated the onset of DM in the OLETF rats at 30 weeks of age. According to previous reports and based on the present data, our subjects were prediabetic, but revealed insulin resistance at 10–20 weeks, and diagnostable DM at 30 weeks.

Remodeling of the Aortic Wall

In the present study, an increase in aortic wall mass in conjunction with an increase in collagen content/dry tissue weight was demonstrated at 15 weeks of age, which was in the stage of prediabetes and insulin resistance. However, the level of collagen content/dry tissue weight of the aorta in OLETF rats did not change at 30 weeks, whereas in LETO rats it tended to slightly increase from 5 to 30 weeks of age. We think that at 30 weeks of age (when the rats are adult), aortic fibrosis and collagen deposition may be related to aging and that the key processes involved in aortic wall remodeling are the natural history of normal rats. Although absolute collagen volume in the aortic wall might be increasing in OLETF rats from 15 to 30 weeks of age, the aortic collagen content/dry weight did not change, which may result from cellular hypertrophy or an increase in another wall constituent. This hypothesis was supported by the reduction in the nuclear number of smooth muscle cells per area.

Alteration in Aortic Wall Stiffness

The elastic properties of the walls of large arteries, especially the aorta, were evaluated by the pressure-diameter relationship, and that relationship was fitted to a logarithmic model, as previously applied. We, therefore, used the stiffness parameter as an index independent of the pressure changes for evaluating wall elasticity.

Arterial wall elasticity is regulated by variations in the structural composition of the wall, including elastic fiber, collagen fiber and vascular smooth muscle tone. Turnover of collagen accumulated in the aortic wall is slow, and may reduce arterial elasticity before morphological changes in the wall can be detected. In the present study, the arterial wall elasticity in OLETF rats reduced from the prediabetic stage and an increase in collagen content was simultaneously observed. Consequently, accumulation of collagen is an important factor in the reduction of arterial wall elasticity in the prediabetic stage.

As a physical factor regulating vessel morphology, wall tension computed as (intraluminal pressure x radius) / (wall thickness) is important. The ratio of medial area to vessel area calculated histopathologically in the present study is equivalent to the reciprocal of the ratio of radius/thickness, and was slightly higher in OLETF than in LETO rats at 15 weeks of age. Therefore, the tension loading on the aortic wall became reasonably small in OLETF rats, which means that the increase in wall thickness itself may be one of the factors reducing aortic stiffness in OLETF rats.

The alteration in aortic wall elastic properties in the prediabetic stage of DM is thought to be related to wall remodeling induced by hyperinsulinemia. Subsequently, aortic stiffness is impaired by the changes in medial wall contractual components with increasing collagen accumulation and without proliferation of smooth muscle cells during hyperinsulinemia and in the normal fasting blood glucose stage of DM.

Study Limitation

The limitation of the present study was the time resolution of the intravascular ultrasound, which has a 1800rpm transducer rotation speed. The frame rate of 30 frames/s of the instrument used yielded only 4–6 images per cardiac cycle at the tachycardia rate (400–300 beats/min) of the study animals. We, therefore, selected carefully the frames with the largest or smallest lumen areas. Using this protocol, we could analyze the relationship between arterial pressure and lumen size and evaluate any alteration in aortic elastic properties.
Clinical Implications

In this study the animal subjects were as young as 5 weeks of age. The serial data of body weight, blood glucose, plasma insulin concentration, triglyceride, cholesterol, aortic stiffness, and histopathology were obtained from 5 until 30 weeks of age, which has implications for the optimal timing of interventional measures. Those individuals who have a genetic background of DM, even though they are young and asymptomatic, should be included in interventional procedures to prevent the development of type II DM and its complications.

Conclusion

In the prediabetic stage of type II DM, OLETF rats showed alterations in aortic wall stiffness as compared with LETO rats. This change can be tracked longitudinally with intravascular ultrasound and is accompanied by serological and pathological changes in the aorta. These changes may be a result of metabolic abnormalities, or a simultaneous alteration parallel to the metabolic abnormalities in genetic DM subjects, and may accelerate the aging process in the aortic wall.

References