Abstract. Plaque rupture and subsequent embolism as well as thrombosis are major causes of acute myocardial infarction and stroke secondary to atherosclerosis. Pai-1, t-PA, TF and ET-1 are thrombosis- and thrombolysis-related factors which play important roles in thrombosis formation and plaque rupture. Since acute myocardial infarction and stroke are more likely to occur between 6 a.m. and 12 p.m. than at another time of the day, we studied the relationship between circadian rhythm and Pai-1, t-PA, TF and ET-1 in normal and atherosclerotic mice. Atherosclerosis was developed in apoE-/- mice fed a normal diet or a high cholesterol diet. The expression of Pai-1, t-PA, TF and ET-1 in the hearts of control C57BL/6J mice and atherosclerotic mice was measured by real-time RT-PCR at different Zeitgeber times (ZT) including ZT0, ZT4, ZT8, ZT10, ZT12, ZT14, ZT16 and ZT20. The expression of Pai-1, t-PA, TF and ET-1 peaked between ZT14 and ZT16 and bottomed at ZT10 in C57BL/6J mice. Their expression in apoE-/- mice fed a normal diet lost circadian rhythm. Their expression in apoE-/- mice fed a high cholesterol diet peaked at ZT4, indicating a reverse circadian rhythm. Our result indicates that circadian changes in the expression of Pai-1, t-PA, TF and ET-1 may be involved in the onset of myocardial infarction and stroke.

Introduction

Acute myocardial infarction and stroke are major complications associated with atherosclerosis. A number of studies have established a relationship between circadian rhythm and these complications. They are more likely to occur between 6 a.m. and 12 p.m. than at another time of the day (1,2). Plaque rupture and subsequent embolism as well as thrombosis are the major causes of these life-threatening complications (3-6). Plaque ruptures in response to local or systemic change in the coagulation system. Several local modulators of plaque rupture are involved in coagulation (7). The factors in the fibrinolytic system such as tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI), increase in advanced human plaques (9). For a systemic perspective, increased plasma levels of PAI-1 (10), tissue factor (11) and tissue plasminogen activator (12) indicate an increased risk of cardiovascular events. In addition, the mechanical stress acting on the plaque and vessel wall plays an important role in plaque rupture (13). For example, endothelin-1 (ET-1) may increase the vessel tone and cause plaque rupture (14).

The present study therefore aimed to explore whether and how Pai-1, t-PA, TF and ET-1 are related to the circadian rhythm in atherosclerotic mice.

Materials and methods

Animals and diet. Twenty-four male C57BL/6J mice ~12 weeks old were purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Science. Forty-eight male apoE knock-out mice ~12 weeks old were purchased from the Department of Laboratory Animal Science Animals of Peking University Health Science Center. The mice were maintained in a daily 12:12 light/dark cycle for 4 weeks. C57BL/6J mice were fed with a normal chow diet. ApoE-/- mice were divided into two groups and fed a normal chow diet and a western-type diet (containing 0.15% cholesterol and 21% fat), respectively. According to Zeitgeber time (ZT) (ZTO is defined as lights-on time and ZT12 as lights-off time), mice were sacrificed at different time points including ZT0, ZT4, ZT8, ZT10, ZT12, ZT14, ZT16 and ZT20. All animal experiments were carried out based on the criteria of the Medical Laboratory Animal Administrative Committee of Shanghai.
Lillie-Ashburnes Oil Red O staining. Mice were deeply anesthetized with 20% ethyl carbamate. The aorta root and heart were rapidly isolated. For frozen sections, the arch of each aorta was removed, stored in buffered formalin (10%) and dehydrated in 20% sucrose solution. Then the aortic segments were embedded in OCT. Cross-sectional serial sections with a 20-μm thickness were prepared for staining. Lillie-Ashburnes oil red O staining was used to show atherosclerotic plaques.

RNA isolation and real-time PCR. The heart was harvested, frozen in liquid nitrogen and stored at -70°C until RNA isolation. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA were reversely transcribed and amplified using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada). The relative mRNA level was measured by real-time PCR using SYBR-Green Realtime PCR Master Mix (Invitrogen, Carlsbad, CA). Two micrograms of total RNA were reversely transcribed and amplified using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada). The relative mRNA level was measured by real-time PCR using SYBR-Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) with SYBR-Green I. Specific primer pairs for t-PA, Pai-1, TF or ET-1 were designed based on published data in GenBank and are listed in Table I.

Statistical analysis. Data are expressed as the means ± SEM and evaluated by one-way ANOVA analysis and the Games-Howell post-test using SPSS 11.5 software. A probability value <0.05 was considered statistically significant.

Results

Formation of atheromatous plaque in the aorta of apoE knock-out mice. Lillie-Ashburnes Oil Red O staining of frozen sections showed no obvious plaque in the aorta root of apoE−/− mice fed a normal chow diet although foam cells were observed under the endothelium. Obvious atheromatous plaques were observed in the aorta root of apoE−/− mice fed a high cholesterol diet for 4 weeks. Oil Red O staining showed obvious lipid depositions in the plaques (Fig. 1).

Daily expression of t-PA, Pai-1 and TF mRNA. The t-PA mRNA in C57BL/6J mice peaked at ZT14 (P<0.01, between ZT0 and ZT14). In apoE−/− mice fed a normal diet, an animal model of the early stage of atherosclerosis, the t-PA expression showed no significant daily circadian rhythm (P>0.05). In apoE−/− mice fed a high cholesterol diet, however, the circadian rhythm of t-PA mRNA reversed. The expression of t-PA peaked at ZT4 (P<0.01 between ZT4 and ZT20) instead of ZT14. At ZT4, the t-PA in the heart of apoE−/− mice fed with a western-type diet was higher than in other groups. In contrast, at ZT14, t-PA in C57BL/6J mice was highest (Fig. 2).

The circadian rhythm of Pai-1 in the hearts of C57BL/6J mice oscillated (Fig. 3) with a clear peak at ZT14 (P<0.01 between ZT0 and ZT14). However, no significant daily fluctuation was observed in apoE−/− mice fed a normal diet. Similar to t-PA, Pai-1 in apoE−/− mice fed a western-type diet was highest at ZT4. At ZT0, Pai-1 mRNA level in apoE−/− mice fed with a western-type diet was higher than that in C57BL/6J mice (P<0.05) or apoE−/− mice fed a normal diet (P<0.01). At ZT8, ZT14 and ZT16, apoE−/− mice fed a normal diet expressed less (P<0.05) Pai-1 than C57BL/6J mice or apoE−/− mice fed a western-type diet. The circadian rhythm curves of C57BL/6J mice and apoE−/− mice fed crossed between ZT10 and ZT12.

The rhythmic expression of the TF gene in the hearts of C57BL/6J mice peaks at ZT14. In the hearts of apoE−/− mice fed a normal diet or a western-type diet, TF circadian expression peaked at ZT20 (P<0.05 between ZT4 and ZT 20) and ZT4 (P<0.01 between ZT4 and ZT20), respectively. At ZT4, TF expression in apoE−/− mice fed a normal diet was highest while at ZT14, TF expression in C57BL/6J mice was highest. At ZT10, ZT12, ZT16 and ZT20, apoE−/− mice fed a western-type diet showed a reduction in the mRNA expression level when compared with that in C57BL/6J mice (Fig. 4).

Daily expression of ET-1 mRNA. A significant circadian rhythm of ET-1 expression was observed in the hearts of all three groups with peaks at ZT16 in C57BL/6J mice, ZT14 in apoE−/− mice fed a normal diet and ZT4 in apoE−/− mice fed a western-type diet. At ZT16, ET-1 expression levels stratified from high to low were found in C57BL/6J mice, apoE−/− mice fed a normal diet and apoE−/− mice fed a western-type diet; while at ZT4, the order was reversed (Fig. 5).

Table I. Primer pairs used to amplify PCR products.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Product size</th>
<th>Annealing temperature</th>
<th>GenBank accession no.</th>
</tr>
</thead>
</table>
| t-PA  | Forward: TGACCGGGATACATGGGAG  
Reverse: CTGAGTTGCGATTGTACCCAGCC | 176 bp | 60˚C | NM_008872 |
| Pai-1 | Forward: CAAGCTCTCCGACTATGGTG  
Reverse: CACTGAGTAGGGGGCATTAC | 176 bp | 65˚C | NM_008871 |
| TF    | Forward: GGACACGGGAGCTGAGAATGG  
Reverse: GCTTGAAGCCTTTCGATAAGTAA | 162 bp | 65˚C | NM_010171 |
| ET-1  | Forward: GCACCGGAGGCTGAAAGTG  
Reverse: GTGGCAGAAGTGAGACACCTC | 119 bp | 65˚C | NM_010104 |
| GAPDH | Forward: ACAGCCGCATCTTCTTGTGACTG  
Reverse: GGCTTGTAGCTGTGGCGTGAATTT | 226 bp | 55˚C | BC_083149 |
Myocardial infarction and stroke are severe complications secondary to atherosclerosis that claim a significant number of lives each year. Local and systemic coagulation disorders are responsible for atherosclerotic plaque ruptures causing these complications. ApoE−/− mice fed with either a normal diet or a western-type diet are proven animal models of atherosclerosis. Our results showed that obvious atheromatous plaque formed in 3-month male apoE−/− mice fed a western-type diet for 4 weeks. Three-month male apoE−/− mice fed a normal diet for 4 weeks meanwhile, had no obvious plaque although many foam cells were observed under the endothelium. Studies showed that atheromatous plaque formation started at 5-6 weeks and formed obvious fibrous plaque at 20 weeks in apoE−/− mice fed a normal diet (15). Therefore, in our investigation, apoE−/− mice fed a normal diet for 4 weeks were at the early stage of atherosclerosis. ApoE−/− mice fed a western-type diet for 4 weeks were considered to be at the advanced stage of atherosclerosis. The biological clock composes several positive and negative transcriptional-translational feedback loops.
including the genes Per, Bmal1, Clock, Cry, Rev-Erbα and CKIε (16). The biological clock is thought to be located in the suprachiasmatic nucleus (SCN) of the hypothalamus (17) and peripheral tissues. The rhythm of the peripheral clock is regulated directly or indirectly by various neural, hormonal and other signaling molecules under the control of the central clock in SCN (18). In addition to clock genes, many other genes show circadian oscillations and are called clock-controlled genes. There are more than 400 genes expressed rhythmically in the hearts of mice (19). Circadian clock genes and clock-controlled genes change in various animal models of cardiovascular disorders. Phase alterations of the clock genes in the heart (bmal1, per2) were demonstrated in streptozotocin-induced diabetic rats (20). In the rat heart with pressure-overload hypertrophy (21), circadian expression of clock-controlled genes (dbp, anp) is significantly reduced.

Myocardial infarction and stroke are more likely to occur in early morning. It may result from the circadian variation in fibrinolytic activity. The circadian fluctuation of fibrinolytic activity, which has lower activity in the morning and higher activity in the evening, has been observed in both healthy individuals and patients with acute or chronic coronary artery disease (22-24). Plasminogen activator inhibitor (PAI-1) is one of the major inhibitors of fibrinolytic activity which contributes to fibrin-rich thrombus formation after plaque disruption and contributes to the progression of atherosclerotic lesions (27-29). In the present study, we found a clear circadian rhythm in the hearts of all three groups. The amount and circadian rhythm of PAI-1, t-PA, TF and ET-1 changed in atherosclerotic lesions (14). Hanai et al (30) found that the ET-1 gene tends to express in rat heart during the night. We confirmed ET-1 rhythmic expression in C57BL/6J mice with a peak at night (ZT16). Its level in atherosclerotic mice decreased in the dark and increased under light. The robust expression increase in the morning in the atherosclerotic mice fed a western-type diet may explain why more cardiovascular events happen in the morning.

In summary, we found that the expression and circadian rhythm of PAI-1, t-PA, TF and ET-1 changed in atherosclerotic mice. The result indicates a role of thrombotic and fibrinolytic factors in the development of atherosclerosis, subsequent plaque rupture and even the onset of myocardial infarction. Further investigations should be conducted to unveil the profound mechanism.

Acknowledgements
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References


