Summary. Elevated plasma levels of C-reactive protein (CRP) are associated with increased risk of cardiovascular disease. CRP immunoreactive protein is also detected in the lesions of atherosclerosis. However, it is not known whether the CRP contents of atherosclerotic lesions are associated with the initiation and progression of atherosclerosis. To examine this hypothesis, we investigated different types of atherosclerotic lesions of rabbits fed with a cholesterol-rich diet for 6, 12, 16, and 28 weeks and examined their relationship with CRP. We measured the aortic atherosclerotic area, macrophages, and smooth muscle cells along with CRP contents in the lesions. Atherosclerotic lesions of aortas began to form at 6 weeks and were characterized by accumulation of macrophages in the intima, and lesions became more fibrotic in the advanced stage. Both plasma CRP levels and the lesional CRP contents were associated with the lesion size. Our results suggest that plasma CRP, as well as lesional CRP, associated with the formation and progression of atherosclerotic lesions.

Keywords: Inflammation, Atherosclerosis, C-reactive protein, Rabbits, Hypercholesterolemia

Introduction

C-reactive protein (CRP) is a classic acute phase protein that has been widely considered as a biomarker of inflammation, infection and tissue damage (Shrive et al., 1996). CRP can also be used as a marker to predict cardiovascular events when combined with high levels of low-density lipoprotein (LDL) or low levels of high-density lipoprotein (HDL) levels in plasma (Ridker, 2003). It is well known that atherosclerosis is a form of chronic vascular inflammation involving abundant inflammatory factors. Several studies suggest that CRP is a crucial mediator for atherosclerosis because CRP is associated with modified LDLs and is involved in uptake of these lipoproteins by macrophages (Kruth, 2001; Zwaka et al., 2001; Libby, 2002; Libby and Ridker, 2004). These studies so far indicate that CRP may directly participate in the development of atherosclerosis, and elevated plasma levels of CRP may constitute a new risk factor for cardiovascular disease (Pasceri et al., 2000; Zwaka et al., 2001; Blaschke et al., 2004). This notion is supported by epidemiological studies showing that there is a close relationship between elevated levels of CRP and an increased risk of cardiovascular events in humans (Ridker et al., 1998, 2003). Furthermore, clinical studies showed that lipid-lowering drugs, statin, can reduce both atherogenic lipoproteins and plasma CRP (Kleemann et al., 2004; Nissen et al., 2005; Voleti and Agrawal, 2006). Pathological studies revealed that CRP is deposited in both human and animal atherosclerotic lesions (Hatanaka et al., 1995; Sun et al., 2005).

Nevertheless, the pathophysiological functional roles of CRP in the development of atherosclerosis have not been completely clarified. Although in vitro studies using different vascular cells showed that CRP may be atherogenic (Pasceri et al., 2000; Blaschke et al., 2004; Labarrere and Zaloga, 2004), animal experiments using either transgenic mice or rabbits failed to demonstrate any pro-atherogenic effects of CRP (Ridker et al., 2003; Koike et al., 2009; Teupser et al., 2011). In fact, it has not been clarified whether plasma or lesional CRP levels are associated with the initiation and progression of atherosclerosis. Because experimental mice do not have endogenous functional CRP, and exogenous human CRP failed to elicit an inflammatory response in mice, it is necessary to address CRP functions using alternative animal models (Siboo and Kulisek, 1978; Reifenberg et
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Materials and methods

Animals

Japanese white male rabbits (2.0-2.5 kg) were provided by the Laboratory Animal Center of Xi’an Jiaotong University. They were randomly divided into two groups: control rabbits (n=10) and cholesterol-diet rabbits (n=50). Control rabbits were fed with a standard chow diet (RM-4), containing 24.5% protein, 3.7% fat, and 13.4% fiber (Table 1) (Vital River, Beijing, China). Cholesterol-fed rabbits were fed with a chow diet containing 0.3% cholesterol and 3% corn oil. Cholesterol was added into the pellet diet by coating at 80°C with corn oil. All rabbits were fed with each diet ad libitum. Cholesterol-fed rabbits were euthanized at 6, 12, 16, and 28 weeks for the evaluation of aortic atherosclerotic lesions (from early-stage lesions to advanced lesions). Rabbits were monitored for plasma total cholesterol (TC) levels weekly and TC levels of cholesterol-fed rabbits were maintained at "atherogenic levels (800~1200 mg/dL)" by adjusting the cholesterol contents in the diet throughout the experiment. The experimental protocols were approved by the Xi’an Jiaotong University Laboratory Animal Administration Committee and performed according to the Xi’an Jiaotong University Guidelines for Animal Experimentation.

Plasma lipids and CRP

After overnight fasting, blood samples were collected via the auricular artery in tubes containing EDTA as an anticoagulant. Blood samples were centrifuged (3000 rpm, 15 min, 4°C) to collect plasma. Plasma TC and triglycerides levels and HDL-cholesterol (HDL-C) were measured using commercial assay kits (Dongou Bioengineering, Beijing, China). The area under the curve (AUC) of plasma TC was calculated to evaluate the degree of cholesterol exposure according to the trapezium rule (Liu et al., 2005). Lipoprotein profiles were determined by gradient sequential ultracentrifugation followed by agarose gel electrophoresis as previously described (Fan et al., 1998). Plasma CRP was measured using high-sensitive rabbit CRP ELISA kits (Immunology Consultants Laboratory, OR, USA) (Hasturk et al., 2007).

Quantification of gross atherosclerotic lesions

Aortic en face atherosclerosis was evaluated after aortic trees were stained with Sudan IV as described previously (Zhao et al., 2008; Zhang et al., 2010). Sudanophilic area was quantified using image analysis software (Pro Plus 6.0, Media Cybernetics, MA, USA) and expressed as percentage of the whole aorta.

Histology and immunohistochemistry

For the microscopic quantification of lesions, the aortic arch of each rabbit was cut into 8 to 10 sections (4 µm) as described previously (Zhang et al., 2010). To evaluate the microscopic lesion area of each aorta, all sections were stained with hematoxylin and eosin (H&E) and Elastica van Gieson (EVG) and measured by an image analysis system described below. For microscopic evaluation of cellular components in the lesions, serial paraffin sections of the aorta were immunohistochemically stained with the following antibodies (Abs) against macrophages (RAM11; dilution 100x; Dako, CA, USA), smooth muscle cells (SMC) (α-actin; dilution 200x; Thermo Fisher Scientific, CA, USA) and CRP (dilution 200x; Immunology Consultants Laboratory, OR, USA) (Engelmann et al., 2006; Huang et al., 2009). All sections were washed 3 times with PBS for 10 min. Endogenous peroxidase activity was blocked with incubation in 0.3% hydrogen peroxide for 10 minutes. Sections were again washed 3 times with PBS for 10 min. Sections were then incubated by primary Abs at 4°C overnight, followed by 3 times with PBS for 10 minutes. Secondary Abs included anti-murine IgG (Beijing Zhongshan Biotechnology, Beijing, China) for macrophage and α-actin staining and anti-chicken IgG (Beijing Zhongshan Biotechnology, Beijing, China) for CRP were incubated for 60 min at room temperature. Sections were washed 3 times with PBS for 10 minutes each and were reacted using an AEC kit (Beijing Zhongshan Biotechnology, Beijing, China).

Table 1. Nutrient composition of Chow and HCD Diets (g/100g).

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<thead>
<tr>
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<th>Chow diet</th>
<th>HCD</th>
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<tr>
<td>Crude protein</td>
<td>24.5</td>
<td>24.5</td>
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<tr>
<td>Crude fat</td>
<td>3.7</td>
<td>6.7</td>
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<tr>
<td>Crude fibre</td>
<td>13.4</td>
<td>13.4</td>
</tr>
<tr>
<td>Ash</td>
<td>8.5</td>
<td>8.5</td>
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<tr>
<td>Carbohydrate</td>
<td>49.9</td>
<td>49.9</td>
</tr>
<tr>
<td>Energy (kcal/g)</td>
<td>3.31</td>
<td>3.52</td>
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</tbody>
</table>
All sections (EVG and immunostaining) for microscopic quantification were captured under an Olympus BX51 light microscope equipped with a digital camera (Olympus, Tokyo, Japan) and were measured with Image-Pro Plus 6.0 image analysis software.

Statistical analysis

All data are expressed as the mean ± SD. Comparisons for multiple groups were conducted by one-way ANOVA followed by Bonferroni test. The correlations between CRP and lesion size were assessed using Pearson correlations. P<0.05 was considered statistically significant.

Results

Plasma TC and CRP levels

As shown in Figure 1 top, plasma TC levels were significantly increased at 2 weeks and remained at high levels throughout the experiment after rabbits were fed with a cholesterol-rich diet. HDL-C levels were decreased in cholesterol-fed rabbits after cholesterol feeding (Fig. 1, bottom). However, plasma triglyceride levels were not changed in cholesterol-fed rabbits compared to normal rabbits (data not shown). Analysis of lipoprotein profiles by agarose gel electrophoresis followed by Fat Red 7B staining revealed that cholesterol diet feeding led to the increased levels of VLDL, intermediate density lipoproteins (IDL), and LDL accompanied by reduced HDL levels (Fig. 1, right). The average plasma CRP levels at the baseline of normal rabbits were 26.02±9.4 mg/L, and after cholesterol diet feeding plasma CRP levels were gradually increased from 6 weeks (Fig. 2), which remained at significantly high levels from 6 to 28 weeks. At 28 weeks, the plasma CRP levels in the cholesterol-fed rabbits were increased by 8.7-fold compared to control rabbits (Fig. 2).

Analysis of atherosclerotic lesions

No spontaneous aortic atherosclerosis was observed in control rabbits but in cholesterol-fed rabbits (Fig. 3), the aortic lesions could be grossly seen in the aortic arch as early as 6 weeks of cholesterol diet feeding. Aortic lesions started to increase with the time of cholesterol feeding from the aortic arch to thoracic aorta.

Fig. 1. Plasma total cholesterol levels (left, upper), HDL-C levels (left, bottom), and lipoprotein profiles (right) of rabbits fed with either a chow diet or cholesterol-rich diet. Plasma lipids were measured as described in the Materials and Methods. Data are expressed as the mean ± SD. **: p<0.01 vs control. Plasma lipoprotein density fractions were isolated by ultracentrifugation and fractionated by agarose gel electrophoresis followed by neutrolipid staining.
Histological examinations revealed that the lesions of aortic arch were composed of macrophage-derived foam cells and a small number of smooth muscle cells by 12 weeks (Fig. 4A). Using EVG staining, we measured the intimal lesions under a light microscope. The intimal lesion area was gradually increased over the four points of experiment accompanied by increased immunoreactive CRP deposition (Fig. 4B). Immunohistochemical staining revealed that the intimal lesions of cholesterol-fed rabbits by 16 weeks were mainly composed of macrophages with a small number of smooth muscle cells. It is worth noting that at 28 weeks the lesions were characterized by increased smooth muscle cells by reduced macrophages, suggesting that these lesions are very fibrotic. Regardless of the lesions formed at different weeks, CRP-immunoreactive protein was consistently found in the intimal lesions: CRP was mainly localized in the extracellular matrix (Fig. 4A). CRP deposition was not observed in normal aorta of both cholesterol-fed and control rabbits.

**Correlation analyses**

To determine the possible physiological significance of plasma CRP levels in lesions in cholesterol-fed rabbits, we analyzed the relationship between CRP values and lesion area and cellular components. First, we found that plasma CRP levels were closely associated with intimal lesion size (Fig. 5 left) as well as lesional SMC contents (r=0.546, p<0.01). However, there was no correlation between plasma CRP and lesional macrophages and plasma TC levels.

Because lesional CRP may affect the progression of atherosclerosis, we also examined the relationship of lesional CRP contents with atherosclerotic lesions. Similar to plasma CRP, lesional CRP was strongly associated with not only intimal lesions (Fig. 5 right), but also AUC of plasma TC (r=0.707, p<0.01). Moreover, there was a weak correlation between lesion CRP and lesion SMC even though they were not statistically significant (r=0.403, p>0.05). In addition, lesional CRP contents were not correlated with the number of the lesion macrophages (r=-0.233, p>0.05) and the plasma CRP levels (r=0.345, p>0.05).

**Discussion**

Although it is still controversial regarding whether CRP is a maker or mediator for the development of atherosclerosis, it is essential to know its pathophysiological significance with the initiation and progression of the lesions. In this study, we generated different types of atherosclerotic lesions (from early stage to advanced lesions) in rabbits fed with a cholesterol diet for different periods (from 6 to 28 weeks). Cholesterol-fed rabbits developed hypercholesterolemia as well as elevated plasma CRP levels. Consistent with our previous studies, we found that the plasma levels of CRP were strongly correlated with the intimal lesion size of aortic arch, indicating that the CRP levels reflect the lesion progression (Sun et al., 2005).
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Fig. 4. Histological and immunohistochemical investigations of the lesions. Serial sections were stained with either HE or EVG or immunohistochemically stained with Abs against macrophage, smooth muscle α-actin, and CRP. Representative micrographs were shown (A) and quantitative data are expressed as the mean ± SD (B). *: p<0.05, **: p<0.01.

Fig. 5. Correlations between plasma CRP levels (left) and lesional contents with intimal lesion size (right).
Because plasma CRP is mainly produced by the liver in response to inflammatory mediators such as IL-6, a vicious circle of “the more lesions, the more cytokines from the lesions, the more CRP production” may occur. In addition, we found that plasma CRP levels are correlated with the lesional smooth muscle cells rather than macrophages. There are two possibilities for this correlation between plasma CRP and lesion SMCs. First, plasma CRP levels are accompanied by the lesion progression from macrophage-rich to SMC-rich lesions, suggesting that this correlation is not secondary. Secondly, it may suggest that CRP is possibly involved in the smooth muscle cell proliferation in the lesions or increased smooth muscle cells may facilitate the CRP deposition or binding in the lesions (Jabs et al., 2003; Vainas et al., 2003). This hypothesis remains to be verified. Neointimal proliferation is known to be a major contributor to vessel stenosis; therefore, it will be interesting to investigate whether CRP can modulate smooth muscle growth in future. In the current study, we also examined lesional CRP contents and found that similar to the plasma CRP, lesional CRP contents are also associated with lesion size. However, it should be mentioned that it is not possible to draw a conclusion whether increased lesional CRP contents lead to increased lesion size. Interestingly, lesional CRP contents were not correlated with plasma CRP but correlated with plasma TC levels. This result indicates the lesional size is largely determined by hypercholesterolemia and local CRP contents rather than plasma CRP levels. In other words, the risk of plasma CRP is much weaker than that of the CRP in the lesions or lesional CRP is independent of plasma CRP levels. Another question that has puzzled researchers for a long time is the source of lesional CRP (Jialal et al., 2006). In our previous study, we have demonstrated that CRP proteins found in the lesions of both human and rabbit are mainly derived from the circulation rather than synthesized de novo by vascular cells, and CRP mRNA expression in the intimal lesions was less than 1% of the liver (Sun et al., 2005). Nevertheless, a small amount of CRP produced by vascular cells in the lesions cannot be responsible for the increased plasma CRP levels, but they may account for the local CRP deposition with the progression of the lesions. It should be pointed out; however, the lesions at advanced stage (28 wks) are characterized by increased smooth muscle cells and decreased macrophages compared to the early stage lesions before 16 weeks. Because lesional CRP was not correlated with macrophage number, it is not likely that CRP is directly involved in lesion development. This notion is supported by our recent study using transgenic rabbits showing that lesional CRP did not affect macrophage accumulation and smooth muscle cell proliferation (Koike et al., 2009).

In summary, our data suggest that elevated CRP in both plasma and lesions is associated with lesion progression. Pathophysiological significance of the dynamic changes in both plasma and lesional CRP observed in cholesterol-fed rabbits deserves further investigation.

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Conflict of interests. None.

References


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