IMUNOHISTOCHEMISTRY METHOD TO DETECT C-REACTIVE PROTEIN IN ATHEROMA PLAQUES OF SPRAGUE DAWLEY RATS FED HIGH LIPID RATION

METODA IMMUNOHISTOKIMIA UNTUK MENDETEKSI C-REACTIVE PROTEIN DALAM PLAK ATEROMA PADA TIKUS *SPRAGUE DAWLEY* YANG DIBERI DIET TINGGI LEMAK

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ABSTRACT

Since Inflammation is believed to have role in pathogenesis of cardiovascular events, measurement of markers of inflammation has been proposed as a method to improve the prediction of the risk of these events. C-reactive protein (CRP) a major acute phase protein, has been associated with the presence and severity of atherosclerosis, and has been found to predict acute vascular events in prospective studies. The aim of this study was to analyse the relationship between appearance of CRP in aorta and atheroma plaque formation in Sprague Dawley rats after 59 days induction with high fat diet. Ten male Sprague Dawley rats, 1.5 months of age were used as experimental animals. Rats were adapted in 10 single cages for 5 days and given basal diet containing normal fat and water *ad libitum*. After adaptation, rats were divided in to 2 groups (group 1 and group 2) of 5 each. Group 1 was fed basal diet containing normal fat (4.5% of fat) and group 2 was fed diet containing high fat (20% of fat). After 59 days, all rats were killed, the heart including aorta were taken out for histophatologic (HE) and immunohistochemistry analyses. The result of this study showed that all rats in Group 1 did not have atheroma plaque and CRP negative, but rats in Group 2, all have atheroma plaque (100%) but only 60% CRP positive. From those result it concluded that although all aortas have atheroma plaque, the CRP may not be detected in the plaque.

Keywords: atheroma plaques, inflammation, C-reactive protein.

ABSTRAK

Radang diduga mempunyai peranan dalam patogenesis penyakit kardiovaskuler. Pengamatan terhadap pemicu terjadinya proses radang saat ini menjadi target utama yang diharapkan dapat digunakan untuk memprediksi kejadian penyakit tersebut. *C-reactive protein* (CRP) merupakan protein fase akut yang keberadaannya dikaitkan dengan kejadian aterosklerosis, dan karena kaitannya dengan proses radang, CRP diduga dapat dipergunakan sebagai sarana memprediksi kejadian penyakit kardiovaskuler. Tujuan penelitian ini adalah untuk menganalisis hubungan kemunculan CRP dalam aorta dan plak ateroma, pada tikus *Sprague Dawley*, setelah selama 59 hari diinduksi dengan diet mengandung lemak tinggi. Sepuluh tikus *Sprague Dawley* jantan, umur 1,5 bulan digunakan sebagai hewan coba. Tikus diadaptasikan terlebih dahulu dalam 10 kandang tunggal percobaan selama 5 hari dan diberi diet basal yang mengandung lemak normal, dan air *ad libitum*. Setelah adaptasi, tikus dibagi menjadi 2 kelompok (kelompok I dan kelompok II), masing-masing 5 ekor tikus. Kelompok I adalah tikus yang diberi diet basal mengandung lemak 4,5% dan kelompok II adalah tikus yang diberi diet lemak tinggi yaitu diet yang mengandung lemak 20%. Setelah 59 hari, semua tikus dimatikan, jantung termasuk aortanya diambil untuk pemeriksaan histopatologi dengan pengecatan HE dan CRP dengan metoda immunohistokimia. Hasil dari penelitian ini menunjukkan bahwa semua tikus yang diberi diet normal (Kelompok I) tidak ditemukan baik plak ateroma maupun CRP, sedangkan tikus yang diberi diet lemak tinggi (Kelompok II) 100% ditemukan plak ateroma pada aortanya, namun hanya 60% dapat diketahui CRP positif. Berdasarkan hasil yang diperoleh disimpulkan bahwa walaupun diet terbukti menyebabkan aterosklerosis (100%), namun CRP belum tentu dapat ditemukan dalam plak ateroma.

Kata kunci: Plak ateroma, radang, C-reactive protein

INTRODUCTION

Inflammation is an important pathogenic feature in atherosclerotic lesion formation. Cellular and humoral inflammatory responses are involved in the initiation and progression of atherosclerotic plaques (Hanssons, 1993). The majority of inflammatory cells infiltrating the arterial wall in early atherogenesis are monocytes, but the fact that hardly any neutrophils are present in the lesion (Hanssons, 1993).

C-reactive protein (CRP) belongs to the pentraxin family of proteins, so called because it has five identical subunits, encoded by a single gene on chromosome 1 (Reeves, 1998). It was so named because it reacts with the somatic C polysaccharide of *Streptococcus pneumoniae*. C-reactive protein is exclusively made in the liver and is secreted in increased amounts within 6 hours of an acute inflammatory stimulus. The plasma level can double at least every 8 hours, reaching a peak after about 50 hours (Reeves, 1998). C-reactive protein is the prototype acute-phase protein in humans. In the acute phase response, its plasma concentration can exceed the normal concentration by 1,000 fold (Gabay and Kushner, 1999).

Evidence is now accumulating to suggest that CRP may contribute to inflammation in atheroma and also may be actively involved in early atherogenesis. The protein displays Ca^{++}-dependent in vitro binding to LDL (Volanakis, 1982) and activates the complement system (Reynolds and Vance, 1987).

Although current study suggests that CRP is a stronger predictor of future cardiovascular events than LDL cholesterol, but its appearance in atherosclerotic lesion formation have yet been clearly reported. The aim of this study was to analyse the relationship between appearance of CRP in aorta and atheroma plaque formation in *Sprague Dawley* rats after 59 days induction with high fat diet, using immunohistochemistry, streptavidin-biotin method.

MATERIAL AND METHODS

Induction of atheroma plaques

Ten male Sprague Dawley rats, 1.5 months of age were used as experimental animals. Rats were adapted in 10 single cages for 5 days and given basal diet containing normal fat and water *ad libitum*. After adaptation, rats were divided in to 2 groups (group 1 and group 2) of 5 each. Group 1 was fed basal diet containing normal fat (4.5% of fat) and group 2 was fed diet containing high fat (20% of fat). After 59 days, all rats were killed, the heart including aorta were taken out for histopathology and immunohistochemistry analyses.

Histopathology

Histopathology of heart and aorta using haematoxilin and eosin staining method was done using standard method at Balai Besar Veteriner (BBV), Wates, Yogyakarta.

Antibodies

The polyclonal rabbit anti rat CRP antibody (used at 20 µg/ 200 µL) was purchased from Bio vision, USA. The secondary goat anti rabbit antibody and the streptavidin-biotin staining kit were purchased from Santa Cruz biotechnology, San Fransisco.

Immunohistochemical staining methods

Immunohistochemical staining for CRP was performed as described (Adji and Mulyata, 2004). All specimens (heart including aorta tissues) were deparafined, rehidrated, and incubated in phosphate buffer saline (PBS) for 10 minutes, after that specimes were incubated in H_2O_2 3% (in absolute methanol) for about 10 minutes. Tissues were incubated again in PBS for 10 minutes and then given blocking solution. Specimens then incubated in PBS for 10 minutes, given streptavidin peroxidase conjugate for 5 minutes, and incubated again in PBS for about 10 minutes. Specimens were

then given chromogen for 5 minutes in room temperature. Wash the specimes with aquadest and incubated in hematoxilin counterstain for 3 minutes. After washed with aquadest, all specimens were given with mounting media solution and covered with covering glass.

RESULTS AND DISCUSSION

From all specimens we made, 5 rats from group 1 all atheroma negative, CRP negatif and 5 rats in group 2, all have atheroma plaques but 3 specimes CRP positif and 2 CRP negatif (Table 1).

Aorta of rats in group 1 did not have atheroma plaque (Figure 1) and CRP negatif (Figure 3), but aorta of rats in group 2 all atheroma positive (Figure 3) and 3 of 5 have CRP positive (Figure 4).

Table 1. Aorta of Sprague Dawley rats after consumed normal fat diet (Group 1) and high fat diet (Group 2), atheroma and CRP in the plaque

No	Group 1: Control (normal lipid diet)		Group 2: High lipid diet	
	atheroma	CRP	atheroma	CRP
1	-	-	+	-
2	-	-	+	-
3	-	-	+	+
4	-	-	+	+
5	-	-	+	+

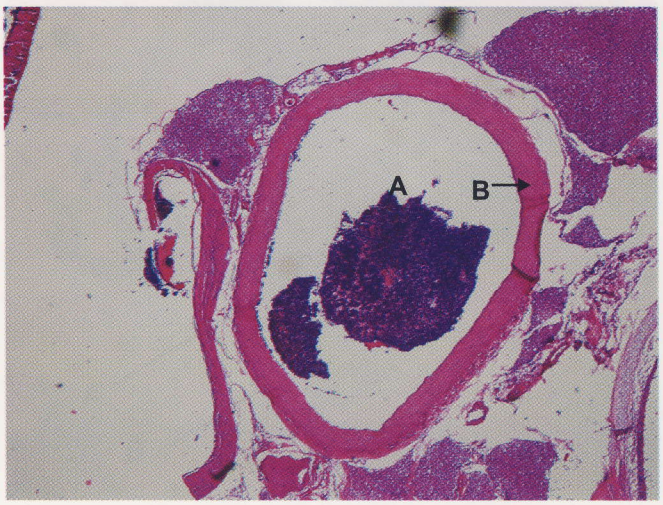

Figure 1. Aorta of Sprague Dawley rat from Group 1, show atheroma negative. A. Lumen, B. Aorta wall (H.E., 4x10).

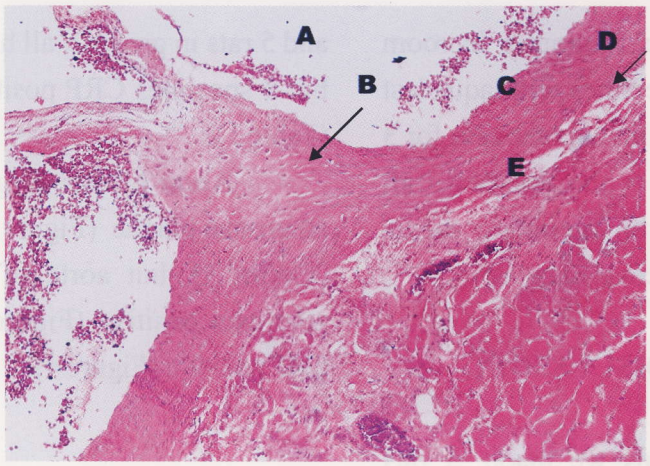

Figure 2. Aorta of Group 2, with atheroma positive. A. Lumen B. Atheroma plaque C. Tunica mucosa D. Tunica muscularis E. Tunica serosa (H.E., 10x10)

The result of immunohistochemistry analyses of all aorta samples seen that all samples from Group 1 were CRP negatif (Figure 3). Animals in Group 2 that fed high lipid diet 3 of 5 (60%) animals were CRP positive (Figure 4). Histopathology analyses of hearts and aortas of all animals showed that basal diet (content 4.5% fat) that given to group 1 for about 59 days did not make atheroma plaque appear. This result was different with animals in group 2. Animals that fed high lipid diet (content 20% fat) all have atheroma plaque. Atherosclerosis is a chronic inflammatory disorder. It is thought to begin with damage to the innermost layer of the artery called endothelium. According to Ross (1999), endothelial cells may be injured not only by modified LDL, but also by many other factors, such as elevated plasma homocysteine concentrations, hypertensions and infectious microorganisms. Endothelial dysfunctions

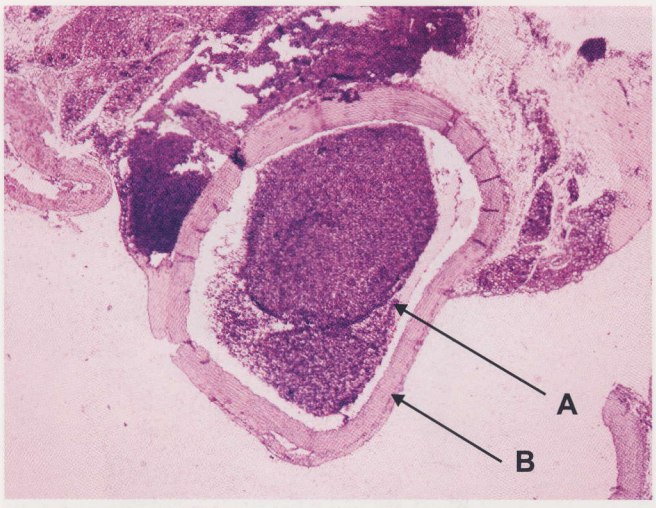

Figure 3. Aorta of Group 1 with CRP negative. A. Lumen B. Aorta wall (Antibody anti CRP, 4x10).

includes increased endothelial permeability to lipoproteins and other plasma constituents, expresiion of growth factors that led to increased adherence of monocytes, macrophag and lymphocytes. These cells may migrate through the endothelium and situate themselves within subendothelial layer. In the vascular wall, macrophages accumulate lipids and become large foam cells. Foam cells in turn, release growth factors and cytokines that promote migration of smooth muscle cells and promote neointimal proliferation, continue to accumulate lipid and support endothelial cell dysfunction. Foam cells, T-cells and smooth muscle cells eventually form the fatty streak.

modified plasma proteins (Pepys and Baltz, 1983), damaged cell membranes (Volanakis and Wirtsz, 1979), a number of different phospholipids and related compounds, small nuclear ribonucleoprotein particles (Du Clos, 1989), and apoptotic cells (Gershov *et al*, 2000). The secondary effects of CRP that follow ligand binding resemble some of the key properties of antibodies, suggesting that under various circumstances CRP may contribute to host defense against infections, function as a proinflammatory mediator, and participate in physiological and pathophysiogical handling of autologous constituents. In this case CRP can be detected (60%), because the situation of

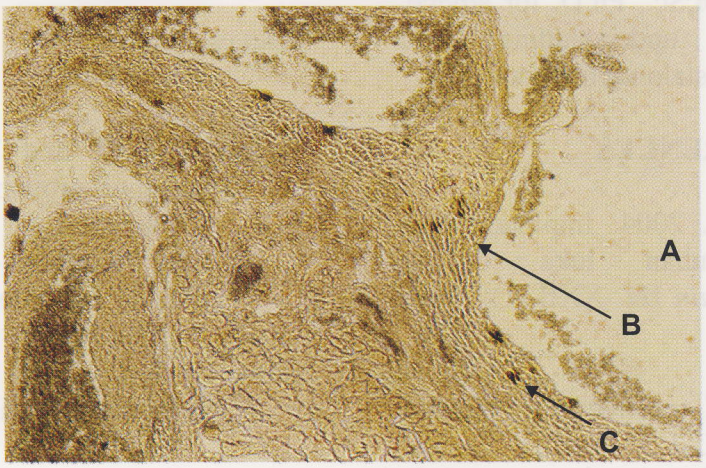

Figure 4. Aorta of Group 2 with CRP positive. A. Lumen B. Atheroma plaque C. CRP (Antibody anti CRP, 10x10).

C-reactive protein will be positive if there were many bounds of antigen (CRP) and antibody anti CRP in tissues. C-reactive protein is protein that may contribute to inflammation in atheroma and also may be actively involved in early atherogenesis (Volanakis, 1982) and activates the complement system (Reynolds and Vance, 1987). Human CRP binds with highest affinity to phosphocholine residues, but it also binds to variety of other autologous and extrinsic ligands, and it aggregates or precipitates the celluler, particulate, or molecular structures bearing these ligands. Autologous ligands include native and

atheromas may be in early stage. Two CRP negatif specimens of our study may appears because the condition of atherogenesis may not be in early stage of atheroma, there was not any inflammation reaction or may because the parrafin-embedded sections process of aorta was not exactly in the inflammation part . The inflammation could be happened only in a small part of arteria then develop to be a plaques. In this case we have tried to find CRP in more than 15 tissue sections every aorta specimen, but the attainment of immunohistochemistry diagnosis in that specimens did not satisfy. We only found 60%

CRP positive in all specimens with atheroma plaques. So, although we did not sure, we decided that the specimen without CRP positive was really negative.

From the result of this study it can be concluded that high lipid diet cause atherosclerosis. To detect C-reactive protein in the plaques we can use immunohistochemistry method, but although we found all specimens positive with atheroma plaques, the attainment of immunohistochemistry analyses still depend on many factors including parafin embedded sections and the stages of atherosclerosis.

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