Original Article

Immunohistochemical Detection of Oxidative Stress Biomarkers, Dityrosine and \(N^\varepsilon\)-(hexanoyl)lysine, and C-Reactive Protein in Rabbit Atherosclerotic Lesions

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Several lines of evidence have demonstrated that C-reactive protein (CRP) is associated with oxidative stress; however, the precise co-localization between CRP and oxidative stress markers in atherosclerotic lesions is not fully established. In this study, we focused on two oxidative stress markers, dityrosine (DY) and \(N^\varepsilon\)-(hexanoyl)lysine (HEL), which had not previously been investigated in relation to CRP in atherosclerotic lesions.

**Aim:** We investigated the production and localization of DY, HEL, and CRP in early-stage and moderately progressed fatty lesions of cholesterol-fed rabbits by immunohistochemistry using specific monoclonal antibodies to examine the co-localization between CRP and oxidative stress in atherosclerotic lesions.

**Methods:** Rabbit atherosclerotic specimens were obtained from New Zealand White rabbits fed a diet containing 1.0% cholesterol for 12 weeks. All specimens were fixed in formalin for histological examinations.

**Results:** CRP-positive cells in rabbit early-stage and moderately progressed fatty lesions were detected mostly in the macrophage-derived foam cell-rich areas. Both DY and HEL were also detected in foam cell-rich areas in both lesions, where they were primarily co-localized with CRP-positive cells.

**Conclusion:** Our results suggest that the generation of oxidative stress markers, DY and HEL, may be mediated by CRP in atherosclerotic lesions, and that CRP may be associated with oxidative stress in rabbit atherosclerotic lesions.


**Key words:** C-reactive protein, Dityrosine, \(N^\varepsilon\)-(hexanoyl)lysine, Atherosclerosis

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**Introduction**

Recently, several lines of evidence have demonstrated that C-reactive protein (CRP) is associated with oxidative stress\(^1\), \(^2\). CRP induces the expression of inducible nitric oxide synthases (iNOS), supporting a proinflammatory role for CRP in human aortic endothelial cells\(^3\). Kobayashi *et al.*\(^4\) have shown that the expression of vascular CRP is closely co-localized with NAD(P)H oxidase, an important enzyme for the origin of reactive oxygen species (ROS) in vessel walls; they have also shown that CRP directly enhances NAD(P)H oxidase p22\(^{phox}\) protein expression and the generation of intracellular ROS in cultured human coronary artery smooth muscle cells. Additionally, in human studies, it has been demonstrated that CRP is positively associated with oxidative stress markers such as urinary F\(_2\) isoprostanes, oxidized-LDL, and the free oxygen radical test level\(^5\), \(^7\). These reports suggest that CRP is not just a bystander to inflammation but may promote oxidative stress; however, the precise co-localization between CRP and oxidative stress markers in
atherosclerotic lesions remains unclear.

In this study, we focused on two oxidative stress markers, which, to our knowledge, had not previously been investigated in relation to CRP in atherosclerotic lesions. The first marker, dityrosine (DY), is formed by the reaction of two tyrosyl radicals, and is generated by ROS, metal-catalyzed oxidation, ultraviolet irradiation, and peroxidases, suggesting that DY may become a universal protein oxidant marker. DY has been detected immunochemically in lipofuscin of pyramidal neurons of aged human brains and in atherosclerotic lesions of apolipoprotein E-deficient mice.

The second marker, \( N^\alpha-(\text{hexanoyl})\text{lysine} \) (HEL), has been found in the reaction between linoleic hydroperoxide and lysine moiety. The formation of HEL has been shown to be a good marker for oxidative modification by oxidized \( \omega-6 \) fatty acids such as linoleic acid and arachidonic acid. The presence of HEL in low-density lipoprotein (LDL) oxidized with copper has been confirmed using the specific anti-HEL polyclonal antibody in vitro, and a previous study demonstrated the production of HEL moiety in human atherosclerotic plaques by immunohistochemistry using anti-HEL polyclonal antibody, but it was not assessed using a monoclonal antibody.

In the present study, to examine the association between CRP and oxidative stress markers, DY, and HEL in vivo, we investigated the production of CRP, DY, and HEL in early-stage fatty lesions and moderately progressed lesions of cholesterol-fed rabbits by immunohistochemistry using monoclonal antibodies and, furthermore, examined the localization of DY and HEL to determine which cells produce these oxidative stress markers.

### Materials and Methods

#### Specimens

In this study, we used rabbit specimens to examine CRP and oxidative biomarker deposition and their relation to other cellular components in early-stage fatty lesions (rich in foam cells) and moderately progressed fatty lesions (rich in foam cells with a thin fibrous capsule). New Zealand White rabbits were fed commercial rabbit diet (LRC4) containing 1.0% cholesterol (Oriental Yeast, Tokyo, Japan) at 100 g/day for 12 weeks; they were then sacrificed by intravenous injection of sodium pentobarbital, and thoracic aorta was quickly removed. Specimens were fixed in formalin for histological examinations. The rabbits were treated in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

#### Oxidative Stress Markers

Monoclonal antibodies specific for DY and HEL have been characterized previously. An antibody specific for protein DY (G6) was prepared using a dimer of 3-\( p \)-(hydroxyphenyl)propionic acid (di-HP) as a hapten, and clones were obtained. The 3C3 antibody to the hexanoyl-lysine moiety was prepared using synthetic hexanoyl protein as an immunogen and then characterized in detail.

#### Histological Examination

All specimens were embedded in paraffin and sectioned at 3.0 \( \mu \)m. The sections were deparaffinized with xylene and ethanol, and were stained with hematoxylin and eosin (HE) for morphological evaluation by light microscopy.

#### Immunohistochemical Staining with Individual Antibodies

The panel of antibodies for immunohistochemical staining to examine rabbit CRP, macrophage, neutrophils, smooth muscle cells (SMCs), DY, and HEL in the lesions is listed in Table 1. CRP was immunostained according to the method of Sun et al. Briefly, sections were immersed in 3% \( \mathrm{H}_2\mathrm{O}_2 \) in methanol for 45 minutes at room temperature to block endogenous peroxidase activity. The sections were then incubated with normal goat serum for 20 minutes to reduce nonspecific background staining and reacted with Table 1. Antibodies for the current study

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Antigen</th>
<th>Species</th>
<th>Working dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-polyclonal CRP</td>
<td>Rabbit CRP</td>
<td>Chicken</td>
<td>× 200</td>
<td>Immunology Consultants Laboratory, Newberg, OR, USA</td>
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<tr>
<td>RAM11</td>
<td>Rabbit monocyte/macrophage</td>
<td>Mouse</td>
<td>× 400</td>
<td>Lab Vision, Fremont, CA, USA</td>
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<tr>
<td>R3</td>
<td>Rabbit neutrophil defensin 5</td>
<td>Mouse</td>
<td>× 20</td>
<td>Hy luc Biotechnolog, Uden, Netherlands</td>
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<tr>
<td>HHF35</td>
<td>Smooth muscle ( \alpha )-actin</td>
<td>Mouse</td>
<td>× 400</td>
<td>Enzo Biochemicals, NY, USA</td>
</tr>
<tr>
<td>G6</td>
<td>DY</td>
<td>Mouse</td>
<td>× 200</td>
<td>Reference 8</td>
</tr>
<tr>
<td>3C3</td>
<td>HEL</td>
<td>Mouse</td>
<td>× 5000</td>
<td>Reference 11</td>
</tr>
</tbody>
</table>


anti-rabbit CRP polyclonal antibody (1:200, v/v) at 4°C overnight. After rinsing with phosphate-buffered saline (PBS), the sections were incubated with anti-chicken peroxidase-conjugated IgY (Abcam plc., Cambridge, UK) as the secondary antibody at room temperature for 60 minutes, and staining was then visualized by reaction with DAB+ (DAKO Japan, Kyoto, Japan).

To stain macrophages, neutrophils defensin 5 (NP-5), SMCs, DY, and HEL, deparaffinized sections were immunostained as follows. To stain macrophages, the sections were predigested with Proteinase K Ready-to-Use (DAKO Japan, Kyoto, Japan) for 5 minutes at 37°C. All sections were incubated with normal serum in PBS for 20 minutes to block nonspecific binding of the secondary antibody and then with each appropriately diluted primary antibody for 60 minutes at room temperature. Immunostaining was performed using the avidin-biotin complex method with the Vectastain ABC-AP (alkaline phosphatase) kit and Vector Aline Phosphatase Substrate Kit I (Vector Laboratories, CA, USA).

Results

Histology

Early-stage fatty lesions were composed of a few layers of macrophage-derived foam cells (foam cell-rich lesions) (Fig. 1A, B), and most moderately progressed lesions were macrophage-derived foam cell-rich lesions with a thin fibrous cap (Fig. 2A, B).

CRP-Positive Cells in Early-Stage and Moderate Lesions

By immunohistochemistry, CRP was found to be localized in both early-stage and moderately progressed fatty lesions. In early-stage fatty lesions, predominant manifestation of CRP was observed in foam cell-rich areas (Fig. 1C), and in moderately progressed lesions, it was also primarily localized in areas rich in foam cells (Fig. 2C). Furthermore, in both lesions, CRP-positive cells were co-localized with macrophages (Fig. 1B, 2B) but not with most SMCs (Fig. 1D, 2D). No CRP staining was seen in areas without any signs of atherosclerotic lesion development. In addition, a similar staining procedure performed by omitting primary antibody yielded negative results with all tissue specimens (data not shown).

Immunohistochemical Detection of DY

DY has been reported to be chemically detected in human atherosclerotic lesions by HPLC and gas chromatography-mass spectrometry. In the present study, we examined the immunohistochemical staining of DY using a monoclonal antibody to examine the distribution of DY in atherosclerotic lesions. As shown in Fig. 1E, 2E, DY immunopositive staining was observed in foam cell-rich areas both in early-stage and moderately progressed fatty lesions, where it almost co-localized with CRP-positive macrophages. The specificity of the antibody was demonstrated by successful absorption of diHP-conjugated bovine serum albumin. In addition, no positive staining was observed in atherosclerotic lesions in response to omitting the primary antibody (data not shown).

Distribution of HEL in Early-Stage and Moderate Lesions

The HEL moiety was immunohistochemically detected in human atherosclerotic plaques using an anti-HEL polyclonal antibody; however, the production and localization of HEL in atherosclerotic lesions has not been revealed using an anti-HEL monoclonal antibody. Thus, in this study, we performed immunohistochemical studies using an anti-HEL monoclonal antibody to investigate the localization of HEL, and examined which cells in atherosclerotic lesions produce HEL. Immunopositive staining was observed in foam cell-rich areas of both early-stage and moderately progressed lesions (Fig. 1F, 2F), essentially similar to the deposition of DY. Furthermore, HEL co-localized with CRP-positive macrophages as well as DY. As a negative control, there was no immunoreactivity when the primary antibody was omitted during the staining procedure (data not shown). In addition, no staining was observed with the anti-HEL antibody preabsorbed with hexanoyl bovine serum albumin. These results indicate that immunopositive materials for anti-HEL monoclonal antibody are present in CRP-positive macrophages in early-stage and moderately progressed fatty lesions.

Discussion

In the present study, we investigated whether the production and localization of oxidative stress markers, DY and HEL, are co-localized with CRP in atherosclerotic lesions of cholesterol-fed hyperlipidemic rabbits based on immunohistochemical detection using specific antibodies. CRP-positive cells in rabbit early-stage and moderately progressed fatty lesions were detected in foam cell-rich areas (Fig. 1C, 2C), and were observed to react with RAM11, identified as macrophages (Fig. 1B, 2B). Both DY and HEL were also detected in foam cell-rich areas in both early-stage and moderately progressed lesions, where they largely co-
Serial sections of early-stage fatty lesions of 1.0% cholesterol-fed rabbit thoracic aorta. (A), HE staining of early-stage fatty lesions. The sections were stained with antibodies against macrophages (Mφ) (B), rabbit CRP (C), SMCs (D), DY (E), and HEL (F). The deposition of CRP is observed in foam cell-rich areas, and is co-localized with macrophages. DY and HEL production was primarily overlapped with CRP-positive macrophages. The lumen is at the top. Upper panel magnification is ×100; lower panel magnification is ×400. The demarcation between intima and media is indicated by an arrow.
Fig. 2.
Serial sections of moderately progressed fatty lesion of 1.0% cholesterol-fed rabbit thoracic aorta. (A), HE staining of moderately progressed fatty lesion. The sections were stained with antibodies against macrophages (MΦ) (B), rabbit CRP (C), SMCs (D), DY (E), and HEL (F). CRP is deposited in macrophage-derived foam cell-rich lesions, and is largely co-localized with DY and HEL. The lumen is at the top. Upper panel magnification is ×100; lower panel magnification is ×400.
localized with CRP-positive cells (Fig. 1E, F, 2E, F).

A number of new oxidative stress biomarkers have been developed, including isoprostanes, 8-hydroxy-2'-deoxyguanosine, DY, 3-nitrotyrosine, oxidized-LDL, lipid hydroperoxide-derived protein modification, such as HEL, oysterseals, and plasma antioxidants, and are widely used to assess the efficiency of antioxidative medicines and dietary antioxidants in humans and animals. Among these, we prepared antibodies specific for DY and HEL, and confirmed the marked deposition of DY and HEL in atherosclerotic lesions. Co-localization between these new oxidative stress markers and CRP in atherosclerotic lesions has not been investigated but, in the present study, their production was likely co-localized with CRP accumulating in atherosclerotic lesions. In addition, our findings of co-localization between these oxidative stress markers and CRP in atherosclerotic lesions extend the basic finding that CRP interacts with oxidative stress and support the previous observation that CRP is likely a key molecule linking inflammation and oxidative stress in the pathogenesis of coronary artery disease, including acute coronary syndrome.

There are various potential sources of ROS generation in vascular cells such as macrophages and lymphocytes: the mitochondrial transport chain, cyclooxygenase, lipooxygenase, xanthine oxidase, and NAD(P)H oxidase. Recent evidence suggests that, of these, NAD(P)H oxidase plays a crucial role in the generation of ROS in vascular cells. NAD(P)H oxidase converts oxygen into superoxide (O$_2^-$) and, O$_2^-$ then dismutates into H$_2$O$_2$, an oxidizing substrate for myeloperoxidase. Myeloperoxidase uses the H$_2$O$_2$ generated by this system to produce an o,o-dityrosine cross-link from the tyrosine residue of the target protein. Recently, Bhattacharjee et al. showed that oxidants derived from the phagocyte NAD(P)H oxidase provide one pathway for generating o,o-dityrosine cross-links in vivo. They showed that neutrophils markedly increase their content of protein-bound DY when they are activated in wild-type animals; however, this increase fails to occur in mice that are deficient in phagocyte NAD(P)H oxidase, and the levels of o,o-dityrosine in urine mirror those in neutrophil proteins. These results suggest that phagocyte NAD(P)H oxidase plays a pivotal role in the production of DY during acute inflammation. In the present study, the localization of DY was almost always found to be overlapped with CRP-positive macrophages. A previous report showed that incubation of cultured coronary artery smooth muscle cells with CRP resulted in enhanced protein expression of NAD(P)H oxidase p22phox and the generation of H$_2$O$_2$ in a dose-dependent manner. Although it remains unclear whether CRP up-regulates NAD(P)H oxidase p22phox expression in other cells, such as endothelial cells and macrophages, we speculate that CRP accumulating in atherosclerotic lesions may mediate the production of DY by increasing NAD(P)H oxidase p22phox expression and ROS generation, since DY was produced in LDL oxidized with tyrosyl radical generated by the myeloperoxidase -H$_2$O$_2$ system.

As mentioned above, DY formation is catalyzed by myeloperoxidase in the presence of H$_2$O$_2$, but it can also be generated by metal-catalyzed oxidation, hypochloric acid, nitrogen dioxide, and peroxynitrite (ONOO$^-$); therefore, we suggest that other pathways generate DY mediated by CRP that are independent of the up-regulation of NAD(P)H oxidase. In inflammation, NO is generated from iNOS and reacts with O$_2^-$, which derives from NAD(P)H oxidase, accompanied by ONOO$^-$ formation. Since a previous study showed that CRP can induce iNOS expression, supporting a proinflammatory role for CRP, CRP may mediate DY generation in atherosclerotic lesions by enhancing iNOS expression. HEL is considered to be an earlier and stable marker for lipid peroxidation-derived protein oxidation, and we have previously shown the production of HEL in human atherosclerotic plaques using the specific anti-HEL polyclonal antibody, but have not investigated using a monoclonal antibody. Thus, we studied the localization of HEL to determine which cells produce HEL in rabbit atherosclerotic lesions by anti-HEL monoclonal antibody. HEL-immunopositive staining was observed in foam cell-rich areas of both early-stage and moderately progressed fatty lesions, where it primarily co-localized with macrophages. Furthermore, no HEL staining could be seen in areas without any signs of atherosclerotic lesion development (data not shown). These findings suggest that the HEL produced in atherosclerotic lesions likely originates from oxidized LDL containing macrophages. Moreover, the localization of HEL was almost always found to be overlapped with CRP-positive cells, suggesting that, as well as DY, CRP may likely mediate HEL generation by increasing the levels of vascular ROS. Moreover, since a recent report indicated that CRP mediates LDL uptake in macrophages via CD32, CRP not only promotes oxidative stress, but also may be involved in HEL production in atherosclerotic lesions.

In conclusion, we have demonstrated in the present study that DY and HEL immunopositive staining is observed in macrophage-derived foam cells in both early-stage and moderately progressed fatty lesions. In
addition, both oxidative stress markers largely colocalize with CRP-positive macrophages. Our results suggest that generation of the oxidative stress markers DY and HEL may be mediated by CRP in atherosclerotic lesions, and that CRP may be involved in oxidative stress in atherosclerotic lesions.

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