Modified C-Reactive Protein is Expressed in Adventitia and Intimal Neovessels from Complicated Regions of Unstable Carotid Plaques

M. Slevin[*,1], N. Rovira[2], M. Turu[2], A. Luque[2], L. Badimon[2], J. Gaffney[1], L.M. Potempa[3] and J. Krupinski[3]

[1]*School of Biology, Chemistry and Health Science, Manchester Metropolitan University, Manchester, United Kingdom;* [2]*Centro de Investigación Cardiovascular, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain;* [3]*Acphazin Inc, Deerfield, Il, USA;* [4]*Department of Neurology, University Hospital of Bellvitge (HUB), Fundació IDIBELL, Barcelona, Spain*

Abstract: *Objective*: Native C-reactive protein (nCRP) is a soluble acute phase reactant whose expression in the vascular wall; in particular, in reactive plaque regions, and circulating levels increase in patients with inflammatory disease. We have recently demonstrated a specific role for the insoluble, monomeric form of CRP (mCRP) in direct stimulation of angiogenesis and therefore decided to investigate its expression in carotid adventitial vasa vasorum and plaque intimal neovessels to determine if it could be involved in the development of unstable plaque lesions.

Methods: We have used immunohistochemistry to examine the expression of both mCRP and nCRP in a series of carotid arterial plaques obtained at transplant (n=20) and employed double immunoflourescent labelling to identify any association of CRP with active-CD105-positive microvessels.

Results: Using characterised and specific antibodies we have identified strong expression of mCRP in adventitial vasa vasorum and angiogenic neovessels from unstable regions of complicated carotid plaques. mCRP was also found to be associated with infiltrating macrophages in inflammatory regions but infrequently with vascular smooth muscle cells. nCRP was expressed much more weakly and only in some regions rich in inflammatory cells. Many of the mCRP-positive vessels also stained positive for CD105 suggesting they were actively involved in the process of angiogenesis.

Conclusions: Based on our previously published observations of the highly angiogenic nature of mCRP in vitro, we hypothesise that mCRP is intimately involved in promotion of neovascularization and possibly, subsequent destabilization of atherosclerotic plaques and could be considered as a possible target for therapeutic manipulation of angiogenesis.

INTRODUCTION

Angiogenesis is a recognized feature of the atherogenic process, intimal neovascularization arising most frequently from the dense network of vessels in the adventitia, adjacent to a plaque, rather than from the main artery lumen. New blood vessels may have an active role in plaque metabolic activity and actively promote its growth beyond the critical limits of diffusion from the artery lumen [1, 2]. The irregular nature of blood vessel formation has been likened to tumour angiogenesis, and hence the factors responsible for their growth may be different from those seen during normal wound healing [3]. Our previous studies and those of others have suggested that haemorrhagic, leaky blood vessels from unstable carotid plaques proliferate abnormally. These relatively large calibre but immature neovessels are poorly invested with smooth muscle cells and possess structural weaknesses which may contribute to instability of the plaque by facilitation of inflammatory cell infiltration and haemorrhagic complications [4]. Immature neovessels may contribute to instability of the plaque by facilitation of inflammatory cell infiltration and haemorrhagic complications. However, our understanding of the mechanisms responsible for induction and maintenance of plaque neovascularization are incomplete and require further investigation.

Increased concentration of high sensitivity of C-reactive protein (hsCRP) are related to increased risk of vascular episodes, and correlated with brain infarct area, with the severity of ischemic episodes, with greater neuronal damage and with a higher risk of future vascular episodes [5, 6]. Increased CRP levels in the plasma arise due to enhanced synthesis by the liver, as a result of interleukin-6 (IL-6) induction. Evidence has shown that CRP is an important biomarker, able to predict the pathogenesis of atherosclerosis and most relevant to this application, may also be a direct participant in the modulation of biological progression of the disease [7-9]. The published data suggests that CRP induces a pro-atherogenic phenotype in the vascular wall. Moreover, it has been shown that vascular cells directly express and secrete CRP [10]. This has been confirmed by *in vitro* studies using EC and VSMC cultures, which expressed CRP in response to various inflammatory stimuli [11, 12]. In human atherosclerotic injuries, CRP co-localizes with SVMC, macrophages and EC and has been detected at high concentrations in plaque 'shoulders' rich in inflammatory cells [13].

CRP is a pentameric oligoprotein composed of identical 23 KDa subunits which can be irreversibly dissociated to

*Address correspondence to this author at the School of Biology, Chemistry and Health Science, Manchester Metropolitan University, Manchester, United Kingdom; Tel: +44 (0) 161 247 1172; Fax:+44 (0) 161 247 6325; E-mail: m.a.slevin@mmu.ac.uk

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form free subunits or mCRP. mCRP has a reduced aqueous solubility and a tendency to aggregate into matrix-like lattices in various tissues, in particular, blood vessel walls [14]. Cell membranes and liposomes can dissociate nCRP to form this more highly biologically active derivative [15]. A distinct difference in the biological activity of these two isoforms has been shown. mCRP is the primary isoform which binds native and modified low/density lipoproteins, it is the most effective activator of the complement cascade when bound to LDL or oxidised LDL, however, fluid phase mCRP can bind to and prevent C1q from subsequent down-stream activation of the complement cascade. mCRP can induce EC activation at concentrations significantly below the CVD risk cut off point of 3.7 (i.e. approximately 1μg per ml) [16, 17]. In this paper we demonstrate expression of the mCRP in adventitial vasa vasorum and intimal neovessels of complicated carotid plaques and hypothesise that it may contribute to adventitial angiogenesis and development of neointimal microvessels therefore enhancing plaque formation and instability.

MATERIALS AND METHODOLOGY

Patient Samples

Human carotid transplant specimens (n=20; Table **1**) were obtained from patients with a significant degree of carotid stenosis as demonstrated by duplex ultrasonography. Extensive neurological examination and histology was used to classify plaque regions into ulcerated, unstable, fibrous, calcified, haemorrhagic, and inflammatory groups. Full clinical and biochemical data were collected (Table **1**). The specimens were opened longitudinally, cut whilst frozen into 2mm segments and fixed in 10% formaldehyde for immunohistochemistry. All patients gave informed consent and the protocol was approved by the appropriate ethical committee. Stages of plaque development were categorized according to the accepted method of Stary [18].

Immunohistochemistry and Immunofluorescent Staining

Paraffin-embedded tissue samples were processed and serial 5μm sections were cut. The Avidin-Biotin Peroxidase (ABC Vectastain kit, Vector Laboratories, Peterborough, UK) method was used and antibodies to nCRP, mCRP were obtained from our collaborator Professor Larry Potempa and have been fully characterized [15, 19]. Anti-CD105 was from AbCAM. All antibodies were used at a dilution of 1:50. Paraffin-embedded sections were deparaffinized, rehydrated and boiled for 10min in an antigen unmasking solution of concentrated citric acid pH 6.0 as described elsewhere [19]. Slides were incubated in 0.5% v/v H_2O_2 in methanol for 30min, with normal serum for 20min and then with a primary antibody (diluted in normal serum) for 30min, followed by 30-min incubation with biotinylated secondary antibody (diluted 1:50) and finally with ABC complex (diluted 1:50) for 30min at RT. Staining was completed after incubation with DAB substrate chromogen solution for 3-10min. Slides were counterstained with haematoxylin, dehydrated, cleared and mounted in DPX. For immunofluorescence, cultured cells were fixed in 4% paraformaldehyde for 20min, permeabilized with 0.2% Triton X100 for 10min, blocked with normal serum and stained with the primary antibody as above, followed by 1h incubation with Alexa-fluor conjugated dyes (FITC/TRITC) at RT. Negative control slides were performed in parallel, where primary antibody was replaced with washing buffer and processed as above (data not included).

RESULTS

mCRP Rarther than nCRP is Expressed in Complicated Carotid Atherosclerotic Plaques and is Associated with Angiogenic Microvessels

20 carotid arteries were examined following transplant surgery and presented at different stages of plaque development (Table **1**). Histological analysis demonstrated intense staining of neointimal microvessels for mCRP in the majority of complicated carotid plaques (Table **2**; Fig. **1B** and insert; Plaque D shown). In contrast, there was no observable staining of nCRP in the same intimal areas or indeed in other areas of these lesions (Fig. **1A**; Plaque D same area on serial section shown). Strong staining of mCRP was also seen in the adventitial vasa vasorum in particular in microvessels from areas associated with inflammation (Fig **1D**; plaque C shown), whilst again, in contrast, there was no visible staining for nCRP (Fig. **1C**; Plaque C same area on serial section shown).

Some staining of nCRP was seen in both intimal and adventitial regions but only in areas of intense inflammation (Fig. **1E** i-iii; Plaque K shown), whilst in the same plaques, the staining of associated microvessels was predominant (Fig. **1F** i-iii; same area on serial section). This pattern was repeated in other arteries, with strongest staining in the microvessels of high grade plaques. Table **2** shows relative expression in EC, SMC and inflammatory areas for all plaques studied.

mCRP Co-Localized with CD105-Positive Adventitial Vessels and Intimal Plaque Neovessels

The majority of mCRP-positive adventitial vasa vasorum (Fig. **2A**; plaque D shown) and intimal neovessels (Fig. **2B**; plaque D shown) stained positive for CD105, a marker of activated and therefore potentially angiogenic EC.

DISCUSSION

Angiogenesis is a recognised mechanism involved in the development of complicated atherosclerotic plaques. Intraplaque haemorrhage results in rapid expansion of the plaque necrotic core, due to the fact that red blood cell membranes are a rich source of free cholesterol and phospholipids and the process occurs in association with excessive macrophage infiltration [20]. The size of the necrotic core directly correlates with the risk of plaque rupture and intraplaque haemorrhage and plaque rupture were proportional to neovessel density in coronary atheroma [21]. Adventitial vessels in unstable plaques contain perivascular smooth muscle cells, however, after plaque rupture, the fibrous cap is disrupted with a luminal thrombus and the newer branches of vasa vasorum close to the necrotic core consist almost entirely of a single layer of EC overlying a ruptured, leaky basement membrane, and associated with remnants of red blood cells [22]. Defects are thought to be caused by proteolytic damage from on-going inflammation and release of signalling molecules affecting cell-cell contact.

Table 1. Patient Details

No	Stary Grade	Age	Sex	Symptomatic Carotid Disease	Hypertension	Hyperchol-esterolemia	Diabetes	Smoking	PVD	CAD	Plaque Morphology
A	*III*	82	M	-	+	-	-	+	-	-	Non-complicated, inflammation and angiogenesis
B	*III*	75	M	-	+	-	-	+	-	-	Lipid core, mild inflammation
C	*VI*	79	M	+	+	+	+	+	-	+	Complicated, haemorrhagic, inflammation, angiogenesis
D	*VI*	66	M	+	+	+	-	+	-	-	Complicated, haemorrhagic, inflammation angiogenesis
E	*VI*	70	M	+	+	+	-	-	-	-	Complicated, with thrombus, angiogenesis and inflammation
F	*VI*	74	M	+	+	+	-	-	+	-	Complicated with thrombus, inflammation and angiogenesis
G	*I*	56	F	-	+	+	+	-	-	-	Thickening of intima
H	*VI*	68	F	+	+	+	-	-	-	-	Complicated, thrombotic, inflammation and angiogenesis
I	*I*	72	F	-	+	-	-	-	-	-	Thickening of intima
J	*IV*	68	F	-	-	-	-	-	-	-	Non-complicated, inflammation and angiogenesis
K	*IV*	75	M	-							Small non-complicated plaque, angiogenesis, inflammation
L	*IV*	49	F	-	-	-	-	-	-	-	Non-complicated plaque, inflammation and angiogenesis
M	*IV*	72	F	-	-	-	-	+	-	-	Non-complicated plaque, inflammation and angiogenesis
N	*IV*	75	M	+	-	-	-	-		+	Non-complicated plaque with angiogenesis
O	*III*	79	M	-	+	-	-	-	-	-	Lipid core with inflammation
P	*II*	74	F	-	+	+	+	-	-	-	Thickening of intima
Q	*IV*	73	M	-	+	+	-	+	-	-	Non-complicated plaque, inflammation and angiogenesis

Table 1. Contd….

No	Stary Grade	Age	Sex	Symptomatic Carotid Disease	Hypertension	Hypercholesterolemia	Diabetes	Smoking	PVD	CAD	Plaque Morphology
R	*V*	83	M	+	-	+	-	-	-	-	Small non-complicated plaque, inflammation and angiogenesis
S	*V*	55	F	+	-	-	-	-	-	-	Non-complicated plaque, inflammation and angiogenesis
T	*V*	64	M	-	+	+	-	-	-	-	Non-complicated plaque, inflammation and angiogenesis

Abbreviations: PVD, peripheral vascular disease; CAD, coronary artery disease.

Table 2. Expression of mCRP and nCRP in Carotid Transplanst

	mCRP				nCRP			
	VSMC	Intimal EC	Macrophages	Adventitial EC	VSMC	Intimal EC	Macrophages	Adventitial EC
A	+	+++	++	+++	-	-	-	++
B	+	+++	+++	+++	-	-	-	-
C	+	+++	+++	+++	-	-	-	+
D	++	++	++	++	+	+	+	+
E	+	+++	+++	+++	-	-	-	-
F	++	+++	+++	+++	++	++	++	++
G	-	-	++	++	-	+	+	++
H	+	+++	+++	+++	-	-	-	-
I	+	+++	+++	+++	-	-	-	-
J	+	+++	+++	+++	-	-	-	-
K	+	+++	+++	+++	-	-	-	-
L	-	+++	++	++	-	-	-	-
M	-	+++	++	+++	-	-	+	-
N	+	+++	+++	+++	-	-	-	-
O	-	+++	++	+++	-	-	+	-
P	+	-	-	++	-	-	-	-
Q	-	++	+	++	-	-	+	+
R	-	+++	+	+++	-	-	-	-
S	-	++	+	+++	-	-	-	-
T	+	-	+	++	-	-	+	-

-, no staining detected; +, <25% of cells/vessels stained positive; ++, 25-50% of cells/vessels stained positive; +++, >50% of cells/vessels stained positive; ++++. >75% of cells/vessels stained positive.

In this work, we have demonstrated for the first time, to our knowledge, that a modified form of CRP, mCRP, is the predominant form of CRP expressed in carotid arterial lesions, and hypothesise that it might have a key role in modu

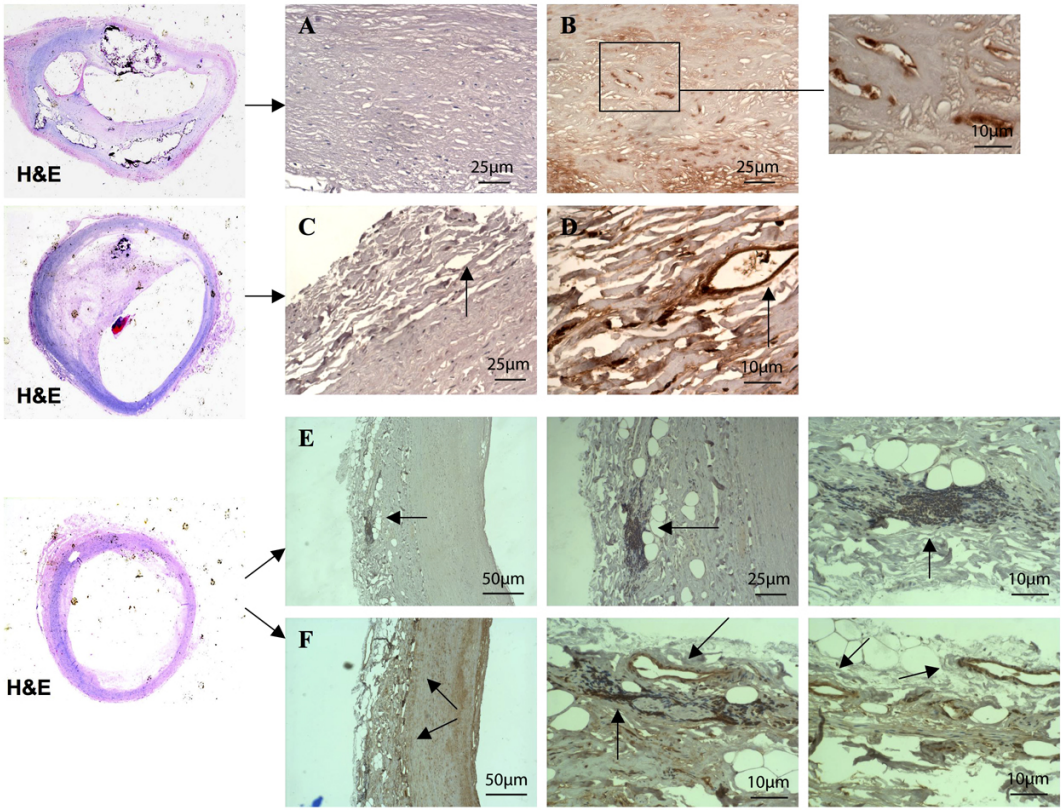

Fig. (1). Expression and localization of CRP in carotid transplant specimens. Left hand pictures show low power (x 10) whole section images stained with haematoxylin and eosin of the arteries in the Figure. (**A**) Shows negative staining for nCRP in the intima of a grade VI complicated carotid artery (plaque D; x 40). (**B**) shows the same area in a serial cut section stained with antibodies to mCRP demonstrating. (**C**) Shows the adventitia negatively stained following IHC using antibodies to nCRP from another grade VI complicated carotid artery (plaque C; x 40; arrow marks specific vessel shown in serial section shown in part D). (**D**) Shows a serial section demonstrating strong staining of the vasa vasorum blood vessel following staining with antibodies to mCRP (plaque C; x 100; arrow). (**E**) Shows a grade III plaque with lipid deposition and microvessel extension from the vasa vasorum into the media and thickened intima. The arrows point to an area infiltrated with inflammatory cells which has stained positive for nCRP (Left x 20; middle x 40; right x 100). (**F**) Shows the same area in a serial section, stained with antibodies to mCRP and showing strong staining of vasa vasorum (arrows; left x 20; middle x 40; right x 100).

lating angiogenesis and therefore be implicated in their progression to unstable, haemorrhagic lesions prone to rupture. Our previous work has shown that mCRP, at concentrations significantly below nCRP levels found in the circulation of patients with active carotid disease, is highly pro-angiogenic both *in vitro*, and *in vivo* [14, 17]. mCRP activated cell signalling pathways associated with mitogenesis and vessel formation in aortic vascular EC suggesting it is a key participant in promotion of neovessel formation. We also showed that only mCRP was able to bind to vascular EC suggesting a possible interaction with the cell membrane and activation of intracellular signalling. Furthermore, mCRP was synthesised and expressed de novo following exposure of the cells to hypoxia suggesting a mechanism where the cells could self-sustain the angiogenic process [17]. Other studies have confirmed the ability of mCRP to induce EC activation. For example, Using carefully purified and synthesised mCRP and nCRP, Bogulawski *et al*, [23] showed that only FITC-labelled mCRP was able to bind to IgG molecules including pentraxins and vitronectin, again, suggesting alternative and increased activity of the modified form. Khreiss *et al*, [24], demonstrated that only mCRP could significantly increase gene expression of IL-8 and MCP-1 within 4h through a p38 MAP kinase dependent pathway in human coronary artery EC at physiological concentrations (1µg/ml).

Previous studies have demonstrated the importance of vasa vasorum activation and extension into the media and intima of actively growing plaques [25], whilst angiogenic blood vessels have been identified in complicated regions of human aortic lesions following immuno-staining with antibodies to CD105 (endoglin; which binds only to active endothelial cells) [26, 27]. We therefore chose CD105 as a marker in order to identify actively growing microvessels. Expression of mCRP but not nCRP was seen predominantly in active CD105-positive adventitial vasa vasorum at sites associated with highest intimal plaque burden suggesting that increased angiogenesis in these areas might enhance plaque growth. Similarly, intimal neovessels from high grade unstable plaques also expressed mCRP which could contribute to their proliferation and the instability of the surrounding lesion.

CONCLUSION

In conclusion, since inhibition of angiogenesis might be an important target for prevention of development of

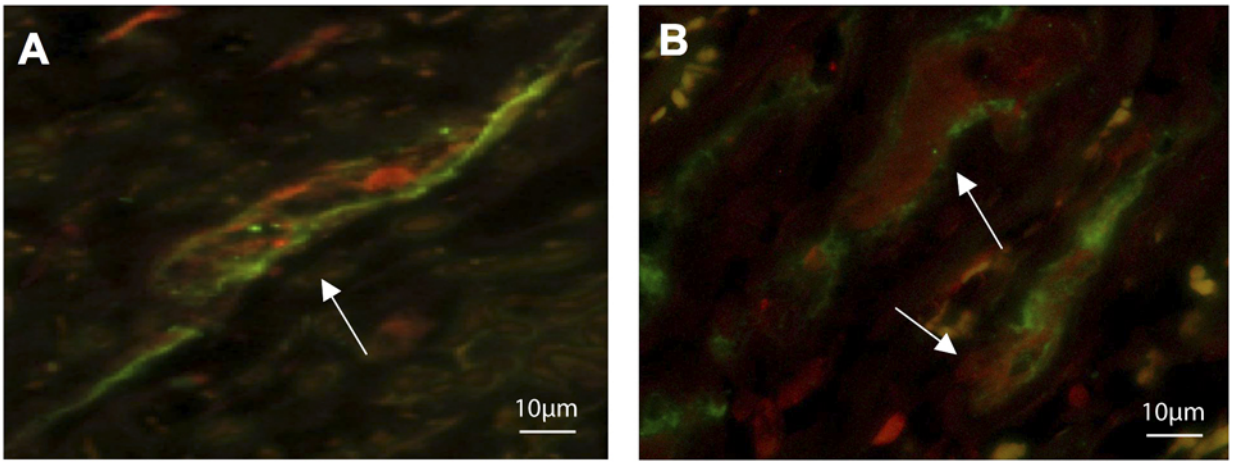

Fig. (2). Co-localization of mCRP with CD105, a marker of endothelial activation. (**A**) Shows co-expression of CD105 and mCRP in the vasa vasorum adjacent to a growing plaque (plaque D shown; arrows). (**B**) Shows a similar staining pattern for intimal neovessels in the same plaque (plaque D shown; arrows). Sections were double-stained and developed with FITC (green; CD105) and TRITC (red; mCRP). Photomicrographs are taken at x 100.

atherosclerosis, identification of key angiogenic determinants of this process such as mCRP, may lead in future to design of novel treatments for inhibition of the formation of active, unstable plaque lesions.

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