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Increased myocardial prevalence of C-reactive protein in human coronary heart disease: Direct effects on microvessel density and endothelial cell survival

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Abstract

BACKGROUND—Elevated plasma C-reactive protein (CRP) is a biomarker of cardiovascular diseases (CVD) but potential roles as a participant of the disease process are not well defined. Although early endothelial cell injury and dysfunction are recognized events in cardiovascular disease, the initiating events are not well established. Here we investigated the local myocardial CRP levels and cardiac microvessel densities in control and CVD tissue samples. Using in-vitro methodologies, we investigated the direct effects of CRP on human endothelial cells.

METHODS—Cardiac specimens were collected at autopsy within 4 hrs of death and were classified as normal controls or documented evidence of CVD. Regional prevalence of CRP, cardiac microvessels (<40µm) were investigated using immunohistochemistry. For in-vitro experiments, human umbilical vein endothelial cells were incubated with CRP. Intracellular oxidant levels were assessed using 2',7'-dichlorofluorescein diacetate fluorescence microscopy and cell survival was concurrently determined. Effects of chemical antioxidants on endothelial cell survival were also tested.

RESULTS—Myocardial CRP levels were elevated in CVD specimens. This was associated with reduced cardiac microvessels and this rarefaction was inversely correlated to adjacent myocardial CRP prevalence. CRP caused concentration dependent increases in oxidant production and cell apoptosis.

CONCLUSIONS—These findings provide evidence supporting myocardial CRP as a locally produced inflammatory marker and as a potential participant in endothelial toxicity and microvascular rarefaction.

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INTRODUCTION

Many recent studies have examined the association between circulating C-reactive protein (CRP) concentrations and increased cardiovascular disease risk. CRP is a classical plasma protein marker that is elevated during acute phase of inflammation, infection, and tissue injury (1, 2). Although, CRP is mainly produced by hepatocytes, there is some recent evidence suggesting that CRP may possibly be produced by macrophages (3), smooth muscle cells (2) or adipose tissue (4). Plasma levels of CRP have emerged as a strong independent risk factor for predicting future cardiovascular events. Several investigations have demonstrated that baseline CRP levels are associated with future coronary events in general population without known pre-existing CAD (5-8).

Recent studies have shown that CRP causes endothelial cell dysfunction and reduced expression and activation of endothelial NOS (NOS3). CRP plays a critical role in the innate immunity pathways and presence of CRP induces important phenotypic changes in the vascular endothelium, including apoptosis (9). NOS3 is a critical regulator of microvascular structure and function. Microvasculature in the heart and other organs is continuously and dynamically regulated by a combination of endogenous pro- versus anti-angiogenic forces (10-14). Local inflammation and oxidant status may play a key role in coronary microvessel abundance. Myocardial levels of CRP have shown to be higher in many cardiovascular diseases. However, the role of locally produced myocardial CRP in cardiac microvascular regulation and cardiovascular disease progression is not completely understood yet.

The mechanistic relevance of various concentrations of CRP on vascular endothelial cells is not completely defined. Moreover, in spite of multitude of evidence of CRP participating in all stages of atherosclerosis, it is currently unknown whether the elevation of CRP in plasma is the cause and/or consequence of atherosclerosis (15, 16). Great interest has been triggered in understanding underlying mechanisms. However, the direct interaction of CRP and endothelial cells has not yet been specifically investigated. For this reason, here we tested the hypothesis that clinical relevant concentrations of CRP can induce oxidant production in endothelial cells. Moreover, local production of CRP by various cells may contribute significantly in the dysfunction associated with increased CRP levels; especially in the cardiac and vascular tissues. Here we tested the hypothesis that myocytes produce CRP locally and that CRP causes direct endothelial cell toxicity. Additionally, we tested the effectiveness of antioxidants in prevention of CRP-induced endothelial cell death.

MATERIALS AND METHODS

Materials

Human Umbilical Vein Endothelial Cell preparations (HUVECs) were used in all the experimental studies. ECs were obtained from Biowhittaker. For all the experiments cells within passages 4-7 were used. Human recombinant CRP (rhCRP) was purchased from Chemicon. ECs were cultured in Endothelial Growth Medium-2 (EGM-2). All the other chemicals were purchased from Sigma.

Human Cardiac Tissue sample collection

Human cardiac tissues (LV anterior myocardium), full thickness was collected at autopsy (OSU pathology/NCI AIDS tissue depository). De-identified patient information was obtained from autopsy reports. Mean age for all the individuals were ~34-38yrs. Patients with preexisting cardiac risk were omitted from all the investigations (diabetes, smoking, therapy involving cardiotoxic medications). Cardiac LV anterior free wall was collected at autopsy within 4 hours of death. These were classified into 2 groups – Normal controls with no evidence of cardiac dysfunction and those with documented evidence of coronary heart

disease (from autopsy report). n=7-12 for each group was used in these studies. These studies were approved by the Institutional review board.

Immunohistochemistry and image analysis

Formalin fixed and paraffin embedded tissues were used for histological analyses. 5 μ M cardiac cross-sections were prepared for immunohistochemistry by standard procedures. Tissues were incubated for 1 hour with appropriate primary antibodies. Staining was visualized with diaminobenzidine, methyl green counterstain. Staining controls were used to demonstrate the specificity of the antibodies used (primary antibody substituted for non-immune rabbit IgG). Cardiac tissue images were captured using Insight high-resolution digital camera, mounted on Olympus microscope. Immunoreactivity was quantified by optical density measurement using an automated digital imaging subroutine (Image Pro software, Media Cybernetics, MD) using automated macros. In all cases the variability in was less than 2% for intra-sample and <10% for inter-sample measurements.

Quantification of microvessel prevalence using image analysis

5 μ m sections were stained with an endothelial cell marker (CD-31). Images were captured using a 40x objective and a standard upright microscope (Olympus, Melville, NY) with a CCD Camera (Diagnostic Instruments, Sterling Heights, MI). Images were analyzed using Image Pro Plus research grade image analysis software (Media Cybernetics, Silver Springs, MD) and a custom written macro. Color segmentation is used to delineate positive endothelial cell (microvessel) staining from the rest of the tissue. Positive objects are filtered based on size and shape to separate vessels from single cells and or inappropriate staining. The final count is approved by the user to ensure that vessels are counted appropriately.

Crystal Violet assay

This is a cell viability assay. Cells are seeded on 96-well plates (in media, w/serum). After 24h, cells are treated with toxicant (or protectant) for required time (low serum). After the treatment cells are washed with PBS and fixed with formalin. The cells are then washed with distilled water and incubated with 0.1% Crystal Violet solution for 10 min. The excess crystal violet was washed and the dye was solubilized with sodium citrate solution. Absorbance of the solution was recorded with spectrophotometer (SpectraMax Plus) at 590 nm. Preliminary experiments involved the production of a standard curve of crystal violet intensity to various dilutions endothelial cells.

Cell Viability Assay

Endothelial cells (1×10^5 cells/well) were seeded in UV transparent 96-well plates, grown to confluence for 24 hours, then treated for 24 hours in medium alone or medium fortified with rhCRP at various concentrations. Heat denatured rhCRP was used as a control treatment. For cell protection assays, cells were incubated with rhCRP with or without L-ascorbic acid and N-acetylcysteine at various concentrations for upto 48 hours. Cells were then washed and fixed in 5% buffered formalin, and stained with crystal violet as a marker of cell viability. Crystal violet signal was assayed spectrophotometrically at 590nm.

Cellular Apoptosis vs. Necrosis

Cells were grown to confluence on 8-well glass slides, and then treated with CRP at 5 μ g/ml and 10 μ g/ml for 48h, and the mechanisms of cell death were investigated using fluorescence microscopy via a commercially available assay kit (Annexin V-Cy3/6-Carboxyfluorescein diacetate kit, Sigma [St. Louis, MO]). After a 10-min incubation in double-labeling solution, the cells were placed on ice and fluorescent images were captured at wide-pass (WB) and infrared (IR) fluorescence using the Optronics Magnafire imaging

system and analyzed using research image analysis software (ImagePro Plus; Media Cybernetics [Silver Spring, MD]). With this method, live cells stain green because of externalization of phosphatidyl serine, whereas necrotic cells stain red because of permeability to propidium-iodide. Early apoptotic cells stain both green and red.

Intracellular Reactive Oxidant Species

Cells were seeded on 8-well chamber slides in EGM at an 80% confluency of the cells. After 8 hours cells were washed with serum-free EBM to remove non-adherent cells. Cells were then treated for 24 or 48 hours with 0-10 $\mu\text{g/ml}$ CRP at 37°C. Prior to loading of the fluorescent dye 5(6)-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA, Molecular Probes), cells were washed with PBS three times. Cells were then loaded with CM-H2DCFDA (10 μM final concentration from a 5mM freshly prepared stock solution in DMSO) at 37°C for 30 minutes in serum-free EBM in the dark. Following washes with dye-free buffer, the chamber was removed and the slide was cover-slipped. Cellular levels of reactive oxygen species were measured immediately by capturing fluorescent and bright field images of the cells at a 400X magnification on an Olympus fluorescent microscope. Cellular boundaries were traced (determined from the bright field images) and relative fluorescent intensity per cell was quantified using a digital image analysis program (Image Pro).

Digital Image Analysis

Digital images were acquired using an Olympus microscope (model BX40) and transferred to Image Pro Plus software (Media Cybernetics, Silver Spring, MD) for both area and intensity analyses. In the DCF experiment, the boundary of individual endothelial cells were traced under bright-field image and defined as AOIs. These AOIs were then be automatically applied to fluorescence images. Relative fluorescent intensity per cell was quantified using a digital image analysis (Image Pro).

Statistical Analysis

All data presented represent 6-12 observations per group and represented as; mean $\pm$ SE. A simultaneous comparison of groups was handled by one-way analyses of variance, with Student-Newman-Keuls post-hoc analysis. Statistical evaluations were performed using GraphPad Prism. Comparisons among treatment groups were performed using student's t-tests or analysis of variance when appropriate. In all cases, significance was defined as $p < 0.05$.

RESULTS

Increased prevalence of C-reactive protein levels in myocardium

Myocardial levels of CRP were investigated in control hearts and those with documented CVD as shown in Figure 1. CRP has been recognized as a 'marker' of inflammation and injury. We observed significantly higher myocardial CRP levels in the CVD tissues. These data provide evidence for myocardium specific local changes in regulation of inflammatory pathways.

Increased myocardial CRP levels associated with microvessel rarefaction

Digital image analyses were carried out for identification and counting of microvessels (10-100 μm^2). We observed a statistically significant reduction in cardiac microvessel prevalence in CVD group relative to controls at sites remote from large vessel lesions (Figure 2). We further tested myocardial CRP prevalence vs. microvessel prevalence for statistical correlation (Spearman's non parametric correlation). There was a statistically

significant negative correlation between myocardial CRP and microvessel density ($p < 0.05$). These data provide evidence of a relationship between local myocyte expression of CRP and prevalence of microvessels in human tissues.

CRP causes direct endothelial cell toxicity

We evaluated direct effects of CRP on endothelial cell survival *in vitro*. Shown in Figure 4 are effects of rhCRP on HUVEC cell survival. Following 48 hours of incubation with rhCRP or heat-denatured rhCRP, cell survival was measured using crystal violet assay. Significant decrease in cell survival was observed following rhCRP treatment ($p < 0.01$). In contrast, heat-denatured rhCRP has no significant effect on cell survival at identical concentrations (Figure 4A). These results suggest that clinically relevant concentrations of rhCRP have direct toxicity to endothelial cells.

CRP treatment causes apoptotic cell death

We investigated potential mechanisms of endothelial cell death after incubation with CRP. Total cell death was determined by crystal violet assay. Out of the total number of cells dead after 48h of CRP incubation, significant proportions of the treated cells were positively stained for the early phase of apoptosis (Annexin-V positive), with minimal evidence of necrosis (Figure 4B). In the control group, endothelial cell death at 48h incubation was less than 5%. Out of these dead cells 50% cells were positive for apoptotic cell death and 50% for necrotic cell death. Following exposure to increasing concentrations of CRP, the percent of dead cells rises primarily due to apoptosis. These data suggest that the primary mechanism for CRP mediated cell death may be apoptosis.

CRP causes increased intracellular oxidants

Given the evidence of apoptosis induced by CRP at clinically relevant concentrations, we investigated potential involvement of reactive oxidant species using DCF fluorescence live cell imaging. CRP caused time- and concentration-dependent increases in intracellular oxidant production relative to control as shown in Figure 5.

Antioxidants protect against CRP toxicity

Given the evidence that both cell death and formation of intracellular oxidants increase significantly with CRP treatment, we then tested the hypothesis that antioxidants could protect against CRP induced endothelial cell death. As shown in Figure 6, simultaneous incubation of cells with the antioxidants protected against CRP induced cell death at 48 hours.

DISCUSSION

It is now well recognized that CRP is a major risk factor for a wide variety of cardiac and vascular disorders, including cardiomyopathies, myocarditis, and vasculopathies. CRP is now shown to play a major role in many cardiovascular events and has complex pathobiology. CRP is known to colocalize with complement in the atherosclerotic plaque due to its local production. CRP also contributes to perpetuation and amplification of inflammatory responses (17). Given the importance of CRP as a critical participant in CVD, the mechanistic understanding of its vascular endothelial toxicity is critical. Recent studies have suggested that CRP has specific vascular effects that include apparent impairment in vascular endothelial function. These vascular changes may be explained by either direct toxicity to the endothelium *in vivo* or indirect effects related pro inflammatory pathways associated or other cytokines. There is some controversy in the literature regarding the direct toxic effects of CRP. It has been thought that CRP induced augmentation of endothelial cell

activation requires CD-14 as a cofactor(18). Conversely, Devraj et. al. have demonstrated that native CRP alone is capable of inducing HAEC activation (19). Molins et. al. demonstrated that monomeric CRP has more potent prothrombotic effects as compared to native CRP (20). Given these complexities *in vivo*, we tested the hypothesis that CRP has direct actions on vascular endothelial cells. Direct toxicity of CRP on endothelial cells has been previously investigated in regards to cellular phenotypes and nitric oxide synthase regulation. However, there have been few studies investigating the direct mechanistic aspects of CRP-induced vascular endothelial cell toxicity. One limitation of many *in-vitro* studies is the concentrations of CRP used which range from 5mg/L to 100mg/L. In a clinical setting 3-5mg/L CRP level is considered to be moderate to high risk. Low-grade chronic elevation in CRP levels may be more significant for direct effects of CRP as compared to acute increases. We have therefore used clinically relevant concentrations of CRP to investigate the direct effects on endothelial cell survival.

We demonstrated for the first time that there is a measurable level of CRP in cardiac tissue from human LV samples with evidence of CVD. In a parallel set of experiments, the presence of CRP in the cardiac tissues was confirmed by western blotting (data not shown here). This observation suggests that CRP might be produced locally in cardiomyocytes. Indeed many recent studies have demonstrated that CRP is produced in multiple sites including kidney and neuronal cells. These increased levels of CRP were directly correlated to reduction in local microvessel density. *In-vitro* investigations with recombinant human CRP caused endothelial cell death and significantly increased intracellular oxidant production. By using general antioxidants, we showed that it prevented CRP induced cell death, indicating the critical role of ROS in CRP induced EC death. Thus, we have demonstrated, that CRP, at concentrations known to predict adverse vascular outcomes *in vivo*, has direct toxic effects on endothelial cells, and that the toxicity is mediated via reactive oxidant species. These adverse effects of CRP on endothelial cell survival may play a critical role in coronary microvessel rarefaction observed in patients with CVD. It is important to note that many confounding factors may also contribute to the coronary microvascular changes observed in this patient population. Myocardial hypertrophy, chronic ischemia and atherosclerosis have all been individually associated with reduction in coronary reserve and may cause rarefaction (21). However, our study establishes a direct association between the increased myocardial CRP levels and decreased microvessel numbers. In this setting, CRP may act as a direct toxicant to the coronary microvascular endothelium and thus act as the major contributor to accelerate the microvascular changes caused by CVD in these patients.

Using a general live/dead assay for adherent endothelial cells, we detected significant loss of cell viability at clinically relevant concentrations of CRP (5 and 10µg/ml) after 48h of incubation. These studies were carried out in low-serum conditions in order to limit the cell growth during incubation period. Control cells in these studies were incubated in low serum in absence of CRP. These concentrations of CRP are within the range of 'moderate to high risk' plasma concentrations in patients with proven CVD disease risk. We then evaluated potential mechanisms of cell death (e.g., early apoptosis vs. necrosis), and found that after CRP exposure, significant increases in apoptotic cell death was detected in a concentration dependent manner. In contrast, the incidence of necrosis was relatively low (<5%) and not related to CRP concentration. Under the same experimental conditions and identical concentrations, heat denatured CRP did not show any significant effect on cell survival. There have been several reports in the literature suggesting that the cell death observed with CRP treatment is solely due to the azide content in the commercially available protein (22-24). Our data with the heat-denatured protein suggests that potential contamination in commercial CRP is not a possible mechanism of our observation. Our findings of CRP induced cellular apoptosis are consistent with those of Zhang et al (25) although they have

utilized higher CRP exposure levels (up to 25µg/ml) as compared to our investigations. We have utilized lower CRP concentrations in our studies because it has been previously demonstrated that even small changes in CRP levels can significantly increase disease risk (26, 27).

Increased reactive oxygen species are often seen in various settings of endothelial dysfunction, and in previous investigations we have demonstrated that this cell type is an avid producer of reactive oxygen and nitrogen species when stressed (28). Therefore, we next evaluated the effects CRP with respect to intracellular oxidant production by using live cell imaging. We observed significant increases in reactive species (as measured by the nonspecific detector of reactive nitrogen and oxygen species, DCF) at 48 h post CRP incubation at 5 and 10 µg/ml. It is important to note that in these experiments the incubation medium containing drug is removed at the time of oxidant detection (e.g., cells are washed and loaded with dye); thus, direct chemical oxidant production from CRP is not a possible mechanism of our observations.

Although at this time the source of intracellular oxidants is unclear, potential sites may include disruption of the mitochondrial electron transport chain or activation of various oxidase enzymes. In previous studies we have demonstrated that antioxidants such as NAC and ascorbic acid can significantly reduce drug induced oxidant production (29). Given these previous observations we evaluated the effects of this combined treatment (CRP plus ascorbic acid and CRP plus NAC) with respect to cell survival. Our aim was to distinguish between two possibilities: first, increased production of cellular ROS and cell death are two separate events induced by CRP, or second, that ROS production is a major cause of CRP induced cell death. We found that the addition of antioxidants significantly attenuated the CRP-induced endothelial cell death at 48 h. These observations are consistent with recent clinical reports suggesting that vitamin C supplementation yielded a 24.0% reduction in plasma CRP levels of healthy individuals exposed to active or passive smoking (30). Moreover these data are consistent with a mechanism of CRP-induced oxidant production, leading to initiation of apoptotic death sequences. Further characterization of the pathways involved in this process and targets involved in initiation are clearly warranted.

Fujii et. al. (31) have recently demonstrated that CRP, at concentrations known to predict cardiovascular events, may serve to impair endothelial progenitor cell (EPC) antioxidant defenses, and promote EPC sensitivity toward oxidant-mediated apoptosis and telomerase inactivation. Thus it would be of clinical relevance to further investigate the possible therapeutic value of the use of antioxidants supplementation in patients with elevated plasma CRP levels.

CONCLUSION

Many previous studies have documented that CRP can cause activation of various proinflammatory pathways including NF-κB activation (32). Here, we provide additional evidence that CRP itself may cause direct toxicity to this important cell type. Further, although the mechanisms of endothelial toxicity are not completely defined, they may be blunted by appropriate antioxidant use. These findings also suggest that the risk of atherosclerosis associated with CRP may not be exclusively related to proinflammatory pathway induction but also to a more vulnerable vascular surface because apoptotic endothelial cells making it more prone to plaque formation and thrombosis. Thus, we have demonstrated, for the first time, that CRP, at concentrations known to predict adverse vascular outcomes *in vivo*, has direct toxic effects on endothelial cells, and that the toxicity is mediated via reactive oxidant species.

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SUMMARY

We investigated the local myocardial CRP levels, cardiac microvessel densities and direct effects of CRP on human endothelial cells. There was significant reduction in number of microvessels and rarefaction was inversely correlated to myocyte CRP prevalence. CRP caused concentration dependent increases in oxidants and cell apoptosis. CRP may be a potential participant in endothelial toxicity and microvascular rarefaction.

Figure 1.

Increased prevalence of C-reactive protein levels in myocardium. Local myocyte production of CRP can be detected in human LV samples and myocardial CRP levels were significantly higher in the CVD samples (* - $p < 0.05$).

Figure 2.

Cardiac microvessel rarefaction in CVD. LV tissues were stained with CD-31, imaged at 400X magnification and microvessels were counted with automated macro. Significant reduction in LV tissue microvessel numbers was observed in the CVD samples as shown in the adjacent graph (* - $p < 0.05$).

Figure 3.

Increased myocardial CRP correlates with microvascular rarefaction. Statistically significant inverse correlation between relationship between local myocyte expression of CRP and prevalence of microvessels was observed in these human tissue samples.

Figure 4.

CRP causes endothelial cell death at these clinically relevant concentrations. There was a concentration dependent increase in cell death (Panel A). Upon further investigations, the major mechanism of cell death was via increased apoptosis (Panel B). Data expressed as mean±SEM of 6-8 individual observations (* - $p < 0.05$).

Figure 5.

CRP treatment causes time dependent increase in cellular oxidants at clinically relevant concentrations. The oxidant levels are measured as DCF signal intensity (Y-axis) and time points are shown on X-axis. All data expressed as mean $\pm$ SEM (* - $p < 0.05$).

Figure 6.

CRP cell toxicity can be prevented by chemical antioxidants. Cells were incubated with antioxidants in presence of CRP. Ascorbic acid and N-acetylcysteine demonstrated a cellular protective activity against CRP induced endothelial cell toxicity. Data expressed as mean $\pm$ SEM of 8 individual observations (* - $p < 0.05$).

Table 1

Population characteristics of patients. LV tissues were obtained at autopsy from normal controls and patients with documented evidence of CVD. The details of all the tissues obtained are shown in this table.

		CONTROL (n=12)	CVD (n=7)
Age (years)	Mean $\pm$ SD	38 $\pm$ 6	34 $\pm$ 6
	Median	39	36
	Range	25-44	26-41
Evidence of cardiac involvement Patient (%)	Myocarditis	0%	29%
	Pericarditis	0%	29%
	Pericardial Effusion	0%	14%
	Atherosclerosis	0%	86%
	Hypertrophy	0%	29%
	Myocardial Infarction	0%	14%