mCRP triggers angiogenesis by inducing F3 transcription and TF signaling in microvascular endothelial cells

Running title: CRP isoforms and angiogenesis

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ABSTRACT

Inflammation contributes to vascular disease progression. However, the role of circulating inflammatory molecules on microvascular endothelial cell (mECs) is not fully elucidated. The aim of this study was to investigate the effects of the short pentraxin CRP on microvascular endothelial cell angiogenic function. Subcutaneously implanted collagen plugs seeded with human mECs exposed to monomeric CRP (mCRP) in mice showed formation of an extended network of microvessels both in the plug and the overlyning skin tissue, while mECs exposure to pentameric native CRP (nCRP) induced a much milder effect. To understand the mechanisms behind this angiogenic effects, mECs were exposed to both CRP forms and cell migration, wound repair and tube-like formation were investigated. nCRP effects were moderate and of slow-onset whereas mCRP induced rapid, and highly significant effects. We investigated how circulating nCRP is transformed into mCRP by confocal microscopy and western blot. nCRP is transformed into mCRP on the mECs membranes in a time dependent fashion. This transformation is specific and in part receptor dependent, and the formed mCRP triggers F3 gene transcription and TF-protein expression in mECs to induce angiogenesis. F3-silenced mECs are unable to form angiotubes. In agreement, mCRP induced upregulation of the TF signaling pathway in mECs with downstream **phosphorylation** of AKT and activation of the transcription factor ETS1 leading to increased CCL2 release. The circulating pentraxin nCRP with little pro-angiogenic effect when dissociated into mCRP on the surface of mECs is able to trigger potent proangiogenic effects by inducing F3-gene upregulation and TF signaling.

Keywords: tissue factor, angiogenesis, vessel wall remodeling, C-reactive protein, endothelial cells.

Abbreviations:

nCRP: pentameric C-reactive protein form

mCRP: monomeric C-reactive protein form

HMEC-1: Human microvascular endothelial cells

F3: Tissue factor gen

TF: Tissue factor

VSMC: Vascular smooth muscle cells

BAEC: Bovine aortic endothelial cells

mECs: microvascular endothelial cells

siRNA: Silencing RNA

INTRODUCTION

C-reactive protein (CRP) is a member of the pentraxin family secreted by the liver and found in the circulation as a pentamer formed by five identical, non-covalently associated 23 kDa monomers (1). Increased CRP levels are associated with increasing risk of cardiovascular events(2, 3).

Circulating pentameric CRP (native CRP (nCRP)) can undergo dissociation into its monomeric form (mCRP), which has different biological properties from those of nCRP. mCRP is localized in fibrous tissues of normal blood vessel intima (4), in inflamed tissues (5) and it accumulates in human atherosclerotic lesions (6) where it colocalizes with macrophages (7) and vascular smooth muscle cells (VSMC) (8). Our group reported that local synthesis of CRP could contribute to carotid plaque neovascularization and to an increased risk of atherosclerotic plaque hemorrhagic transformation(6), even contributing to the likelihood of atherosclerotic plaque rupture(9). Angiogenic function evaluated in vitro with bovine aortic endothelial cells (BAECs) indicated that mCRP could be a potent inducer of angiotube formation(10). However a mechanistic analysis on how the inflammatory pentraxins induce angiogenesis was still missing.

In vivo angiogenesis is predominantly mediated by microvascular endothelial cells (mECs) sprouting from preexisting vessels, leading primarily to new capillaries (11). Angiogenesis and also inflammation appear to play a role in the process of plaque instability and plaque disruption. Angiogenesis is often found in the early vessel wall remodeling, and it favors leukocyte and macrophage infiltration in growing atherosclerotic lesions. Previous studies have reported that microvessel density was increased in coronary plaques prone to rupture (AHA-classification coronary type VI plaques) (12) and in ruptured and lipid-rich plaques when compared with fibrocalcific lesions (13).

Endothelial cells have a heterogeneous phenotype. Indeed, large vessel luminal ECs in direct contact with circulating blood, such as the ECs in the coronary arteries, do not express tissue factor (TF), whereas vasa vasorum in normal plaques and intraplaque mECs show endogenous TF expression(14). TF is a multifunctional signaling receptor involved in blood coagulation and in intraluminal arterial thrombosis triggered when inflammatory plaque content is exposed to circulating blood(15, 16). Beyond its thrombotic effects TF is involved in cell signaling(14, 17, 18).

We previously reported that mCRP, but not native pentameric CRP, significantly enhanced platelet activation, adhesion, and thrombus growth under arterial flow conditions (19). Further, we have recently shown that mCRP generated by the dissociation of nCRP on the surface of platelets attached to collagen (20) or to purified biological substrates has immediate prothrombotic effects(21).

Here our objective was to identify the mechanisms behind the angiogenic effect of CRP. We report that a) mECs promote CRP dissociation into mCRP; and, b) operative intracellular TF-signaling is required to induce the pro-angiogenic mCRP effects in mECs.

MATERIAL AND METHODS

CRP isoforms obtention

High purity human native CRP (Calbiochem) was stored in 10 mmol/L Tris, 140 mmol/L NaCl buffer (pH 8.0) containing 2 mmol/L $CaCl_2$ to prevent spontaneous formation of mCRP. mCRP was obtained by urea chelation from purified human CRP as previously described in the same low ionic strength buffer (19, 22). CRP preparations were processed and tested to avoid contaminants; as such, preparations were dialyzed in large Tris-HCl buffer volumes to fully remove sodium and endotoxin levels were below the detection limit of the limulus assay.

Blood collection

Freshly drawn venous blood from non-smoking healthy volunteers with informed consent was collected in 10 UI/mL sodium heparin. Blood samples were kept at room temperature, and used within 2 hours of collection. Donors claimed not to have taken any medication during 2 weeks prior to blood extraction. Procedures were approved by the Clinical Research Committee of the Hospital de la Santa Creu i Sant Pau.

Cell culture

Human microvascular endothelial cells (HMEC-1) were a kind gift from the Centre of Disease Control (Atlanta, GA).(23) mECs (HMEC-1) were cultured in MCDB131 media (Invitrogen) and supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine (Invitrogen) and 50 mg/mL gentamicin (Invitrogen) as previously reported (14, 18, 24). mECs were cultured in normoxic (21% O_2) conditions in an incubator with standard gas mixture (74% N_2 and 5% CO_2). Additionally, mECs were cultured in hypoxic (1%O_2) conditions in a Hipoxic/Anoxic Workstation:H35 (Don Whitley Scientific LTD.) with 94% N_2 and 5% CO_2.

Angiogenesis assay *in vivo*

All animal experiments were performed under approved protocols of the institutional animal use and care committee. Angiogenesis was investigated as previously described(14, 18, 25). Briefly, 1 x 10^7 mECs were suspended in 300 µl of Matrigel containing nCRP or mCRP (at 1, 3 or 10 µg/mL) and injected subcutaneously in nude mice. All mice were euthanized at seven days post-injection. Plugs and the surrounding skin were visualized macroscopically (Leica AF 6000LX Stereomicroscope with digital camera DFC, 8 bits of resolution, objective 1.0x0.03/1.0x0.09). Skin and plugs were fixed, embedded and stained with appropiate antibodies, or extracted for hemoglobin quantification. Negative control plugs contained only Matrigel. When indicated the experiment was performed with siRNA-random and siRNA-TF-mECs.

Hemoglobin quantification

Hemoglobin quantification was performed as previously described (26). Briefly, the Matrigel plugs were homogenized in 500 µl water on ice and cleared by centrifugation at 200g for 6 min at 4ºC. The supernatant was collected and used in triplicate to measure hemoglobin content with Drabkin's reagent (Sigma-Aldrich) according to manufacturer instruction. The absorbance was measured at 540 nm.

Angiotube formation in a 3D basement membrane

Three-dimensional (3D) cultures were prepared on three-dimensional basement membrane (BD Matrigel™) (BD Biosciences) as described (18). Cells were cultured for different times. Control and treated mECs migration was investigated by real-time

acquisition of images at 30-minute intervals for 8 hours (Leica DMIRE2). Images of 5 randomly selected areas of each plate were acquired, digitalized, and processed with Leica Software TCS-AOBS. The total tub length and the area covered by tubes from 3 independent experiments were measured.

Transwell migration assay

Chemotaxis assays were carried out with Transwell chambers. mECs were plated into modified Boyden chambers (Corning Costar Corporation) as described (14). Briefly, 6.5 nm transwell chambers with 8 µm pores (Corning) were coated with 10 µg/mL of type I collagen (Sigma) for 2 hours at 37º C. 5×10^4 cells/well were placed in the upper chamber and the lower chamber was filled with 500 µL of media containing nCRP (1, 3 or 5 µg/mL) or mCRP (1, 3 or 5 µg/mL). After 4 hours at 37º C, cells that migrated to the bottom of the membrane were fixed and stained with Diff-Quick (VWR Scientific Products). The total number of migrated cells was determined by counting five fields in each well per experimental condition using a phase-contrast microscope (magnification, x20).

Cell migration assay

Cells were seeded in 35 mm glass bottom sterile culture dishes until reaching confluence. The confluent cell monolayer was wounded by manually scraping with a soft sterile 200 µL pipette tip to create a double-sided wound. Confluent cultures (after scapping) of mECs were incubated with mCRP (0, 3 and 5 µg/mL) and with nCRP (0, 3, 5 µg/mL). Cell migration and wound repair were analyzed over a period of 8 hours (37°C) with 10% FBS. Images were taken under a 10x lens, using an inverted microscope (Leica DMIRE2). The area free of cells in each field was calculated using ImageJ software, and values were averaged. Measurements were made at time 0 and during cell migration. Cell migration and wound repair were quantified by measuring the residual cell-denuded area. Quantization was done by measuring the distance to the wound edge of the migrating as previously reported (14). Values were expressed as a percentage of the cell-covered area at time 0 and at the time to wound closure, as previously reported (27).

In a second approach, a multi-scratch wound-repair assay was performed. mEC were seeded on 90 mm diameter dish grown in the presence of serum and grown to confluence. The medium was changed 16 h before wounding. Confluent cells were injured with multiple parallel scratches by scraping with 0.1–2 µl tips across the dish (more than 50% of cells were detached from the dish). Cells were incubated in the presence or absence of 3µg/mL mCRP. Cells were allowed to migrate repairing the wound for 4 hours and RNA and protein were isolated. The objective was to collect cells in migration and we produced scratches to have more than 60% of wounded area (28, 29).

The experiments were performed in normoxic and hypoxic conditions.

CRP dissociation assay

Briefly, 2×10^5 mECs were seeded on 35 mm glass bottom sterile culture dishes. Cells were kept in MCDB131 supplemented with 10% FBS for 14 h. Cells were then incubated with nCRP (3 µg/mL) for 8 hours (37°C). The experiments were performed under normoxic and hypoxic conditions. Cells were processed for immunofluorescence, or extracted for protein and RNA analysis.

In order to study cell receptors involved in CRP dissociation, mECs were incubated for 1 hour with blocking antibodies against CD32 (100 µg/mL) (AF1330 and AF1257 respectively, R&D Systems). Then, cells were treated with nCRP (3 µg/mL) and incubated over a period of 8 hours (37°C). These experiments were performed under normoxic conditions. Cells were processed for immunofluorescence to detect mCRP expression on the cell membrane.

Immunofluorescence staining

Cells were rinsed with PBS pH 7.4 and fixed with 3.8% paraformaldehyde for 15 min. Fixed slides were washed with PBS and incubated in blocking buffer. Then, they were incubated in Signal Enhancer for 30 min and washed again. Immunodetection of CRP isoforms was performed with the monoclonal antibodies against nCRP (clone 1D6) and mCRP (clone 8C10), kindly provided by Dr LA Potempa. Cell nuclei were counterstained with Hoechst 33342.
Five-micrometer-thick frozen Matrigel plug sections were prepared for immunostaining as described(30). Controls were stained with secondary antibodies only.
Images were recorded by Leica inverted fluorescence confocal microscope (Leica TCS SP2-AOBS, Wetzlar, Germany) and coverslides were viewed with HCX PL APO 20X/0.7 IMM Corr and HCX PL APO 63x/1.2 W Corr/ 0.17 CS objectives. Fluorescent images were acquired in a scan format of 1024 x 1024 pixels in a spatial data set (xyz or xzy) and were processed with the Leica Standard Software TCS-AOBS. Controls with no primary antibody and controls without cells showed no fluorescence labeling.

Protein Isolation

Briefly, supernatants were collected and cells were washed twice with PBS. Cells were scraped and stored frozen (-80 ºC) and supernatants were precipitated with acetone during at least 1 hour at -20ºC.
Endothelial cells were sequentially extracted based on differential protein solubility (31). Tris-soluble (cytosolic) fraction and urea-detergent soluble (cytoskeleton/membrane) fractions were obtained. Sample contaminants (salts, lipids, etc.) of protein extracts from cells and supernatants were removed with a commercial kit (2D-CleanUp Kit, Amersham Biosciences, UK) and protein concentration was measured with 2D-Quant Kit (Amersham Biosciences, UK) as indicated by the manufacturer.

Western Blot Analysis

Cell supernatants and membranes (25 µg protein) were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes. Detection was performed with: monoclonal antibodies against nCRP (clone 1D6) and mCRP (clone 8C10), rabbit TF antibody (American Diagnostica), rabbit anti-phospho-Akt Ser473 antibody, rabbit anti-Akt antibody (Cell Signaling), rabbit anti-phospho-Ets-1 Thr38 antibody (Novus Biological), and mouse Ets-1 (1G11) (Abcam). Band densities were determined with the ChemiDoc™ XRS system (Bio-Rad) in chemiluminescence detection mode and analyzed with Quantity-One software (Bio-Rad).

Real time PCR

RNA from cell lysates extracted by RNeasy isolation kit (Qiagen) was reverse transcribed. mRNA levels were analyzed by real time PCR. Assays were used for TF (Hs00175225m1), and CD32 (Hs01634996_s1 and hCG1642498 (Thermo Fisher scientific) as described(14). Human GAPDH (4326317E) (Thermo Fisher scientific) was used as an endogenous control. Taqman real time PCR was performed as previously described(32). mRNA expression was quantified and normalized by GAPDH.

Silencing RNA (siRNA)

F3 and CD32 silencing by siRNA were performed with a Nucleofector device and its corresponding kit (Amaxa, Inc.). The transfection protocol was performed following manufacturer's instructions using the T16 program, as we have previously reported(14). The scrambled siRNA or annealed pre-designed siRNA against TF (s4932), and CD32 (s194408 and s5073) were purchased from Thermo Fisher scientific. In all experiments, a scrambled siRNA was used as control.

Tissue Factor procoagulant activity

Briefly, 200 x 10^3 cells were seeded on 35 sterile culture dishes with 500 µL of media containing nCRP (0, 1, 3 or 5 µg/mL) or mCRP (1, 3 or 5 µg/mL). After 2, 4, 6, 8, and 24 hours cells supernatant was collected and tissue factor procoagulant activity (TF-PCA) was measured by using a commercially available chromogenic FXa generation assay (Actichrome TF, American Diagnostic) following the manufacturer's instructions.

CRP proliferation assay

To determine CRP dependent mECs proliferation cells were incubated with **nCRP (0,1, 3 or 5 µg/mL) or mCRP (1, 3 or 5 µg/mL). After 2, 4, 6, 8, and 24 hours** cells were harvested, ethanol (70%) fixed overnight at -20ºC, stained with propidium iodide (50 µg/mL) followed by flow cytometry analysis.

Statistical analysis

Results are expressed as mean ± SEM and tested for statistical significance by the two-tailed Student's *t* test or ANOVA, followed by the Dunnett's test, to determine statistical significance between treatments. $P<0.05$ was considered statistically significant.

RESULTS

Angiogenesis assay *in vivo*

To investigate the in vivo effects of CRP we subcutaneously inoculated nude mice with mECs suspended in Matrigel. Three conditions were investigated: control cells, nCRP exposed (1, 3 and 10 μg/mL)-mECs and mCRP exposed (1, 3 and 10 μg/mL)-mECs. Angiogenesis, both in the Matrigel plug and in the overlying skin tissue, was evaluated after 7 days. nCRP at low concentrations (1 and 3 μg/mL) did not stimulate the formation of blood- filled microvessel (Fig 1A Matrigel plug; Fig 1B hemoglobin concentration). Only the highest nCRP concentration (10 μg/mL) induced the development of thin microvessels (P<0.01). mCRP induced a statistically significant formation of thin and long blood-filled microvessels from the lowest tested dose of 1 μg/mL. Quantification of hemoglobin content in the retrieved plugs indicated competent neovessels (Fig 1B). mCRP-mECs plugs had a significantly increased blood-perfused neovessel network formed at all tested concentrations (P<0.01 at 1, and P<0.001 at 3 and 10 μg/mL). The statistical comparison between the two CRP forms demonstrated that the effect of mCRP was significantly higher at all concentrations (P<0.05 at 1 and 3, and P<0.01 at 10 μg/mL).

As shown in Figure 1A (bottom), the skins overlying the plugs were analyzed. There was little vascularization over plugs containing nCRP-mECs. Only the highest nCRP dose (10ug/ml) induced a mild but significant vascularization (p<0.05). Instead vascularization over mCRP-mECs plugs was highly significant (Figure 1C). These results indicate that mCRP is able to induce angiogenesis in vivo rapidly and efficiently. The mechanisms behind this significant *in vivo* pro-angiogenic effect of mCRP were further investigated in in vitro experiments.

Endothelial tube formation assay

To understand mCRP-induced angiogenesis we first tested mCRP effects in 3D-BM cultures (Fig 2A). Both CRP forms induced formation of angiotubes but with different patterns and density. The appearance and distribution of the angiotubes in mCRP treated mECs was very different from that obtained in untreated mECs or in mECs treated with nCRP (Fig 2A). mCRP induced newly formed tubes that connected in a very complex way to form fairly extensive networks while nCRP produced individual capillary-like tube formation. In fact, the quantification of the area covered by tubes revealed that mCRP, at any tested concentration, induced a significantly larger network of angiotubes than control cells (P<0.01 at 1 μg/mL and P<0.001 at 3 and 5 μg/mL) and nCRP-mECs (P<0.01 at 3 μg/mL and P<0.001 at 1 and 5 μg/mL) (Fig 2B-2C). Representative larger images of nCRP and mCRP-induced tube formation, shown in Figure 2D, evidence the different angiotube network density induced by the two CRP forms.

Chemotaxis and wound healing assay

In the chemotaxis assay, we observed that whereas nCRP did not induce mECs migration, mCRP significantly induced mEC motility, achieving a significant difference from controls at 3 μg/mL of mCRP (P<0.05) (Fig 3A). The difference between the two CRP forms was significant at all tested concentrations (P<0.05 at 1 μg/mL and P<0.001 at 3 and 5 μg/mL).

Wound-repair analysis indicated that the time to closure and the kinetics of cell repair were also differentially affected by both isoforms (Fig 3B-3C). Representative images of phase-contrast micrographs after 4 hours of incubation are shown in Figure 3B. The analysis of cell coverage rate (covered surface/time) during the first two hours of

incubation showed that both CRP forms induced a faster repair of the wounded area than untreated cells. However, mCRP induced a faster repair than nCRP (P<0.01 and P<0.001 at 3 and 5 μg/mL) (Fig 3C). Accordingly, time to wound closure was significantly shorter with mCRP (Fig 3D). The short time period in the migration assay (interval of 4 to 6 hours) is below cell doubling time and along with Boyden chamber results evidence that both CRP forms affect migration rather than proliferation.

In a parallel experiment with multi-scratch wounding, to analyze the effect of hypoxia, we evidenced that mCRP (3 μg/mL) also induced rapid repair (within 4 hours) in hypoxic conditions (Fig 3E).

CRP induces tissue factor expression in mECs

We had previously shown that in mECs autocrine TF-signaling induced migration and angiogenesis (18) . Indeed, upregulation of F3 transcription in migrating mECs was already detectable at 2 hours and peaked at 4 hours(18), times compatible with the functional changes evidenced by treating mECs with mCRP. Therefore we investigated whether the effects of mCRP on mECs-angiogenesis could be mediated by triggering F3 upregulation and TF-signaling. Real time PCR experiments showed that mECs treated with mCRP (3 μg/mL) for 4 hours significantly increased TF mRNA levels both in normoxic and hypoxic conditions (P<0.05) (Fig 4A; 4D). Interestingly TF mRNA levels were induced by mCRP but not by nCRP (3 μg/mL /4hours) (P<0.05) (Fig 4B; 4E). Accordingly, TF protein levels expressed on mECs cells were significantly increased only after mCRP treatment (P<0.05) (Fig 4C; 4F). We had previously shown that autocrine TF-signaling in mECs induced phosphorylation of Akt and Ets and upregulation of CCL2 expression. Accordingly mCRP 3μg/ml treated mECs, after 0, 2, 4, 6, 8 h of culture showed upregulation of phospho-Akt and of phospho-Ets-1 (Fig 4G). The release of CCL2, marker of TF-induced mEC-angiogenesis(18), was significantly increased in mCRP exposed microvascular endothelial cells (Figure 4H).

mCRP, TF and mECs migration

To determine whether upregulation of *F3* transcription and TF protein signaling mediated CRP- induced migration, the *F3* gene was silenced in mECs and migration analyzed as described above by wound-repair analysis. Silencing of the F3 gene by siRNA-TF in mECs induced a reduction of 88+-2.5% in mRNA transcripts and disappearance of the TF-protein band (tested by western blot), as previously reported. Apoptosis in response to TF silencing was investigated by TUNEL and there were negligible levels of TUNEL-positive cells after silencing (3+-1% in scrambled siRNA treated cells and 2+-1% in siRNA-TF treated cells), similarly to what we have previously shown (14, 18).

Migration was analyzed as described above and the area under curve (AUC) after 6 hours showed that mCRP treated cells repaired the wound at a significantly faster rate than the controls (scrambled-siRNA-mECs) in normoxic and hypoxic conditions (P<0.001 and P<0.05 respectively) (Fig 5A-B). The cell growth kinetics (measured by cell covered area) evidenced that mECs exposed to mCRP migrated faster from the beginning (Suppl. Fig I). Silencing TF abrogated the effects of CRP on mEC-migratory function, in both normoxia and hypoxia (Fig 5A-B). The evaluation of time to wound closure (TWC) similarly showed that silencing-TF prolonged TWC. All together these results indicate that the effects of CRP on migration are mediated by *F3* gene expression (Fig 5C-D).

mCRP and mECs procoagulant activity

We determine the procoagulant effect of nCRP and mCRP on mECs (Suppl. Fig I). The cell supernatant exhibited increased procoagulant activity (PCA) only in incubations at the highest nCRP concentration (5μg/mL), PCA was increased in a time-dependent fashion reaching significance at 6 hours (*P*<0.05). On the contrary, PCA of mECs exposed to mCRP was increased in a concentration and time-dependent fashion at all concentrations (1, 3, and 5 μg/ml) from 2 hours until 8 hours. At 24 hours mECs procoagulant activity (nCRP and mCRP exposed cells) were at levels of the control cells.

mCRP and mECs-proliferation

We evaluated the modifications in the cell cycle induced by nCRP and mCRP (1, 3, and 5μg/mL) in a time dependent fashion. Cell cycle analysis by flow cytometry did not show significant differences in the expression patterns between CRP forms, concentrations, or incubation times (Suppl. Fig II).

mCRP, TF and angiogenesis

In vivo angiogenesis was tested as above, by subcutaneously inoculating nude mice with matrigel containing F3-silenced-mECs or with random-siRNA- controls, with and without mCRP (3μg/mL) (Fig 5E). mCRP induced angiogenesis in F3-expressing cells but could not induce angiogenesis in F3 silenced cells. Additionally, mCRP did not rescue angiotube formation in TF silenced mECS. Quantification of hemoglobin content showed that competent neovessel formation was not induced when F3 was silenced in mECs (Fig 5F).

All together these results suggest that functional tissue factor gene expression is required for mCRP-induced pro-angiogenic effects *in vivo* as well as previously seen *in vitro.*

CRP is dissociated by mECs-membranes

nCRP showed the potential to induce migration but at a delayed time with respect to mCRP **(Suppl. Fig III).** Therefore, we investigated whether nCRP **undergoes** dissociation into mCRP by interacting with mECs and then triggering neovessel formation.

mECs were exposed to nCRP, afterwards supernatants were removed and cell membranes were blotted with antibodies against nCRP and mCRP. Whereas nCRP was reduced (and almost disappeared from mECs membranes) in a time-dependent fashion (Fig 6A), mCRP (absent up to 2hours) appeared on the mECs membrane in a time-dependant fashion. The cells and supernatants collected at 2-hours intervals for up to 8 hours confirmed the confocal microscopy observations. Whereas nCRP on supernatants were slowly disappearing with prolonged incubation time, reaching a statistically significant reduction after 6 hours ($p<0.05$) (Fig 6B), mCRP was increasingly appearing with prolonged incubation time in the cell membrane (*P*<0.05) (Fig 6C). Similar results were obtained when cells were cultured under hypoxic conditions **(Suppl. Fig IV).**

mCRP dissociation on the mECs membranes was found partially dependent on CD32. Pre-incubation of mECs with CD32 blocking antibodies significantly reduced nCRP to mCRP transformation as seen by confocal microscopy (Fig 6D). The computer assisted morphometric image analysis indicated that blocking CD32 significantly reduced nCRP to mCRP transformation (*P*<0.01) (Fig 6E). In fact at long incubation times (from 4 to 8 hours) monomerization into mCRP was abrogated in mECs without available membrane CD32 receptor (Fig 6D). The same results were obtained when CD32 was silenced (Fig 6D-6F).

DISCUSSION

Inflammation plays a crucial role in atherosclerosis (33), and it has been associated to exacerbated angiogenesis although the mechanisms are not known (34-36). Inflammation might specifically exert distinct effects on the microcirculation of different target organs affecting their function when acute phase proteins, as CRP are released to plasma from the liver (1).

CRP seems to be not only a predictive biomarker but also a causal factor on cardiovascular disease progression (37). As an effector molecule, growing data suggests that CRP may directly contribute to endothelial dysfunction (38). However, the mechanism by which CRP may affect angiogenic function has not been elucidated. In addition, we are starting to evidence that the pentameric liver-released form and the monomeric CRP form have different effects. In the present study we demonstrate that mCRP exerts potent angiogenic effects on microvascular endothelial cells. **We also describe a link between mCRP and procoagulant activity.** In addition we report for the first time that CRP dissociates into mCRP on the endothelial cell membrane and that the mCRP induces angiogenic effects by upregulation of F3 gene transcription and TF signaling.

Endothelial cells could be directly activated by mCRP through its interaction with lipid raft microdomains to mediate pro-inflammatory cellular responses (39) . mCRP is the bioactive conformational form triggering proinflammatory effects on the endothelium(40, 41). Growing evidence indicated that generation of mCRP can occur spontaneously by oxidative stress, low pH, and bioactive lipids from activated or damaged cells, but it can be accelerated in vivo under pathological conditions by bioactive lipids such as lysophosphatidylcholine exposed on the surface of activated or damaged cells. Previous studies described the effect of native CRP on endothelial cells with no detectable changes before 4 hours (38, 42, 43). The CRP induction became detectable only after 6 to 12 hours of incubation, reaching maximal effects at 24 hours coinciding with in vitro kinetics of dissociation into subunits(44). Our study shows that nCRP can induce TF expression after a 4-hour incubation period. In human phagocytes and in endothelial cells, native CRP binds primarily to Fcγ receptor IIa (CD32) and to some extent to FcγRI (CD64) (41, 45). It has been reported that CRP increases endothelial expression of CD32 in human and bovine aortic endothelial cells (HAECs and BAECs) (41, 46), and that CD32 can contribute to the proatherogenic effects of CRP (47). In mECs the expression of CD64 is very low (data not shown), therefore we studied the relative role of CD32 in CRP-monomerization. We demonstrate that mCRP transformation was abolished when CD32 Fcgamma receptor was blocked (by siRNA or by a specific antibody) in the microvascular endothelial cells. We found a significantly reduced CRP monomerization indicating a receptor-regulated process. In platelets we previously showed that the receptor involved in CRP monomerization was the heterodimer GPIIb-IIIa (21).This mechanism should occur mainly in loci with chronic or persistent inflammation (e.g., atherosclerotic lesions), where CRP is significantly up-regulated and interaction with microvascular endothelial cells is increased.

We hypothesized that the pro-angiogenic effects of mCRP in mECs were dependent on an operative F3 gene. TF the protein encoded by the F3 gene is a transmembrane signaling receptor expressed in ECs(48) and involved in inflammation (18) and angiogenesis (49) being a central player in arterial wall remodeling (25). Previous studies showed that in mECs autocrine TF signaling (14) as well as monocyte-derived Wnt-5 (50) contributes to neovessel formation. The maturation of the newly formed microvessels was facilitated by TF- induced CCL2 secretion that recruited HVSMCs

toward ECs (14). This mature neovessel formation via CCL2 expression and release was mediated by transcription factor Ets-1 upregulation and activation(18). Wu et al(51) observed TF expression in carotids of CRP-transgenic mice, associated the protein to vascular smooth muscle cells and concluded that TF expression is triggered by native CRP-induced ROS generation and Cirillo et al (52) reported native CRP-induced proliferative effects on the vessel wall, both ECs and VSMCs. Here we are specifically studying mCRP-effects on microvascular endothelial cells and have identified that only mCRP is able to induce F3 upregulation and to significantly elevate TF protein levels in mECs. **We also provide evidence that mCRP are associated with an increased pro-coagulant activity in the microvascular endothelial cells secretome**. The pro-angiogenic effects of mCRP are abrogated by F3 gene silencing, clearly showing the importance of TF in the process. Finally, mCRP induces pAKT, pETS1 and CCL2 secretion, following the pattern of activation of TF-induced signaling. Therefore, activation of the axis F3-TF-ETS1-CCL2 is required for the pro-angiogenic function of mCRP.

Conclusion

In conclusion, our data show that in microvascular endothelial cells mCRP induces angiogenesis *in vivo* and promotes migration, wound repair and tube-like formation.

The circulating CRP pentameric form that circulates in blood after its secretion by the liver can be dissociated into monomers **on** the surface of microvascvular endothelial cells partially via the Fcγ receptor CD32 and once monomerized CRP triggers angiogenic effects by inducing TF-signaling.

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FIGURE LEGENDS

Figure 1: Effect of CRP in angiogenesis assay *in vivo*. Blood vessels formed in Matrigel- plugs an skin tissue *in vivo*. Seven days after implantation the tumor and the surrounding skin were removed. **(A)** Top: Control human mECs and mECs treated with 1, 3 or 10 µg/mL of either nCRP or mCRP. Scale bar: 5 µm. Bottom: A macroscopic view of representative skin tissue 7 days after injection of Matrigel plugs. Scale bar: 1 mm. Images are representative from 3 animals for each group. **(B)** Colorimetric determination of hemoglobin concentration in Matrigel plugs. Seven days after implantation, plugs were removed and hemoglobin content analyzed. **(C)** Quantification of tube % covered area. Bars represent the mean ± SEM (N=3). Statistical analysis was performed by ANOVA followed by the Dunnett's test to determine statistical significance between doses and Student's *t* test between treatments (**P*< 0.05, ***P* < 0.01 and ****P* < 0.001 vs control).

Figure 2: mCRP induces microvessel formation in mECs. (A) Phase-contrast micrographs showing the morphology of human mECs treated with different concentrations of nCRP or mCRP for 8 hours in 3DBM culture Original magnification x10 (N=3). Scale bar: 100 µm. **(B)** The area covered by tubes and **(C)** average of total tube length were measured. Three independent experiments were run with each plate being measured at 5 randomly selected areas. Values are mean ± SEM. Statistical analysis was performed by ANOVA followed by the Dunnett's test (*P<0.05, **P<0.01 and ***P<0.001 vs control). (D) 3 µg/mL of nCRP or mCRP for 8 hours hours in 3DBM culture. Original magnification x10 (N=3). Scale bar: 100 µm.

Figure 3: Effect of CRP in mEC migration. (A) Chemotaxis assay in Boyden chamber. Bars represent the mean ± SEM (N=3). Statistical analysis was performed by ANOVA followed by the Dunnett's test (**P*<0.05, ***P*<0.01 and ****P*<0.001 vs nCRP or control). **(B)** Phase-contrast micrographs showing the wounded area covered by mECs treated with different concentrations of nCRP or mCRP after 4 hours of incubation. Original magnification x10 (N=3). Scale bar: 100 µm. **(C-D)** Migration wound-repair assay. Growth kinetics measured by **(C)** the velocity of coverage during 2 hours of incubation (cell covered area (%) / Time (h)) and **(D)** the time to wound closure (N=4). Statistical analysis was performed by ANOVA and Student's *t* test between treatments (**P*< 0.05, ***P* < 0.01 and ****P* < 0.001 vs ctrl). **(E)** Migration assay after multi-scratch wounding. Confluent cultures were wounded by a multi-scratch procedure and incubated for 4 hours in the presence or absence of 3 µg/mL mCRP. Experiments were performed in normoxic and hypoxic conditions. Results are expressed as cells coverage area (%) relative to initial point. (N=4). Statistical analysis was performed by Student's *t*-test (****P*<0.001 vs control).

Figure 4: CRP induces *F3* transcription and TF signaling in human microvascular endothelial cells. mECs were incubated with nCRP (3 µg/mL) or mCRP (3 µg/mL) in **(A-B-C)** normoxic and (**D-E-F)** hypoxic conditions. In **A-D**, *F3* transcript levels were measured in mECs before and after 4 hours of incubation with mCRP In **B-E,** *F3* transcription regulation by nCRP and mCRP. Results are expressed as relative (mean± sem; N=4). **(C; F)** TF protein levels by Western blot at 4 hours normalized by β-actin (control ± sem; N=3). Statistical analysis was performed (A; D) by Student's *t*-test and (B-C; E-F) by ANOVA test (**P*<0.05 vs ctrl or time 0). **(G)** Western blots of mCRP

3µg/ml treated HMEC-1 after 0, 2, 4, 6, 8 h of culture. Western blots show phospho-Ets, Ets protein, phospho-Akt and the Akt protein. To test for the equal loading, western blots were reprobed for β-actin. n = 4. **(H)** Concentration of CCL2 in the supernatants of endothelial cells incubated with mCRP (3µg/ml).

Figure 5: Tissue factor (TF) is required for CRP pro-angiogenic effects in human microvascular endothelial cells (mECs). Confluent cultures of HMEC-1 or HMEC-1 F3-siRNA monolayers were wounded by scraping and incubated with nCRP (3 µg/mL) or mCRP (3 µg/mL) in **(A; C)** normoxic and **(B; D)** hypoxic conditions. Growth kinetics **(A-B)** by area under the curve of the cell covered area relative to initial point after 6 hours and **(C-D)** by the time to wound closure. (N=3). Statistical analysis was performed by ANOVA followed by the Dunnett's test (**P*<0.05; ***P*<0.01 and ****P*<0.001 vs scr-siRNA). **(E)** Blood vessels formed in Matrigel plugs *in vivo*. Vascularity of HMEC-1 Scr-siRNA or HMEC-1 F3-siRNA with and without 3 µg/mL mCRP was photographed. Images are representative from 3 animals for each group. Original magnification, 10X; scale bar 1000 mm. **(F)** Colorimetric determination of hemoglobin concentration in Matrigel plugs. Seven days after implantation, Matrigel plugs were removed to analyze the hemoglobin content. Bars represent the mean ± SEM (N=3). Statistical analysis was performed by Student's *t* test (****P* < 0.001 vs ctrl).

Figure 6: nCRP is dissociating into mCRP in mECs membrane. (A) Representative confocal images of the nCRP treated mEC cultures after 2, 4, 6 and 8 hours of incubation in normoxic conditions. Images show double immunofluorescence staining of nCRP or mCRP (green) as indicated and Hoechst dye to stain nuclei (blue). Scale bar: 10 µm. **(B-C)** Western blot analysis of (B) nCRP on supernatant and (C) mCRP in mEC. Data are given as mean (normalized with Ponceau staining or with β-actin) ± SEM. (N=3). Statistical analysis was performed by ANOVA followed by the Dunnett's test (**P*<0.05 vs time 0). **(D)**Confluent cultures of mECs were preincubated for 1 hour with anti-CD32 (100µg/mL) or siRNA CD32 in normoxic conditions. Then, all were treated with nCRP (3µg/mL) and mCRP was detected after 4 and 8 hours of incubation Representative confocal images of double immunofluorescence staining of mCRP (green) and Hoechst (blue). Scale bar: 10 µm. **(E)** mCRP in mECs was quantified by computer assisted image morphometric analysis. Data are given as mCRP positive area per cell (μm^2/cell) ± SEM. (N=3). Statistical analysis was performed by Student's *t*-test (**P*<0.05; ***P*<0.01 vs control). **(F)** mCRP formation from 4 hours to 8 hours of incubation (N=3). Statistical analysis was performed by Student's *t*-test (****P*<0.001 vs control).