Published in final edited form as:

Inflamm Res. 2009 January ; 58(1): 30–37. doi:10.1007/s00011-008-8156-9.

Activation by C5a of endothelial cell caspase 8 and cFLIP

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Abstract

Objectives and design—In this study, we examine the relationship between C5a and activation of cysteine aspartic acid protease 8 (caspase 8) in human umbilical vein endothelial cells (HUVEC).

Materials or subjects—Primary cultures of HUVEC were used.

Treatments—Recombinant human C5a (50 ng/ml) was used in the presence or absence of 10 µg/ml cycloheximide (CHX).

Methods—HUVEC were treated with C5a alone and in the presence of CHX, then monitored for cell viability, poly-ADP-ribose 1 (PARP-1) and caspase 8 activities. Gene and protein expressions were assessed for caspase 8 and the caspase 8 homologue, FLICE –inhibitory protein (cFLIP).

Results—We found a 43.1 ± 6.9 percent reduction in viability of HUVEC stimulated for 18 h with 50 ng/ml C5a in the presence of 10 µg/ml CHX ($p < 0.05$). In contrast, the cell viability of cells stimulated for 18 h with 50 ng/ml C5a or 10 µg/ml CHX alone was not significantly different compared to the non-stimulated control. Treatment of HUVEC with C5a induced an increase in caspase 8 activity but did not significantly affect cFLIP levels.

Conclusions—These data suggest caspase 8 activation induced by C5a leads to cell death if protein synthesis of anti-apoptotic protein(s) is blocked.

Keywords

Complement factor 5a (C5a); Cycloheximide (CHX); Caspase 8; Caspase 8 homologue FLICE; Inhibitory protein (cFLIP)

Introduction

The endothelium plays an important role in inflammatory syndromes, such as sepsis and atherosclerosis [1,2,3,4,5]. In the case of sepsis, high levels of pro-inflammatory mediators (C5a, TNF α , LPS, IL6, and FasL) are produced and can be linked to altered apoptotic cell death. Although research has focused on myeloid cells (e.g., neutrophils, lymphocyte, macrophages), recent reports suggest that non-myeloid cells play an important role during inflammatory syndromes. For example, apoptosis of endothelial cells has been reported in regions of atherosclerotic plaques, suggesting endothelial cells are capable of apoptosis under specific stimuli [6]. Other studies have found elevated levels of shed apoptotic endothelial cells or endothelial cell microparticles in the sera of septic and multiple sclerosis patients [7,8].

Primary cell culture studies have shown that endothelial cells are resistant to cell death when stimulated with TNF α , IL-1 or LPS [9,10,11,12]. This resistance has been attributed to cytoprotective proteins, which modulate apoptotic reactions at proximal (e.g. cFLIP), intermediate (e.g., Bcl-2, Bcl-x), and distal (e.g. BIRC2, TNFAIP3) apoptotic cascade locations. This has been supported with cecal ligation and puncture (CLP) models showing endothelial apoptosis corresponds to suppressed levels of Bcl-2 [13]. The protein cFLIP has received attention because of its upstream inhibition of apoptosis. Although the mechanism by which cFLIP attenuates cell death has not been fully elucidated, it has been suggested that cFLIP acts as a competitive inhibitor to caspase 8 by blocking caspase 8 binding to the death induced signaling complex (DISC) [14]. This is supported by other reports suggesting that cFLIP is cleaved into a p43 subunit that attaches to DISC, preventing the processing of caspase 8 into its active form [15,16]. Overall, this action results in a reduction of the long or “inactive” form of cFLIP. However, several cell types (HUVEC, human aortic endothelial cells, HL60, Jurkat) stimulated by either TNF α , FasL, or LPS initiate activation of MAP kinase 1 (MEK1), phosphatidylinositol 3-kinase/Akt, and NF κ B pathways, resulting in gene transcription and protein synthesis of cFLIP so that cytosolic levels remain constant [17,18,19]. It appears that maintaining cytosolic levels of cFLIP, along with other anti-apoptotic proteins, is important for endothelial cell survival after exposure to apoptosis inducing agents.

There is increasing evidence that anaphylatoxins (C5a, C3a) promote pro-inflammatory conditions contributing to altered cellular apoptosis [20,21,22,23]. C5a is known as a potent stimulus, inducing chemotaxis, release of granules, and the generation of reactive oxygen species from phagocytic cells. Yet, recent studies have suggested that C5a plays a role in the apoptotic process during systemic inflammatory conditions. C5a is thought to have an anti-apoptotic effect on neutrophils, during sepsis. Studies suggest that the ligation of C5aR (CD88) activates PI-3K and MAPK survival signaling pathways in neutrophils, thus suppressing the apoptotic response [23]. Expression of C5aR was initially thought to be restricted to monocytes/macrophages and polymorphonuclear cells, but it now appears to include several other cell types, including endothelial cells [24,25,26]. The actions of C5a-C5aR in non-myeloid cells *in vivo* are less clear. Although, it has been demonstrated that C5a produces robust gene expression response in human umbilical vein endothelial cells (HUVEC), including genes associated with cell adherence (E-selectin, ICAM-1, VCAM-1), chemokines (IL-1, IL-6), growth factors (e.g. VEGF, PDGF), and apoptosis (caspase 3, caspase 8, cFLIP) [27].

For this study we chose to investigate the relationship between C5a induced stimulation of HUVEC and activation of caspase-8 and cFLIP. Our data suggest that C5a stimulation alone did not significantly affect cell viability compared to non-stimulated controls. However, preincubation of cells with cycloheximide (CHX) followed by C5a stimulation significantly reduced cell viability. In addition, we found caspase 8 activity increased after C5a stimulation, which coincided with reduced cytosolic levels of cFLIP.

Material and Methods

Endothelial Cell Culture

Primary HUVEC were isolated and plated in T-75 flasks coated with 1% gelatin (Sigma, St. Louis, MO). Cells were cultured in medium 199 (Gibco BRL, Grand Island, NY) containing endothelial growth supplement (Sigma, St. Louis, MO), 20 % fetal bovine calf serum (Hyclone, Logan, UT), 5 % penicillin/streptomycin (Gibco BRL, Grand Island, NY) and 25 μ g/ml heparin (Sigma, St. Louis, MO). All experiments were performed using the first 3–4 cell passages. A CHX pre-incubation period of 30 min was used for experimental conditions where CHX and C5a were used in combination.

Cell Viability Assay

HUVEC were plated into 12 well plates at 1×10^5 cells/well and allowed to attach for 24 h. Prior to experimentation, media was removed and replaced with 0.5 ml of fresh media. Confluent, HUVEC were stimulated with C5a (50 ng/ml), C5a (50 ng/ml) with CHX (10 μ g/ml). Controls included non-stimulated cells and cells incubated with CHX (10 μ g/ml) for 18 h. After 18 h, the media was removed, replaced with 0.5 ml fresh media. Next, 100 μ l of CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI) was added to each well, incubated at 37 °C for 2 h, then read at 490 nm.

Caspase-8 Activity Assay

Caspase-8 activity was detected using a caspase-8 fluorescent assay kit (BD Bioscience, Palo Alto, CA). HUVEC were plated into 12 well plates at 1×10^5 cells/well and allowed to attach for 24 h at 37 °C prior to experimentation. Cell supernatant fluids were removed and replaced with 0.5 ml of fresh medium then stimulated with C5a (50 ng/ml) alone or with CHX (10 μ g/ml) for 18 h. Controls included non-stimulated cells and cells incubated with CHX (10 μ g/ml) alone for 18 h. After stimulation, the cells were washed with 37 °C HBSS, lysed with lysis buffer (BD biosciences, San Jose, CA) and incubated on ice for 10 min. Cell lysates were centrifuge at 10000 rpm for 3 min and 250 μ l of the supernatant was added to 250 μ l of reaction buffer containing 25 μ l of Caspase-8 substrate. This solution was incubated for 1 h and 30 min at °C, then read in a microplate reader with a 400 nm excitation and 550 emission filter. Quantitation of sample was performed as described in the kit protocol, using a 7-amino-4-trifluoromethyl coumarin (AFC) standard curve. Samples were normalized by calculating the relative fluorescent units per microgram of protein (RUF/ μ g).

RT-PCR

HUVEC were stimulated with C5a alone or in the presence of CHX for 0, 4, and 8 h. CHX control involved incubating HUVEC with CHX for 8 h. One microgram total RNA was reversed transcribed with superscript reverse transcriptase (Invitrogen, Carlsbad, CA) using random and poly dT primers for first-strand cDNA synthesis. This was repeated for each timepoint. Primers for each gene of interest were designed using Primer Select software and included: cFLIP: forward primer, 5'-GGGGACTTGGCTGAACTGCTCTA-3', reverse primer, 5'-AAATCCTCACCAATCTCTGCCATCA-3'; Caspase-8: forward primer, 5'-CACTAGAAAGGAGGAGATGGAAAG-3', reverse primer, 5'-CTATCCTGTTCTCTTGGAGAGTCC-3'; GAPDH: forward primer, 5'-CGGAGTCAACGGATTTGGTCGTAT-3', reverse primer, 5'-AGCCTTCTCCATGGTGGTGAAGA-3'. Polymerase Chain Reactions (PCR) were run on Gene Amp PCR system 9700 (Applied Bio-systems, Foster City, CA) using a "hot start" method with the following conditions: 94 °C for 5 min, 94 °C for 1 min, 60–65 °C for 1 min, 72 °C for 1 min, 72 °C for 10 min. GAPDH reaction mixture was run for 26, 28, and 35 cycles to ensure that the reaction product was in the linear range. PCR products were examined in 1 % agarose gel. The RT product volumes were readjusted and the reaction was repeated with GAPDH primers at 28 cycles. After RT product adjustment, the selected gene primers were amplified with 28 and 35 cycles, and then examined by agarose gel electrophoresis to confirm product size.

Western Blots

To examine the ability of C5a to induce proteins in HUVEC, we employed Western-blotting techniques. HUVEC were grown to confluence, then stimulated with human C5a (50 ng/ml) in the presence or absence of CHX (10 μ g/ml) at 37 °C for 2, 8 or 18 h. After stimulation, the supernatant fluids were removed and the monolayer was washed twice with PBS. Monolayers were then placed on ice and lysed using NP40 buffer (50 mM Tris-HCl pH 8.0, 1 % NP40)

containing protease inhibitors (Roche, Mannheim, Germany). The lysate was then collected with sterile cell scrapers and sonicated. The resulting cell lysate was centrifuged at 13,500x g for 10 min and the supernatant fluid removed. This was repeated for each time point. The protein concentrations were estimated using a BioRad protein colorimetric assay. The standardized supernatants were combined with sample buffer, sterile glycerol and stored at -80°C . The cell lysates were subjected to 10 and 15 % sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) and transferred to methanol activated PVDF membranes. The membranes were blocked for 1 h with 5 % non-fat dry milk dissolved in TBST (1 M Tris pH 8, 1 M NaCl, 0.1 % Tween 20). A dilution of 1:1000 for anti-rabbit antibodies to full length PARP-1 (Cell Signaling Technology, Danvers, MA) was used for immunoblotting. Detection of cFLIP was performed using a 1:2000 dilution of anti-rabbit antibodies to full length cFLIP (Gene Therapy Systems, San Diego, CA) or a 1:1000 dilution of anti-rabbit antibodies (AVIVA Systems Biology, San Diego, CA) to short and long forms of cFLIP. Caspase 8 was immunoblotted using a 1:2000 dilution of anti-mouse antibodies (Cell Signaling Technology, Danvers, MA) which detected both the inactive (55 kDa) and active (p41/43 kDa) fragments or anti-mouse antibodies which detect only the inactive form of caspase 8 (NeoMarkers, Fremont, CA). After an overnight incubation at 4°C , the membranes were washed thrice with TBST and incubated with their respective secondary anti-rabbit or anti-mouse antibodies coupled to horseradish peroxidase for 2 h at room temperature. The membranes were then washed twice with TBST followed by an additional wash with TBS. The immunoreactive bands were visualized using enhanced chemiluminescence (ECL) Western blotting detection agents according to manufacturer's directions.

Statistical Analysis

Statistical analysis of the responses obtained from cell viability and caspase 8 activity were carried out using unpaired student t test and InStat 3 (Graphpad, San Diego, CA) software. P values of < 0.05 were considered to be significant.

Results

C5a promotes cell death in HUVEC when in the presence of cycloheximide

Several apoptotic (e.g., caspase 3, NF κ B1, caspase 8) and anti-apoptotic genes (e.g., BIRC3, TNFAIP3, cFLIP) are induced in HUVEC when stimulated by C5a [27]. With this in mind, we chose to examine more closely the ability of C5a to induce an apoptotic response in HUVEC. To do this, we used CHX, a known inhibitor of protein synthesis. We tested the cytotoxicity of CHX on endothelial cells (data not shown) and determined a CHX concentration of 10 $\mu\text{g}/\text{ml}$ would be used for the duration of the study. In addition, we used a C5a concentration of 50 ng/ml, based on data from our previous study [27].

HUVEC stimulated with 50 ng/ml C5a or 10 $\mu\text{g}/\text{ml}$ CHX for 18 h maintained cell viability of 95.7 ± 0.49 and 90.7 ± 6.2 %, respectively. These results were not significantly different compared to the non-stimulated control (Fig. 1A and B). In contrast, cells stimulated with C5a in the presence of CHX showed a significant reduction (43.1 ± 6.9 %) in cell viability compared to non-stimulated cells. This result is complemented by brightfield images (Fig. 1B), where cells stimulated with C5a in the presence of CHX displayed apoptotic morphology (e.g., cell rounding) (frame 1d). Cells stimulated with C5a (frame b) or CHX alone (frame c) maintained cobble-stone appearances, similar to the non-stimulated cells (frame a). Next, we subjected HUVEC to increasing concentrations of C5a (1–100 ng/ml) for 18 h in the presence of CHX (10 $\mu\text{g}/\text{ml}$). Figure 1C shows the dose dependant loss of cell viability in HUVEC.

PARP-1 protein levels are rapidly decreased in response to C5a and CHX, while caspase 8 activity increases

The loss of cell viability when HUVEC are co-incubated with C5a and CHX, suggests initiation of apoptotic cascades, which are usually constrained by anti-apoptotic proteins. To examine this possibility, we monitored caspase 8 enzymatic activity and PARP-1 protein levels under similar experimental conditions. Caspase-8 serves as an upstream apoptotic initiator in the CD95/Fas and TNF α pathways. Accordingly, it is possible under the experimental conditions that C5a initiates apoptotic cascade(s). PARP-1, a regulator of DNA repair, was selected as a complementary marker of apoptosis [28]. Western blot analysis was used to examine full length PARP-1 levels, with the antibody which was specific for the full length form of PARP-1. Cell lysates were collected 0, 2, 8 and 18 h after C5a addition, in the presence or absence of CHX (Fig. 2). PARP-1 levels remained constant after stimulation with C5a alone (Fig. 2A upper frame). In contrast, cells stimulated with C5a and CHX showed reductions in or loss of PARP-1 at all timepoints, while treatment of cells with CHX alone still resulted in the presence of PARP-1.

Next, we measured caspase-8 activity in HUVEC using 7-amino-4-trifluoromethyl coumarin (AFC) under the same experimental conditions. The active form of caspase 8 targets the AFC complex, so that caspase activity may be measured by fluorescent emission. Cell lysates were collected after 18 h and assayed for the active fragment (p41/43) of caspase 8 (Fig. 2B). Non-stimulated HUVEC demonstrated a low level of caspase 8 activity (6.3 ± 2.9 RFU/ μ g). Cells stimulated with C5a for 18 h produced 18.3 ± 0.9 RFU/ μ g of protein, compared to 10.3 ± 1.7 RFU/ μ g for cells stimulated with C5a and CHX. This data suggests that C5a induces caspase 8 activity in HUVEC, but does not result in PARP cleavage. Although cells stimulated with CHX alone or in combination with C5a showed elevated levels of caspase 8 activity, they were not significant compared to the non-stimulated control.

C5a induces increase in gene expression of caspase 8 and cFLIP

The influence of C5a on the gene expression of caspase-8 and cFLIP was assessed. We wanted to determine if the elevated caspase-8 activity observed in Figure 2B was linked with increased caspase-8 gene expression. In addition, we monitored the gene expression of cFLIP, which has been identified as a competitive inhibitor for caspase-8. We predicted that changes in caspase-8 gene expression would correspond to similar changes in cFLIP gene expression. As depicted in Figure 3A, caspase-8 gene expression increased 4 and 8 h after C5a stimulation. The gene expression for cFLIP was unchanged after 4 h, but dramatically increased after 8 h of C5a stimulation. Cells stimulated with C5a in the presence of CHX demonstrated a robust increase in caspase-8 gene expression after 4 and 8 h (Fig. 3B). Interestingly, cells treated with C5a and CHX had a greater increase in caspase-8 gene expression at 4 h compared to those stimulated with C5a alone. In contrast, C5a and CHX treated cells showed limited increase in cFLIP gene expression at 8 h. This suggests that C5a induces caspase-8 gene more rapidly than cFLIP, but cFLIP gene expression is moderately repressed in the presences of CHX. Basal gene expression levels of cFLIP were unchanged by CHX alone.

C5a promotes activation of caspase-8 and cFLIP

We investigated the activity of caspase-8 and its known competitive inhibitor, cFLIP, after cell stimulation with C5a. To better understand the apoptotic response elicited by C5a, we collected cell lysates after 18 h incubation with C5a (50 ng/ml) in the presence or absence of CHX (10 μ g/ml). We selected antibodies which recognized only the inactive (long) form of cFLIP so that we could monitor the loss of the proforms more closely (Fig. 4A and B). Controls included cells treated with CHX alone for 18 h and nonstimulated cells (Fig. 4A). Cell stimulation with C5a promoted a slight loss of full length cFLIP (Fig. 4B) compared to non-stimulated controls. HUVEC stimulated with C5a and CHX demonstrated a nearly total loss of cFLIP after 18 h.

To monitor changes in the cytosolic levels of caspase 8, we utilized an antibody that recognizes the inactive (55 kDa) and active (p41/p43) fragments of caspase 8. HUVEC stimulated with C5a alone showed slight increase in the precursor form, correlating with increased levels of “active” p41/p43 fragments. This caspase activation complemented the AFC assay data (Fig 2B) showing increased caspase activation after 18 h of C5a and CHX stimulation. To follow the relationship between cFLIP and caspase 8 activation over time, cell lysates were collected following 2, 8, and 18 h after stimulation with C5a (50 ng/ml). First, we utilized antibodies that recognize pro-forms (inactive) and fragmented (active) forms of caspase 8 and cFLIP, to determine if C5a stimulation co-activated both caspase 8 and cFLIP. HUVEC stimulated with C5a showed a progressive increase in the active caspase 8 fragment (p41/43 kDa) (Fig. 5). In addition, there was a clear increase in the pro-form of caspase 8, especially at 8 and 18 h. Thus, it appears, that C5a induced gene activation, (described in Figure 3A), leads to increased caspase 8 production and activation. Similarly, C5a stimulation initiated cFLIP cleavage, which increased the 43 kDa active fragment levels over time. This suggests that C5a stimulation promotes caspase 8 and cFLIP activation (cleavage) events.

Discussion

During an inflammatory response, escalating levels of pro-inflammatory mediators such as IL-1, IL-6, TNF α , and C5a interact with endothelial cells, causing robust gene activation and the initiation of apoptotic intracellular signaling. Although early classification of an apoptotic state involves increased caspase 8 activity, other indicators such as external expression of phosphatidylserine or mitochondrial damage may be used to fully characterize an apoptotic state.

Several reports have shown that endothelial cells are resilient to apoptosis induced by IL-6, LPS and TNF α unless the cells are also co-incubated with either cycloheximide or actinomycin D [29,30,31,32]. But the relationship between C5a receptor ligation and the initiation of apoptotic cascades is less clear. Thus, we chose to investigate the relationship between C5a receptor ligation on endothelial cells and the activation of caspase 8 and cFLIP. HUVEC were stimulated with 50 ng/ml of C5a, based on an earlier study demonstrating that this concentration provided optimal transcription that could not be attributed to contaminating LPS [33].

The present study provides evidence that C5a in the presence of CHX, much like other potent inflammatory mediators, reduces cell viability. Figure 1 shows the phenotypic morphology and dose dependent relationship between cell viability and C5a concentration. After 18 h, C5a in the presence of CHX caused a significant reduction in cell viability (Fig. 1A). In addition, the dose dependent relationship between C5a and cell death suggested that C5a may be activating apoptotic signaling cascades. To examine this, we assayed the activity of early and late enzymes of apoptosis (caspase 8 and PARP-1). We found endothelial cells stimulated with C5a/CHX rapidly lost full length PARP-1 after 2 h, while PARP-1 level of cells stimulated with C5a alone remained unchanged (Fig. 2A), suggesting that upstream inhibition of apoptotic signaling may be occurring, but not leading to DNA damage unless anti-apoptotic mechanisms are inhibited. To determine if caspase activity was present, we stimulated the endothelial cells with C5a alone and with C5a in the presence of CHX for 18 h then assay caspase 8 activity using an AFC fluorescent probe. We predicted that suppression of anti-apoptotic protein synthesis by CHX would cause elevated levels of caspase activity. Our results showed an increase in caspase activity for both C5a treated and CHX/C5a treated cells. We found the caspase 8 activity of cells stimulated with C5a alone, was higher than C5a/CHX stimulated cells. This may represent the decrease in viability indicated in figure 1, thus as cells quickly progress toward complete cell death the activities of the caspase begin to decline. In contrast, C5a alone stimulation does not induce down stream apoptotic responses and continued apoptotic progression allowing for an extended elevation of caspase activity. The CHX control

(Fig. 2B) showed increase caspase activity compared to the non-stimulated control, suggesting that CHX does contribute to caspase 8 activation and can be cytotoxic to HUVEC, even at low concentrations.

Next, we wanted to determine if C5a stimulation of HUVEC induces the activation of the key upstream apoptotic modulators, caspase 8 and cFLIP. Changes in gene activation of cFLIP suggest that C5a is activating apoptotic signaling in a caspase dependent manner. More recent studies have suggested that caspase activity and their corresponding inhibitors have non death functions [34,35]. For example, caspase-8 has been shown to be involved in T-cell proliferation. This function appears to be dependent on the intracellular location of the active caspase 8 molecule. Thus, caspase activity resulting from endothelial C5aR ligation, may also include non-death associated functions.

We found an increase in caspase 8 for cells stimulated with C5a alone and in the presence of CHX (Fig 3). The gene expression of caspase 8 increased after 4 h and continued through 8 h. Similarly, C5a and C5a /CHX elevated the gene expression of cFLIP after 8 h of stimulation. The time course of gene activation show that caspase 8 and cFLIP gene expression are staggered, with changes in cFLIP activation lagging behind caspase 8. Thus, the consumption of caspase 8 and cFLIP may include signals to replace or elevate cytosolic protein levels of these apoptotic proteins. Several studies have demonstrated that endothelial cells exposed to TNF- α , or Fas-ligand initiate survival pathways involving NF- κ B and phosphatidylinositol 3-Kinase/Akt [17,18,19]. Each pathway has been implicated in controlling cFLIP levels, and critical for endothelial cell survival. C5a, like other inflammatory mediators, may induce similar responses in endothelial cells. Evidence for this comes from our previous study, where we found that C5a stimulated HUVEC displayed robust gene activation, including NF- κ B and NF- κ BIA [27]. Complementary to this, Schraufstatter et al., reported that C5a receptors expressed on HUVEC are capable of activating EGFR [36], which has been shown to induce NF- κ B. Thus, transactivation of EGFR by C5aR, may provide an additional route of NF- κ B signal transduction in endothelial cells.

To evaluate protein changes for caspase 8 and cFLIP, we collected cell lysates after 18 h of C5a stimulation with or without CHX. The antibody utilized in Figure 4A and B recognized the full length of cFLIP, thus a loss of protein expression would indicate C5a directly or indirectly targeted cFLIP activation. We found C5a moderately reduced cFLIP protein levels after 18 h. Similarly, HUVEC stimulated with C5a in the presence of CHX showed a greater loss of cytosolic full length cFLIP (Figure 4B). Although cFLIP is known to be CHX sensitive, our controls demonstrated that at CHX concentrations $\geq 10\mu\text{g/ml}$ the levels of cFLIP remain unchanged (Fig 4A).

To examine the changes in cytosolic caspase 8 protein expression we used an antibody that recognizes the inactive and active form of the proteins (Fig. 4C and D). The data suggest that stimulation with C5a alone induces moderate increases in the active form (p43/41) through the 18 h time point. Changes in the levels of caspase 8 stimulated with C5a/CHX for 18 h was less obvious. This pattern complemented our AFC fluorometric assay result suggesting that C5a combined with CHX causes a progressive decrease in active and inactive caspase 8. Additionally, we monitored the activation of caspase 8 and cFLIP in the presence of C5a alone. Figure 5 shows the simultaneous activation of caspase 8 and cFLIP over time. Formation of the active fragments of each molecule occurs in a progressively and also corresponds to an increase in full length caspase 8. This activity highlights the importance of cFLIP as an anti-apoptotic molecule in HUVEC during C5a stimulation.

Interestingly, C5a induces both the activation of apoptotic and anti-apoptotic signaling cascades. Other apoptosis studies involving TNF- α follow this same pattern, where elevated

caspase activity, caused anti-apoptotic signaling to be reduced by inhibiting NF- κ B [37,38]. However, inhibiting the synthesis of new anti-apoptotic proteins such as A20, cIAP2, and TRAF1, by CHX may precede influences on gene expression.

We have previously shown in experimental sepsis that C5a induces apoptosis of thymocytes [39] and adrenal medullary cells [40]. C5a also induces dysfunction of cardiomyocytes [41], signaling paralysis in blood neutrophils [42]. Although we have not examined the status of endothelial cell apoptosis, we have previously shown that C5a reacting with endothelial cells will induce upregulation of genes related to cell adhesion molecules and cytokines/chemokines [27], which would collectively be expected to convert the endothelial cell to a proinflammatory state. In addition, we found that several proapoptotic and anti-inflammatory genes were also upregulated. In the setting of sepsis, evidence for endothelial and epithelial cell apoptosis has been reported [43]. We have shown that C5a blockade can improve burn-induced cardiac dysfunction [44] and it is possible that C5a blockade can prevent endothelial cell death during sepsis.

Collectively, this study suggests that HUVEC stimulated with C5a alone may initiate apoptotic and anti-apoptotic proteins. Previous studies have suggested that *de novo* synthesis of anti-apoptotic proteins is essential for endothelial cell survival when exposed to pro-apoptotic molecules [45,46,47]. Our results suggest that C5a stimulation may initiate similar events.

Acknowledgments

Grant Support: NIH, GM-29507, GM-61656, HL-31963

References

1. Reinhart K, Bayer O, Brunkhorst F, Meisner M. Markers of endothelial damage in organ dysfunction and sepsis. *Crit. Care Med* 2002;30:302–12.
2. Hack C, Zeerleder S. The endothelium in sepsis: source of and a target for inflammation. *Crit. Care Med* 2001;29:21–7.
3. Hotchkiss R, Tinsley K, Swanson P, Karl I. Endothelial cell apoptosis in sepsis. *Crit. Care Med* 2002;30:225–8.
4. Srinivasan SR, Radhakrishnamurthy B, Vijayagopal P, Berenson GS. Proteoglycans, lipoproteins, and atherosclerosis. *Adv Exp Med Biol* 1991;285:373–81. [PubMed: 1858569]
5. Bäck M. Inflammatory signaling through leukotriene receptors in atherosclerosis. *Curr Atheroscler Rep* 2008;10(3):244–51. [PubMed: 18489853]
6. Kaplanski G, Marin V, Fabrigoule M. Thrombin-activated human endothelial cells supported monocyte adhesion in vitro following expression of intercellular adhesion molecule-1(ICAM-1;CD54) and vascular cell adhesion molecule-1 (VCAM-1; CD106). *Blood* 1998;92:1259–67. [PubMed: 9694714]
7. Mutunga M, Fulton B, Bullock R, Batchelor A, Gascoigne A, Gillespie J, Baudouin S. Circulating endothelial cells in patients with septic shock. *Am J Respir Crit Care Med* 2001;163:195–200. [PubMed: 11208646]
8. Minagar A, Jy W, Jimenez J, Sheremata W, Mauro L, Mao W, et al. Elevated plasma endothelial microparticles in multiple sclerosis. *Neurology* 2001;56:1319–24. [PubMed: 11376181]
9. Hull C, McLean G, Wong F, Duriez P, Karsan A. Lipopolysaccharide signals an endothelial apoptosis pathway through TNF receptor-associated factor 6-mediated activation of c-Jun NH₂-terminal kinase. *J Immunol* 2002;169:2611–8. [PubMed: 12193732]
10. Hu X, Ester Y, Harlan J, Wong F, Karsan A. Lipopolysaccharide induces the antiapoptotic molecules A1 and A20 in microvascular endothelial cells. *Blood* 1998;92:2758–65.
11. Pohlman T, Harlan J. Human endothelial cell response to lipopolysaccharide, interleukin-1, and tumor necrosis factor is regulated by protein synthesis. *Cell Immunol* 1989;119:41–9. [PubMed: 2784080]

12. Harlan J, Harker L, Striker G, Weaver L. Effects of lipopolysaccharide on human endothelial cells in culture. *Thromb Res* 1983;29:15–26. [PubMed: 6836543]
13. Zhou M, Simms H, Wang P. Adrenomedullin and adrenomedullin binding protein-1 attenuate vascular endothelial cell apoptosis in sepsis. *Ann Surg* 2004;240:321–30. [PubMed: 15273558]
14. Rasper D, Vaillancourt J, Hadano S, Houtzager V, Seiden I, Keen S, et al. Cell death attenuation by ‘Ursurpin’, a mammalian DED-caspase homologue that precludes caspase 8 recruitment and inactivation by the CD-95 (Fas, APO-1) receptor complex. *Cell Death Differ* 1998;5:271–88. [PubMed: 10200473]
15. Scaffidi C, Schmitz I, Krammer P, Peter M. The role of c-FLIP in modulation of CD95-induced apoptosis. *J Biol Chem* 1999;274:1541–8. [PubMed: 9880531]
16. Thome M, Schneider P, Hofmann K, Fickenscher H, Meinel E, Neipel F, et al. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* 1997;386:517–21. [PubMed: 9087414]
17. Bortol R, Tazzari P, Cappellini A, Tabelini G, Billi A, Baregge R, et al. Constitutively active Akt1 protects HL60 leukemia cells from TRAIL-induced apoptosis through a mechanism involving NF- κ B activation and cFLIP_L up-regulation. *Leukemia* 2003;17:379–89. [PubMed: 12592338]
18. Okada Y, Masahiko K, Hisanori M, Yoshinari I, Akihiro Morkawa, Kunio O, Kimura H. Reduced expression of Flice-inhibitory protein (FLIP) and NF B is associated with death receptor-induced cell death in human aortic endothelial cells (HAECs). *Cytokine* 2001;15(2):66–74. [PubMed: 11500081]
19. Suhara T, Mano T, Oliveira B, Walsh K. Phosphatidylinositol 3-kinase/Akt signaling controls endothelial cell sensitivity to fas-mediated apoptosis via regulation of FLICE-inhibitory protein (FLIP). *Circ Res* 2001;89:13–9. [PubMed: 11440972]
20. Reidemann N, Gou R, Neff T, Laudes I, Keller K, Sarma V, et al. Increased C5a receptor expression in sepsis. *J. Clin. Invest* 2002;110:101–8. [PubMed: 12093893]
21. Wesche D, Lomas-Neira J, Perl M, Chung C, Ayala A. Leukocyte apoptosis and its significance in sepsis and shock. *J Leuk Biol* 2005;78:325–37.
22. Perianayagam M, Balakrishnan V, King A, Pereira B, Jaber B. C5a delays apoptosis of human neutrophils by a phosphatidylinositol 3-kinase signaling pathway. *Kidney International* 2002;61:456–63. [PubMed: 11849385]
23. Guo R, Sun L, Hongwei G, Shi K, Rittirsch D, Sarma V, et al. In vivo regulation of neutrophil apoptosis by C5a during sepsis. *J Leuko Biol* 2006;80:1575–83. [PubMed: 16997861]
24. Gasque P, Singhrao S K, Neal JW, Gotze O, Morgan BP. Expression of the receptor for complement C5a (CD88) is up-regulated on reactive astrocytes, microglia, and endothelial cells in the inflamed human central nervous system. *Am. J. Pathol* 1997;150.
25. Foreman KE, Vaporciyan AA, Bonish BK, Jones ML, Johnson K J, Glovsky MM, et al. C5a-induced expression of P-selectin in endothelial cells. *J. Clin. Invest* 1994;94:1147–55. [PubMed: 7521884]
26. Monsinjon I, Gasque P, Chan P, Ischenko A, Brady J, Fontaine M. Regulation by complement C3a and C5a anaphylatoxins of cytokine production in human umbilical vein endothelial cells. *Faseb J* 2003;17:1003–14. [PubMed: 12773483]
27. Albrecht E, Chinnaiyan A, Sooryanarayana V, Kumar-Sinha C, Barrette T, Sarma V, et al. C5a-induced gene expression in human umbilical vein endothelial cells. *Am J Pathol* 2004;164:849–59. [PubMed: 14982839]
28. Bouchard V, Rouleau M, Poirier G. PARP-1, a determinant of cell survival in response to DNA damage. *Exp Hematology* 2003;31:446–54.
29. Zen H, Karsan A, Stempien-Otero A, Yee E, Tupper J, Li X, et al. NF- κ B activation is required for human endothelial survival during exposure to tumor necrosis factor- but not to interleukin-1 or lipopolysaccharide. *J Biol Chem* 1999;274:28808–15. [PubMed: 10497254]
30. Duriez P, Wong F, Dorovini-Zis K, Shahidi R, Karsan A. A1 Functions at the mitochondria to delay endothelial apoptosis in response to tumor necrosis factor. *J Biol Chem* 2000;275:18099–107. [PubMed: 10849436]
31. Eissner G, Kohlhuber F, Grell M, Ueffing M, Scheurich P, Hieke A, et al. Critical involvement of transmembrane tumor necrosis factor- in endothelial programmed cell death mediated by ionizing radiation and bacterial endotoxin. *Blood* 1995;86:4184–93. [PubMed: 7492776]

32. Miura M, Friedlander R, Yuan J. Tumor necrosis factor-induced apoptosis is mediated by a CrmA-sensitive cell death pathway. *Proc. Natl. Acad. Sci. USA* 1995;92:8318–22. [PubMed: 7667287]
33. Schindler R, Gelfand J, Dinarello C. Recombinant C5a stimulates transcription rather than translation of interleukin-1(IL-1) and tumor necrosis factor: translational signal provided by lipopolysaccharide of IL-1 itself. *Blood* 1990;76:1631–8. [PubMed: 2207333]
34. Maelfait J, Beyaert R. Non-apoptotic functions of caspase-8. *Biochem Pharmacol.* Aug 6;2008 [Epub ahead of print]
35. Koenig A, Russell JQ, Rodgers WA, Budd RC. Spatial differences in active caspase-8 defines its role in T-cell activation versus cell death. *Cell Death Differ.* Jul 11;2008 [Epub ahead of print]
36. Schraufstatter IU, Trieu K, Sikora L, Sriramarao P, DiScipio R. Complement c3a and c5a induce different signal transduction cascades in endothelial cells. *J Immunol* 2002;169(4):2102–10. [PubMed: 12165538]
37. Irmeler M, Martinon F, Holler N, Steiner V, Ruegg C, Wajant H, Tschopp J. Caspase-induced inactivation of the anti-apoptotic TRAF1 during Fas ligand-mediated apoptosis. *FEBS Lett* 2000;468:129–33. [PubMed: 10692572]
38. Levkau B, Scatena M, Giachelli CM, Ross R, Raines E. Apoptosis overrides survival signals through a caspase-mediated dominant negative NF-kappa B loop. *Nat. Cell Biol* 1999;1:227–33. [PubMed: 10559921]
39. Guo RF, Huber-Lang M, Wang X, Sarma V, Padgaonkar VA, Craig RA, Riedemann NC, McClintock SD, Hlaing T, Shi MM, Ward PA. Protective effects of anti-C5a in sepsis-induced thymocyte apoptosis. *J Clin Invest* 2000;106:1271–80. [PubMed: 11086028]
40. Flierl MA, Rittirsch D, Chen AJ, Nadeau BA, Day DE, Sarma JV, Huber-Lang MS, Ward PA. The complement anaphylatoxin C5a induces apoptosis in adrenomedullary cells during experimental sepsis. *PLoS ONE.* 2008On-Line Vol 3/issue 7/e2560
41. Niederbichler AD, Hoesel LM, Westfall MV, Gao H, Ipaktchi KR, Sun L, Zetoune FS, Su GL, Arbabi S, Sarma JV, Wang SC, Hemmila MR, Ward PA. An essential role for complement C5a in the pathogenesis of septic cardiac dysfunction. *J Experimental Medicine* 2006;203(1):53–61.
42. Huber-Lang MS, Yonkin EM, Sarma JV, McGuire SR, Lu KT, Guo RF, Padgaonkar VA, Curnutte JT, Erickson R, Ward PA. Complement-induced impairment of innate immunity during sepsis. *J Immunol* 2002;169(6):3223–31. [PubMed: 12218141]
43. Weche DE, Lomas-Neira JL, Perl M, Chung C-S, Ayala A. Leukocyte apoptosis and its significance in sepsis and shock. *J. Leukoc. Biol* 2005;78:325–7. [PubMed: 15817707]
44. Hoesel LM, Niederbichler AD, Schaefer J, Ipaktchi KR, Gao H, Rittirsch D, Pianko MJ, Vogt PM, Sarma JV, Su GL, Arbabi S, Westfall MV, Wang Sc, Hemmila MR, Ward PA. C5a-blockade improves burn-induced cardiac dysfunction. *J. Immunol* 2007;178:7902–10. [PubMed: 17548628]
45. Kay J, Korner A. Effects of cycloheximide on protein and ribonucleic acid synthesis in cultured human lymphocytes. *Biochem J* 1966;100:815–22. [PubMed: 5969294]
46. Kreuz S, Siegmund D, Scheurich P, Wajant H. NF-kB inducers up-regulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. *Mol Cell Biol* 2001;21:3964–73. [PubMed: 11359904]
47. Choi K, Wong F, Harlan J, Chaundhary P, Hood L, Karsan A. Lipopolysaccharide mediates endothelial apoptosis by a FADD-dependent pathway. *J Biol Chem* 1998;273:20185–8. [PubMed: 9685365]

Fig 1.

C5a induces loss of HUVEC viability in the presence of CHX. **A)** MTS assay for cell viability. Cells were treated with C5a (50 ng/ml), CHX (10 µg/ml), or C5a (50 ng/ml) plus CHX (10 µg/ml) for 18 h. * $P < 0.05$ vs. C5a treated. **B)** Brightfield images, 200X magnification: a) non-stimulated control b) CHX (10 µg/ml) 18 h c) C5a (50 ng/ml), 18 h d) C5a (50 ng/ml) with CHX (10 µg/ml), 18 h. Percentage of viability was expressed as the proportion of untreated cells. **C)** Dose dependent response with MTS assay. Cells were stimulated with C5a (1–100 ng/ml) for 18 h in the presence of CHX (10 µg/ml). * $P < 0.05$ vs. control conditions (CHX 10 µg/ml); ** $P < 0.01$ vs. control conditions (CHX 10 µg/ml). The results shown are the mean $\pm$ S.E. of experiments done in triplicate and are representative of three separate experiments.

Fig 2.

C5a stimulation of HUVEC initiates apoptotic pathway activity. **A)** Western blots for protein expression of PARP. Cell lysates were collected after 2, 8, 18 h following stimulation with C5a (50 ng/ml) alone or with C5a (50 ng/ml) and CHX (10 μg/ml). Control lysates were collected from cells without C5a stimulation (Non-stimulated). Loading conditions were confirmed by β-tubulin content. **B)** Caspase-8 activity assay. Cell lysates are collected after 18 h and analyzed for Caspase-8 activity following stimulation with C5a (50 ng/ml) alone or with C5a (50 ng/ml) and CHX (10 μg/ml). Control lysates were from cells without C5a stimulation (Non-stimulated) and cell stimulated with CHX (10 μg/ml) for 18 h. *P < 0.05 vs. C5a; **P < 0.05 vs. Non Stimulated. Data are ± S.E. of three separate experiments.

Fig 3.

C5a induces increased gene expression of caspase-8 and cFLIP in HUVEC. **A)** RT-PCR of Caspase-8 and cFLIP gene expressions after 0, 4, and 8 h of stimulation by C5a (50 ng/ml). **B)** RT-PCR of Caspase-8 and cFLIP gene expressions after 0, 4, and 8 h of stimulation by C5a (50 ng/ml) with CHX (10 μ g/ml). RT- samples were run under identical conditions for each time course, but without enzyme (Superscripts) for 8 h. GAPDH was used as a loading control. Data are representative of three separate experiments.

Fig 4.

Western blotting of Caspase-8 and cFLIP. **A**) Cell lysates were collected 18 h following C5a (50 ng/ml) alone or in the presence of CHX (10 µg/ml). Control included non-stimulated (-) and 18 h CHX (10 µg/ml) treatment (+). The activity of cFLIP was monitored by the loss of the full length form. C5a treatment caused diminished cytosolic cFLIP levels compared to untreated controls. The combination of C5a and CHX caused cFLIP cytosolic level to be virtually undetectable **B**) Cell lysates were collected at 18 h following stimulation with C5a (50 ng/ml) alone or in the presence of CHX (10 µg/ml). Control included nonstimulated (-) and 18 h CHX (10 µg/ml) treated (+). Activation of caspase 8 was monitored by the appearance of the cleaved forms (p43/41). Data are representative of three separate experiments.

Fig 5. Western blotting of Caspase-8 and cFLIP from HUVEC lysates. Cell lysates were collected 2, 8, 18 h following C5a (50 ng/ml). The active fragment of caspase 8 and cFLIP increased over time.