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Complement C3a and C5a Induce Different Signal Transduction Cascades in Endothelial Cells

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In leukocytes, C3a and C5a cause chemotaxis in a \( G_i \)-dependent, pertussis toxin (PT)-sensitive fashion. Because we found that HUVECs and immortalized human dermal microvascular endothelial cells express small numbers of C3aRs and C5aRs, we asked what the function of these receptors was on these cells. Activation of the C3aR caused transient formation of actin stress fibers, which was not PT-sensitive, but depended on rho activation implying coupling to \( G_{12/13} \). Activation of the C5aR caused a delayed and sustained cytoskeletal response, which was blocked by PT, and resulted in cell retraction, increased paracellular permeability, and facilitated eosinophil transmigration. C5a, but not C3a, was chemotactic for human immortalized dermal microvascular endothelial cells. The response to C5a was blocked by inhibitors of phosphatidylinositol-3-kinase, src kinase, and of the epidermal growth factor (EGF) receptor (EGFR) as well as by neutralizing Abs against the EGFR and heparin-binding EGF-like factor. Furthermore, immune precipitations showed that the EGFR was phosphorylated following stimulation with C5a. The C5aR in endothelial cells thus uses a signaling cascade—transactivation of the EGFR—that does not exist in leukocytes, while the C3aR couples to a different \( G \) protein, presumably \( G_{12/13} \).


Materials and Methods

Purification of C3a and C5a

Human C3a and C5a were produced after cleavage of their parent proteins, C3 and C5, respectively, using a fluid phase C3/C5 convertase, CVF, and Bb (28). C3a and C5a were collected after direct passage through DEAE-Sephacel equilibrated in 20 mM imidazole HCl (pH 7)/0.075 M NaCl. Then, the anaphylatoxins were adsorbed onto CM-Sephadex (Amershams Pharmacia Biotech, Piscataway, NJ), and eluted with 0.25 M NaCl. The synthetic polypeptide consisting of the 21 terminal amino acids of C3a, C3a (57–77), was a kind gift of Dr. T. E. Hugli (La Jolla Institute for Molecular Medicine, San Diego, CA).

Cell culture

HUVECs were obtained from Clonetics (San Diego, CA) and used between passage 5 and 7. Human immortalized dermal microvascular endothelial cells (HMECs; Ref. 29) were obtained from the Center for Disease Control (Atlanta, GA). The cells were grown in endothelial growth medium as recommended by the supplier (Clonetics).

HMECs or HUVECs were seeded at low density on collagen-coated coverslips and grown in endothelial growth medium (Clonetics) containing 10% FCS. On the day of the experiment, cells were serum starved for 2 \( h \) (HUVECs) or 3–4 \( h \) (HMECs) and stimulated with C3a or C5a for the time indicated for each experiment. For immunoprecipitations, HMECs were serum starved for 16 \( h \). All experiments were performed at 37 °C in a tissue culture incubator.

To block \( G_{12/13} \), endothelial cells were incubated for 16 \( h \) with 100 ng/ml of PT (List Biological Laboratories, Campbell, CA). Rho was inhibited with C3 botulinum toxin (10 \( \mu \)g/ml; List Biological Laboratories) for 24 \( h \),
which leads to inactivation of rho in ~90% of endothelial cells (30). Rho kinase, a downstream target of rho, was inhibited with 10 μM Y27632 (gift of Yoshitomi Pharmaceuticals, Osaka, Japan) added 30 min before the addition of the stimulus (31). Similarly, tyrphostin AG 1478 (1 μM in DMSO; Calbiochem, La Jolla, CA), a specific inhibitor of the epithelial growth factor (EGF) receptor (ERGFR; Ref. 32), and PP2 (Calbiochem), a specific inhibitor of src kinase, (33) were added for 30 min before the addition of stimulus. All blocking Abs were used at 10 μg/ml.

**FACS analysis**

For FACS analysis, HMECs or HUVECs were trypsinized shortly, placed in PBS containing 2% FCS, incubated for 30 min on ice with anti-C5aR or anti-C3aR Ab (1/200 dilution; both from BD Pharmingen, San Diego, CA), washed three times with PBS/FCS, and labeled with mouse- or rabbit-antibiotinylated IgG for 30 min. Following another three washes, the cells were incubated with streptavidin-PE (1/250 dilution; BD Pharmingen) for 30 min, washed once more, and analyzed on a FACSscan using CellQuest Pro software (BD Biosciences, Mountain View, CA). The specific Ab was replaced with anti-rabbit (C5aR) or anti-mouse (C3aR) IgG for negative controls.

**RT-PCR of C3aR and C5aR**

RNA was purified from confluent HUVEC or HMEC cells with the RNeasy kit (Qiagen, Valencia, CA) and poly(T)-tailed cDNA was produced using the Omniscript RT kit (Qiagen). The primers to detect the C3aR were derived from the sequences between aa 161 and 340. Forward primer: 5'-GGC GCT GAT GGT GGC CAC GAG CAG GAT GCT-3'. Reverse primer: 5'-TCA CCT AGT GAT CGT TAT TGC CAC GA-3'. The primers to detect the C5aR were derived from aa 1-132. Forward primer: 5'-AAT TCC TTT TGC TAT CTA TAC ACC ACC ACC TGT G-3'. Reverse primer: 5'-GGC GCT GAT GGT GCC GAC CAG CAG CAG GCT-3'. The PCR was conducted for 40 cycles using a PerkinElmer Cetus instrument (PerkinElmer, Wellesley, MA). Melting was for 40 s at 94°C, annealing was for 1 min at 56°C, and chain elongation was conducted at 72°C for 2 min.

**Fluorescence microscopy**

Filamentous actin (F-actin) formation was visualized as previously described for HMECs activated with IL-8 (34). In short, HMECs or HUVECs were seeded at low density on collagen-coated glass cover slips, grown to confluence, serum starved for 2 or 4 h, and stimulated with the indicated concentration of C3a or C5a for the times stated. In some experiments, HMECs were used just before reaching confluence. The cells were fixed with 3% paraformaldehyde in PBS, put on ice and permeabilized for 5 min in 0.2% Triton X-100 incubated with 5 μM/mL of Alexa 488-phalloidin (Molecular Probes, Eugene, OR) for 30 min, washed three times with PBS, and mounted with Antifade (Molecular Probes). Fluorescence microscopy was performed on a Leica DM IRBE microscope using a ×100 oil immersion objective. Images were obtained with a Hamamatsu digital camera (Hamamatsu Photonics, Hamamatsu City, Japan) and analyzed with Improvision OpenLab 3.0 software (Improvision, Boston, MA), which allows the determination of fluorescence intensity and cell area of individual cells. A minimum of 30 cells in three different experiments was analyzed for each data point. To determine cell retraction, HMECs or HUVECs were grown to confluence, stimulated and stained as above, and retracted cells were counted in the digital image representing a 1-mm² area.

**Haptotaxis of endothelial cells on collagen**

The assay was performed as described previously for IL-8 (34). The bottom side of Transwell filters (8-μm pore size; Costar, Cambridge, MA) was coated for 30 min with 230 μl bovine collagen (100 μg/mL; Cohesion, Franklin, MA) in PBS and blocked for 30 min with 1% BSA. Endothelial basal medium (500 μL) containing 0.1% BSA, 0.5% hydrocortisone, and 50 μg/mL gentamicin was pipetted into the bottom well, and 5 × 10⁴ HMECs or 2.5 × 10⁵ HUVECs in the same media were added to the inserts. Following the addition of C3a or C5a to the bottom wells, the cells were incubated for 4 h at 37°C in a tissue culture incubator and stained for 10 min with 1 μM calcine-AM (Molecular Probes). Cells in the upper well were carefully removed with a cotton swab and transmigrated cells were counted at ×5 magnification on a Leica DM IRBE microscope (Leica, Deerfield, IL) using FITC excitation and emission. Results represent the mean ± SEM of four experiments in triplicate.

**Immunoprecipitations of the EGFR**

HMECs were grown to confluence on 100-mm² tissue culture plates, serum starved for 18 h, incubated with 5 ml fresh serum-free media, stimulated with 50 nM C5a, 500 nM C3a, or 30 ng/ml of EGF for the indicated times at 37°C. Placed on ice, washed once with PBS, and lysed in 400 μl lysis buffer (150 mM NaCl, 25 mM Tris (pH 7.5), 1 mM EDTA, 2 mM sodium vanadate, 2 mM sodium pyrophosphate, 1% Nonidet P-40, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 mM PMSF, 10% glycerol). Following centrifugation for 10 min at 10,000 × g, the protein content in the supernatants was determined using the bichinchoninic acid reagents (Pierce, Rockford, IL). Ab (rabbit polyclonal anti-EGFR Ab, 5 μg/sample; Upstate Biotechnology, Lake Placid, NY) was added to each sample containing 0.8 mg protein, and the samples were rotated for 2 h at 4°C. Protein A/G Sepharose (30 μl of a 50% slurry; Amersham Pharmacia Biotech) was added for 30 min, and the samples were washed three times in lysis buffer and once in PBS. The resulting pellets were suspended in 30 μl SDS sample buffer, boiled for 3 min, and loaded onto a 7% SDS gel. Western transfers and blots were performed according to standard protocols, using 5% nonfat dry milk to block the blot, followed by a 1/2000 dilution of the anti-pTyrosin Ab (PY20; BD Transduction Laboratory, Mountain View, CA) and goat anti-mouse HRP-IgG conjugate (BioSource International, Camarillo, CA) as the second Ab (1/8000 dilution) followed by detection by ECL (ECL plus reagent; Amersham Pharmacia Biotech). The blots were stripped with Re-Probe (Geno Technology, St. Louis, MO), and redeveloped with the anti-EGFR Ab. The densitometric ratio between the two blots was determined and values were compared with those in unstimulated cells (n = 4).

**Determination of cell barrier function**

HMECs (2 × 10⁴ cells/well) were seeded on 0.4 μm pore-size Transwell filters (Costar) and grown to confluence for 7 days. FITC dextran (m.w. 70,000; Sigma-Aldrich, St. Louis, MO) was added to the upper wells along with no stimulus, 1 μM C3a, or 100 nM C5a; and as a function of time, aliquots were withdrawn from the lower chamber and fluorescence measured using a Packard fluorocount plate reader (Packard Instrument, Meriden, CT). Maximal flux was determined using filters not covered with cells.

**Eosinophil transmigration**

Eosinophils were prepared from mildly allergic but otherwise healthy human donors by Percoll centrifugation followed by negative selection of CD16 positive cells by using a magnetic separation technique (35, 36). The cells were labeled fluorescently using 1 μM calcine-AM (Molecular Probes).

HMECs were grown as monolayers on 5-μm pore-size inserts (Costar). In some cases, cytochalasin B (5 μg/ml) was added to the cells for 5 min, followed by extensive washing with DMEM. Subsequently, 200,000 fluoroescnently labeled eosinophils were added to each upper well and no mediator. C3a (1 μM), or C5a (50 nM) were pipetted into the lower wells. After 4 h, the number of transmigrated cells in the lower well was counted in a central 1.1-mm² area using a Leitz Fluovert FS microscope (Leica Microsystems, Deerfield, IL). All values are indicated as relative to those seen in the presence of 1 μM C3a, which caused an intermediate level of chemotaxis.

**Results**

**Expression of the C3aR and the C5aR by endothelial cells**

The presence of C3aRs and C5aRs on endothelial cells was determined by FACS analysis with receptor-specific Abs. Both HMECs and HUVECs expressed receptors for both the C3aR and the C5aR (Fig. 1A), although apparent receptor numbers were low in all cases. Because the shift was monophasic, it appears that all cells expressed a small number of receptors. This was confirmed by the uniform response of all cells in the actin polymerization assay (Fig. 2).

To verify the results obtained by FACS, messages for C3aR and C5aR mRNA were amplified using RT-PCR. Products for the C3aRs and the C5aRs were detected in both HUVECs and HMECs (Fig. 1B).
Cytoskeletal effects of C3a and C5a on endothelial cells

To show that the C3aRs and C5aRs on endothelial cells were functional, the effect of C3a and C5a on the cytoskeleton of endothelial cells was determined. The addition of C3a or C5a caused increased F-actin formation in both HMECs (Fig. 2, top left panel) and HUVECs (Fig. 2, bottom left panel). In unstimulated serum-starved endothelial cells, F-actin staining of low intensity was concentrated in the cell periphery, where adjacent cells touched each other. After the addition of C3a, prominent stress fibers appeared within 1 min of activation, which led to an increase in F-actin staining and to stretching of the cells on the substratum. In HMECs, this behavior could best be quantified by determining the surface area covered by individual cells, which more than doubled at this early time point (Fig. 2, top right panel). The response to C3a was transient and almost completely reversed by 5 min. In contrast, the response to C5a was prolonged lasting for almost 1 h (Fig. 2) and leading to a 3-fold increase in F-actin content (Fig. 2, middle right panel). Starting between 5 and 10 min, it was observed that a fraction of the cells retracted, leaving denuded surface areas between adjacent cells. The affected fraction of cells was not large enough to result in a significant decrease of mean cell surface area (Fig. 2), but when retracted cells were counted on a confluent layer of HMECs, an increase of retracted cells from 6 ± 3 cells/mm² in unstimulated cells to 63 ± 31 cells/mm² following stimulation with 10⁻⁷ M C5a for 20 min was observed (see also Fig. 4, C and D). No such effect was noted with C3a.

Because it was problematic to achieve healthy quiescent HUVECs by prolonged serum starvation, these cells were grown to confluence, serum starved (2 h), and stimulated. Because cell borders were difficult to assess using confluent cells, the mean phalloidin staining intensity was determined for a 2000-μm² area covered by several cells. The response to C3a and C5a in HUVECs (Fig. 2, M–O, and bottom right panel) was very similar to that seen with HMECs, including the transient time course for C3a and the protracted response to C5a. In the confluent monolayer formed by HUVECs, C5a stimulation induced gap formation between adjacent cells (see Fig. 2O), leaving denuded substratum in between cells. The number of contracted cells increased from 4 ± 1 cells in unstimulated HUVECs, to 30 ± 3 cells after a 20-min exposure to 10⁻⁸ M C5a.

Cytoskeletal changes were induced by concentrations of C3a or C5a within the low (C5a) to high (C3a) nanomolar range as expected from the dissociation constants of the two receptors for their respective ligands (Refs. 37 and 38; Fig. 3). A 21-aa synthetic peptide derived from the C terminus of human C3a is reported to have similar specific activities for smooth muscle contraction and induction of vascular permeability as full-length C3a (1). In vitro, this peptide is one to two orders of magnitude less active than C3a.

FIGURE 1. Expression of C3aRs and C5aRs by HMECs and HUVECs. A, FACS analysis of the expression of the C3aR and the C5aR by human endothelial cells. Samples were prepared as described in Materials and Methods. Top panel, Results obtained for HMECs; bottom panel, Results for HUVECs. B, RT-PCR of C3aR and C5aR message by HMECs and HUVECs. Lane 1, C3aR in HMECs (expected size: 0.54 kb); lane 2, C5aR in HMECs (expected size: 0.4 kb); lane 3, 1-kb ladder; lane 4, C3aR in HUVECs; and lane 5, C5aR in HUVECs.

FIGURE 2. Time course of the cytoskeletal response to C3a and C5a. Subconfluent HMECs grown on collagen were stimulated with 10⁻⁷ M C3a, 10⁻⁸ M C5a, or 100 ng/ml LPA at 37°C, fixed with paraformaldehyde, stained with Alexa 488 phalloidin, and processed as described in Materials and Methods. A, Unstimulated HMECs; HMECs stimulated with C5a for 1 min (B), 5 min (C), 20 min (D), 45 min (E), and 60 min (F), or with C3a for 1 min (G), 5 min (H), or 20 min (J). LPA caused a similar response shown at 1 min (J), 5 min (K), and 20 min (L) (all ×100 objective). Changes similar to those induced by C3a and C5a in HMECs were observed in HUVECs (×60 objective). M, Unstimulated HUVECs; N, HUVECs stimulated with 10⁻⁷ M C3a for 1 min; and O, HUVECs stimulated with 10⁻⁸ M C5a for 20 min. Right panels, Quantification of F-actin response; C3a stimulation caused a transient increase in stress fiber formation. In HMECs, this response was best quantified by determining the area covered by individual cells (top right panel, mean ± SEM of 30–40 cells for each data point). The time course of C3a activation in HUVECs closely resembled that seen in HMECs (bottom right panel, mean ± SEM of 30 regions; 2000 μm²). The response to C5a was delayed and prolonged, and the increase in polymerized actin was characterized by a combination of stress fiber formation and cell contraction. Quantification of these results showed increased F-actin content in both HMECs (middle right panel) and HUVECs (bottom right panel).
In accordance with these reports, the C3a-peptide (57–77) caused the same response as C3a in HMECs, but at ~30-fold higher concentrations (Fig. 3).

Effect of C3a and C5a on cell retraction and monolayer permeability

As noted above, C5a, but not C3a, caused retraction of the two endothelial cell types used (Fig. 4, A–E). Because stimulation with C5a caused cell retraction and gap formation between cells, monolayer permeability was determined next. Paracellular flux was measured using FITC-dextran passage through filters bearing monolayers of HMECs stimulated with C3a or C5a. In the absence of the anaphylatoxins, endothelial cells created a nearly impermeable boundary between the two compartments. After stimulation with C5a, an increased amount of the labeled dextran was able to pass through the cell barrier (Fig. 4E). In contrast, C3a did not perturb the cell barrier function (Fig. 4E).

Participation of endothelial cell anaphylatoxin receptors in eosinophil transmigration

Because eosinophils can transmigrate through an endothelial cell monolayer in response to C3a and C5a (36), and because the presence of endothelial cells augments the anaphylatoxin-mediated chemotaxis of the eosinophils, it was important to determine whether endothelial cell activation contributed to this process. To prevent the cytoskeletal response of the endothelial cells, cytochalasin B was added to either naked filters or filters with monolayers of endothelial cells as described previously for epithelial cell transmigration (39). Following thorough washing, eosinophils were added to the upper chambers and anaphylatoxins to the lower chambers, and the assemblies incubated for 4 h at 37°C. Untreated monolayers of endothelial cells were used for comparison. As reported previously for HUVECs (36), the presence of HMECs increased the chemotaxis of eosinophils toward C3a and C5a compared with naked filters (Fig. 5). To assure that any residual traces of cytochalasin B that were left after thorough washing were insufficient to perturb the migratory machinery of the eosinophils, transmigration of eosinophils through cytochalasin B-treated filters was determined and did not result in a negative effect on naked filters (Fig. 5). In contrast, cytochalasin B treatment of the HMEC monolayers reduced eosinophil chemotaxis induced by C5a to levels seen in the absence of endothelial cells (Fig. 5), indicating that the cytoskeletal response of the endothelial cells contributed to the C5a-mediated transmigration of eosinophils. The contribution of the endothelial cell cytoskeletal response to C3a-dependent chemotaxis was only minor.

Signal transduction cascade used by the C3aRs and C5aRs

The relative ease of the actin staining assay and the availability of specific inhibitors for components of the signal transduction machinery allowed to screen for downstream elements of the response
to C3a and C5a. In leukocytes, both C3αRs and C5αRs couple to Gαi, which is abundant in hematopoietic cells. Endothelial cell C5αRs similarly coupled to Gαi, as indicated by the blocking effect of PT on the cytoskeletal response to C5a in both HMECs and HUVECs (Fig. 6, C and H). In contrast, the effect of C3a was if anything accentuated following preincubation with PT (Fig. 7, F and X), implying use of a different G protein for the C3αR in endothelial cells.

Because stress fiber formation has been associated with activation of rho (40), the effect of C3 botulinum toxin, which blocks rho (30), and Y27632, which inhibits rho kinase (31), were determined next. Both of these inhibitors blocked the C3α-mediated cytoskeletal response in HMECs (Fig. 7, B and D), but failed to prevent the C5α-dependent effect (Fig. 6B), indicating that only the C3αR activates the rho cascade. The same was seen in HUVECs, where Y27632 abrogated the response to C3a (Fig. 6W). Similarly, C3 botulinum toxin blocked this response (data not shown). However, it was noticed that in the presence of C3 toxin, the integrity of the HUVEC cell monolayer was disturbed. Although we used conditions that were the same as those used previously to block rho in HUVECs (30, 41), the C3 toxin results in these cells should be seen with caution.

Recent reports indicate that there is considerable cross talk between GPCRs and receptor tyrosine kinases (42, 43), and that protein tyrosine kinase (PTK) inhibitors not seen in control cells.

**FIGURE 6.** Effect of inhibitors on the C5α-mediated cytoskeletal response to C5α: HMECs (A–E) or HUVECs (F–J) were incubated with inhibitors as described for Fig. 6, and then stimulated with 10 nM C5α for 20 min. A and F, +C5α, no inhibitor; B and G, +C5α + Y27632; C and H, +C5α + PT; D and I, +C5α + AG 1478; E and J, +C5α + PP2. Middle and bottom panels. The results were quantified for HMECs and HUVECs, respectively. Left panels. The mean F-actin fluorescence intensity (mean ± SEM, n = 30–40) of the cytoskeletal response to C5α. For comparison, LPA (100 ng/ml) known to couple to Gα12/13 (61) was used in the same assay (W–Z). HUVECs stimulated with 100 nM C5α for 1 min. V, no inhibitor; preincubation with 10 μM Y27632 for 30 min (W), with 100 ng/ml PT for 16 h (X), with 1 μM AG1478 (Y), or 1 μM PP2 (Z) for 30 min. Bottom panel. The mean ± SEM of 30 2000-μm² rectangles each is shown. There is a statistical difference for Y27632-treated cells (p < 0.01).

**FIGURE 7.** Effect of inhibitors on the C3α-mediated cytoskeletal response in HMECs (A–J) and HUVECs (V–Z) at 1 min. A and B, Inhibitor, 10 μM Y27632; A, no stimulus; B, +100 nM C3α; C and D, inhibitor, 10 μg/ml C3 botulinum toxin for 24 h (30); C, no stimulus; D, +100 nM C3α; E and F, inhibitor, 100 ng/ml PT for 16 h; E, no stimulus; F, +100 nM C3α; G and H, inhibitor, 1 μM AG1478; G, no stimulus; H, +100 nM C3α; I and J, inhibitor, 1 μM PP2; I, no stimulus; J, +100 nM C3α. In the middle graph, the effect of the various inhibitors is quantified (mean ± SEM of 30–40 cells each) using cell area determination as the parameter measured. C3 botulinum toxin and Y27632 showed statistically significant inhibition (p < 0.01) of the cytoskeletal response to C3α. For comparison, LPA (100 ng/ml) known to couple to Gα12 (61) was used in the same assay (V–Z). HUVECs stimulated with 100 nM C3α for 1 min. V, no inhibitor; preincubation with 10 μM Y27632 for 30 min (W), with 100 ng/ml PT for 16 h (X), with 1 μM AG1478 (Y), or 1 μM PP2 (Z) for 30 min. Bottom panel. The mean ± SEM of 30 2000-μm² rectangles each is shown. There is a statistical difference for Y27632-treated cells (p < 0.01).

Tracted responses of GPCRs often involve the secondary activation of receptor tyrosine kinases such as the EGFR. Therefore, AG1478, a specific inhibitor of the EGFR tyrosine kinase activity (44), and PP2, a specific inhibitor of src kinase (33), were tested in the actin polymerization assay. In the two endothelial cell types tested, both inhibitors blocked the actin response to C5α (Fig. 6, D, E, I, and J), but not to C3a (Fig. 6, H, J, Y, and Z). All inhibitors of the C5α actin response also prevented cell retraction (Fig. 6, right middle and bottom panels).

**Effect of C3α and C5α on endothelial cell migration**

Because actin polymerization is a prerequisite for cell migration, we questioned whether HMECs and HUVECs were chemotacted by C3α or C5α. Although C3α failed to cause endothelial cell migration, C5α showed a chemotactic effect on the endothelial cells (Fig. 8A). Cell migration was blocked by the same inhibitors that prevented the C5α-mediated actin response, i.e., PT (Fig. 8, C
and D), PP2, and AG1478 (Fig. 8, C and D), indicating that C5a-mediated cell migration of endothelial cells—in contrast to C5a-mediated chemotaxis of leukocytes—depends on EGFR transactivation. The effect of PP2 appeared to be distal of the EGFR activation, as this inhibitor also blocked EGF-mediated cell migration. The effect of PT is also shown (E). Mean ± SD of 6–12 filters. E and F, Phosphorylation of the EGFR following stimulation with C5a. HMECs were stimulated with 100 nM C5a or 50 ng/ml EGF for the indicated times and immunoprecipitated with anti-EGFR Ab (rabbit polyclonal, 10 μg/ml; Upstate Biotechnology). α, 1 μM PP2. In addition, the effect of PT is also shown (E).

Discussion

This study shows that endothelial cells express functional C3aRs and C5aRs and delineates the different signal transduction pathways induced by the two stimuli. This active role of the endothelial cells in inflammatory processes has not been sufficiently appreciated. A number of receptors involved in leukocyte chemotaxis, e.g., the CXCR1, CXCR2, CXCR4, and CCR1, are also expressed by endothelial cells, especially by microvascular endothelial cells (34, 46–48). C3a and C5a are another pair of GPCRs expressed both by the endothelium and by leukocytes, and the relative role of receptors on both cell types deserves further investigation. Interestingly, it has been described that only C5a, but not C3a, induced eosinophil transmigration in vivo in the rabbit mesenteric artery model (36), although both C3a and C5a were chemotactic for endothelial cells in vitro. It is possible that endothelial cell retraction caused by the stimulation of endothelial cells with C5a played a role in this process, while stimulation with C3a did not allow leukocyte penetration. Both C3a and C5a are known to increase vascular permeability, and although this may in part be an indirect effect resulting from fluid cell degranulation (7, 8, 49), our data suggest that endothelial cell retraction may also contribute to this phenomenon.

Generally, factors which cause increases in endothelial cell monolayer permeability and cell migration are also angiogenic (50). This has been shown for vascular endothelial cell growth factor (51), thrombin (52), and IL-8 (53, 34). Our results suggest that it is possible that C5a may have angiogenic properties, which have not been recognized. However, a putative angiogenic effect of
C5a could only operate in a situation of chronic inflammation with sustained complement activation, because the activity of C5 convertase is short-lived (36, 54), and because C5a is rapidly converted by plasma carboxypeptidase N to C5a desArg (55), which has a lower affinity for the C5aR.

In this study, the cytoskeletal response to anaphylatoxins was studied extensively in large vessel endothelial cells (HUVECs) and a dermal microvascular endothelial cell line (HMECs), but it was not limited to these cells. It could in fact be seen in all endothelial cell types tested, which included human brain microvascular cells (a gift from Dr. M. Fiala, University of California, Los Angeles, CA) and pulmonary microvascular endothelial cells (HMVECs; Clonetics; data not shown).

In leukocytes, both the C3aRs and CSRs couple to G\textsubscript{12} and G\textsubscript{13} (56, 57, 21, 26). G\textsubscript{16} expression is limited to hemopoietic cells, and G\textsubscript{16} is unusually abundant in these cells. Because G protein coupling appears to be far less specific than initially recognized, it is not surprising that G protein usage appears to vary for different cell types. Specifically, activation of the C3aR on endothelial cells was not inhibited by PT, implying that the receptor couples to a different G protein in these cells. Because inhibitors of the rho cascade blocked C3a function, these G proteins are likely G\textsubscript{12} and/or G\textsubscript{13}, which are both known to activate rho (58). Interestingly, a recent report indicated that activation of the thrombin receptor in the same endothelial cell line used in this study could cause coupling of that receptor to G\textsubscript{12} and G\textsubscript{13} and, alternatively, and signal through rho stimulation (59). Importantly, activation of HMECs with C3a closely resembled the response to LPA (Figs. 2 and 6). Coupling of the C3aR to G\textsubscript{12} or G\textsubscript{13} could prevent stress fiber formation (59). Furthermore, the response to C3a was short-lived, as has been described previously for lysophosphatidic acid (LPA) and endothelin, two mediators that couple to G\textsubscript{13} and G\textsubscript{12}, respectively, and signal through rho stimulation (60, 61).

Activation of endothelial cell C5aRs in contrast led to coupling to G\textsubscript{13} as supported by the inhibitory effect of PT and the migratory response induced by C5a. Interestingly, a large portion of the endothelial cell response to C5a was blocked by various modes of inhibition of the EGFR. In leukocytes, which lack EGFRs, the signal transduction machinery of the C5aR is sufficient to cause cell migration directly. It is not clear at this point why there is this difference in the cell activation response of these different cell types. Transactivation of the EGFR by activation of GPCRs has been described over the last few years for a number of GPCRs (63, 42, 45), but it is certainly not a ubiquitous finding for all GPCRs. It has not been described previously for the C5aR, but may have long-reaching consequences in vivo. First, activation of the EGFR is known to be mitogenic (64), and mitogenesis is another hallmark of angiogenesis (65). Secondly, it causes activation of NF-\text{kB} (66), and NF-\text{kB} activation induces the expression of TNF-\alpha, IL-\text{1B}, and chemokines of the IL-8 family (IL-8, growth-related oncogene-\alpha, macrophage inflammatory protein-2, and others; Refs. 67 and 68), and will greatly amplify any inflammatory response. Interestingly, it was reported in a recent article that stimulation of mouse microvascular endothelial cells with C5a, especially in combination with other inflammatory stimuli, caused up-regulation of macrophage inflammatory protein-2 (69).

Because transactivation of the EGFR by several GPCRs has been shown to involve HB-EGF as an intermediate (45), we determined whether it was involved in C5a-dependent endothelial cell activation, which it was. HB-EGF is a heparin-binding member of the EGF family, which exists as a membrane-anchored precursor that becomes activated by proteolytic cleavage during cell activation (70) and goes on to activate the EGFRs HER1 and HER4. Expression of HB-EGF is up-regulated in cells undergoing stress or exposed to proinflammatory stimuli (71–73). Although it is not clear whether the concentration of HB-EGF is the rate-limiting factor during GPCR-mediated transactivation of the EGFR, it is appealing to speculate that its up-regulation may amplify the response to various GPCRs during the inflammatory response.

Complement activation leads to the concomitant generation of C3a and C5a in vivo, and concentrations of C3a exceed concentrations of C5a by about an order of magnitude. Because endothelial cell activation was similarly an order of magnitude more sensitive to C5a than C3a, the C3aR and C5aR pathways would be activated concomitantly. Similar dual endothelial cell activation pathways exist for IL-8 and for thrombin, although they are regulated at different levels. Instead of activation by two ligands, C3a and C5a, IL-8 activates two receptors, the CXCR1, which behaves like the C3aR and activates rho, and the CXCR2, which behaves like the C5aR (34) and stimulates the EGFR cascade (I. U. Schraufstatter, K. Trieu, D. Rose, R. A. Terkeltaub, and M. Burger, manuscript in preparation). Thrombin achieves the same responses (74) by concomitant coupling to several G proteins, including G\textsubscript{12} and G\textsubscript{13} (75, 76, 45).

An active role of the endothelium in leukocyte transmigration that goes beyond the presentation of adhesion molecules has only been recognized recently (77, 78). As in the current study, these reports found that the cytoskeletal response of endothelial cells assisted in the passage of leukocytes (77–79). In the case of lymphocyte transmigration through brain endothelial cell monolayers, rho activation was essential (78), which was not the case in our experiments. Future experiments will have to show whether this difference was determined by the type of leukocyte, the type of endothelial cell, or a difference in the duration of the activation, which was more transient in our case.

In summary, C3aRs and C5aRs on endothelial cells may contribute in various ways to the up-regulation of inflammatory processes observed following complement activation.

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