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An Alternative Role of C1q in Cell Migration and Tissue Remodeling: Contribution to Trophoblast Invasion and Placental Development

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Fetal trophoblast cells invading the decidua in the early phase of pregnancy establish complex interaction with the maternal extracellular matrix. We discovered that C1q was widely distributed in human decidual stroma in the absence of C4 and C3 and was actively synthesized by migrating extravillous trophoblasts. The cells expressed the messages for the three chains of C1q and secreted this complement component that interacted with the proteins of the decidual extracellular matrix. Solid phase-bound C1q promoted trophoblast adhesion and migration, and cell binding to C1q resulted in activation of ERK1/2 MAPKs. Ab inhibition experiments showed that the receptors for the globular head of C1q/p33 and α6β1 integrin were both involved in this process and were colocalized on the cell surface following binding of C1q to trophoblasts. We also found that C1q<sup>-/-</sup> mice manifested increased frequency of fetal resorption, reduced fetal weight, and smaller litter sizes compared with wild-type mice. C1q deficiency was associated with impaired labyrinth development and decidual vessel remodeling. Collectively, these data suggest that C1q plays an important role in promoting trophoblast invasion of decidua and that defective local production of C1q may be involved in pregnancy disorders, such as pre-eclampsia, characterized by poor trophoblast invasion. The Journal of Immunology, 2010, 185: 4420–4429.
migration at sites of anchoring villi and in the decidual tissue (17). Decidual stroma is also rich in laminins that surround the decidual cells as a distinct pericellular layer (18, 19) and interact mainly with α5β1 and α6β1 on EVTs (20, 21).

Elastin microfibril interfacer 1 (EMILIN1) is a connective tissue glycoprotein associated with elastic fibers (22) previously identified as another candidate ECM molecule involved in EVT invasion. A characteristic feature of this protein is to display a gradient distribution in decidua with a progressive increase in its expression toward remodeled spiral arteries that favors EVT migration from the anchoring columns to the perivascular areas (23). EMILIN1 shares a structural homology with the globular head of C1q (gC1q), the first component of the complement system (24), raising the possibility that C1q may contribute to trophoblast invasion of decidua. The main function of this complement component is to act as a recognition molecule of the complement system and trigger the activation of the classical pathway. This results in the release of biologically active products primarily involved in the removal of immune complexes and infectious and other harmful agents through the activation of various cellular responses, including enhancement of phagocytosis (25), stimulation of neutrophil, eosinophil, and mast cell chemotaxis (26), and clearance of apoptotic cells (27). However, C1q has also been shown to exhibit activities apparently unrelated to its defensive functions, such as promotion of EC adhesion and spreading (28) and regulation of fibrillogenesis of tropoelastin (29). Over the last few years, we have been analyzing human placenta for the presence and distribution of complement components with the aim to define their role in embryo implantation. Recently, we have shown that C1q is expressed on the surface of decidual ECs (DECs) and acts as a molecular bridge that favors adhesion of endovascular trophoblasts and endothelial re-placement (29). Data are now presented suggesting that invasive trophoblast cells synthesize and secrete C1q that binds to ECM proteins and is used by EVTs to invade the decidua.

Materials and Methods
Abs and reagents

Goat antiserum anti-human C1q, mAb anti-human C1q γ, and purified human C1q γ were purchased from Quidel (San Diego, CA). Two mAbs anti-gC1q receptor (gC1qR; clones 74.5.2 and 60.11) recognizing distinct domains of the molecule were previously reported (30); rabbit IgG anti-human cC1qR, calreticulin and anti-gC1qR/p33 were previously reported (31). The neutralizing mAb M89D3 to CD31 was kindly provided by M. R. Zocchi (San Raffaele Hospital, Milan, Italy) (32, 33); rabbit IgG and mAb P1H4 to human β1 integrin, mAb P5D2 to α2 integrin, mAb CGA7 to human smooth muscle α-actin, and mAb 349 anti-paxillin were from Chemicon (Milan, Italy). Rabbit IgG to human C1q and to cow cytotkeratin widen spectrum, mAb W6/32 anti-HLA, mAb OV-TL 12/30 anti-cytokeratin 7, and mAb 349 anti-paxillin were from Chemicon (Milan, Italy). Rabbit IgG to human C1q and to cow cytotkeratin widen spectrum, mAb W6/32 anti-HLA, mAb OV-TL 12/30 anti-cytokeratin 7, and mAb 349 anti-paxillin were from Chemicon (Milan, Italy). Rabbit IgG to human C1q and to cow cytotkeratin widen spectrum, mAb W6/32 anti-HLA, mAb OV-TL 12/30 anti-cytokeratin 7, and mAb 349 anti-paxillin were from Chemicon (Milan, Italy).

Immunohistochemical staining

Tissue samples were fixed in 10% buffered formalin and paraffin embedded. Four-micrometer-thick sections of placental tissue were stained with H&E and examined for the presence and distribution of invasive trophoblasts. For immunohistochemical analysis, the slides were microwaved three times in Tris-HCl/EDTA (pH 9) buffer (DakoCytomation) for 5 min, brought to room temperature, and washed in PBS. Postneutralization of the endogenous peroxidase with H2O2 for 10 min, the sections were first incubated with protein block (Novocastra) for 10 min. Sections were incubated with the appropriate primary Ab for 30 min at room temperature. Binding of anti-C1q IgG or anti-cow cytotkeratin widen spectrum were detected using the Labeled StreptAvidin Biotin + HRP kit and diaminobenzidine as substrate (DakoCytomation) and that of mAb anti-cytokeratin 7 was revealed with the Alkaline Phosphatase Anti-Alkaline Phosphatase Complex (DakoCytomation) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt/NBT or Fast Red (DakoCytomation) as chromogens. The sections were then covered with a Leica DM3000 optical microscope (Leica Microsystems, Mannheim, Germany), and captions were collected using a Leica DFC320 digital camera (Leica Microsystems).

Cell isolation and culture

CTBs were purified from placental specimens postremoval of decidual tissue and fetal membrane as previously described (2). Briefly, placental tissue was incubated with HBSS containing 0.25% trypsin and 0.2 mg/ml DNase (Roche, Milan, Italy) for 20 min at 37°C. Postfractionation through Percoll gradient, the leukocytes were totally removed by immuno-nomagnetic beads coated with mAb to CD45 (Dyna, Invitrogen, Milan, Italy). CTBs collected by negative selection were seeded in a 25-cm² flask coated with 5 μg/cm² FN (Roche), cultured overnight in RPMI (Life Technologies, Invitrogen) supplemented with 10% FCS, and finally detached by trypsin-EDTA treatment. The cells obtained under these conditions contained 95% cytokeratin 7-positive CTBs and a few vimentin-positive decidual stromal cells (DSCs). The presence of contaminating leukocytes and ECs was excluded by RT-PCR assay for CD45 and FACS analysis with anti-vWF and anti-cD31 Abs, respectively.

HLA-G+ and HLA-α+ cells were purified by incubating freshly isolated CTBs (3 × 10⁷) with Dynabeads M-450 (Dyna, Invitrogen) conjugated with mAb anti-human HLA-G at 4°C for 45 min with gentle rotation. The cells adsorbed to magnetic beads were collected with a magnetic collector, washed three times with Dulbecco’s PBS containing 0.1% BSA (Sigma-Aldrich), and used as a source of HLA-G-positive CTBs. The unadsorbed cells were incubated with CD45 magnetic beads (Invitrogen) to remove contaminating leukocytes and subsequently with Dynabeads M-450 conjugated with mAb Wb/32 that recognized a conserved epitope of human HLA-A, -B, and -C at 4°C for 45 min with gentle rotation. The negatively selected cells were 100% HLA-negative.

DSCs were isolated as described by Carlino et al. (39). Briefly, decidual tissue was digested with 0.5% trypsin (Sigma-Aldrich), 50 μg/ml DNase1 (Boehringer Mannheim, Mannheim, Germany) in HBSS overnight at 4°C and then treated with collagenase type I (3 mg/ml; Worthington Biochemical, Milan, Italy) for 30 min at 37°C. After Ficol (Roche) density gradient centrifugation, the decidual cells were cultured for 24 h in RPMI 1640 supplemented with 10% FCS, and the decidual cell-conditioned medium (DCCM) was collected, stored at ~80°C, and used as source of chemotactic stimuli in the migration assays. Nonadherent cells were removed by extensive washing, and adherent cells were used at the third passage of culture.
Under these conditions, the cells were negative for CD14, CD45, cytokeratin 8-18, vWF, or CD31 and positive for α-actin, vimentin, CD13, CD10, and CD105.

Peripheral blood-derived macrophages (PBDMs) were obtained from Ficoll-purified PBMCs cultured for 7 d in RPMI supplemented with 10% FCS as previously described (40).

**RT- and real-time quantitative PCR**

Cells and tissues were harvested in Omniloz (Euroclone, Milan, Italy) according to the supplier’s instructions. Total RNA was extracted and reverse transcribed as previously described (29). RT-PCR was performed in a Techne TC-312 thermal cycler (Bowany Scientific, Milan, Italy) using Taq DNA polymerase (AccuTaq, Sigma-Aldrich).

Real-time quantitative PCR (RQ-PCR) was carried out on a Rotor-Gene 6000 (Corbett, Experla, Ancona, Italy) using iQ SYBR Green Supermix (Bio-Rad, Milan, Italy). Table I lists the primers used for PCR following Kaul and Loos (41). Amplification conditions, sequences, and concentrations of the primers are similar to those of RT-PCR. The melting curve was recorded between 55°C and 99°C with a hold every 2 s. Purified Clq/C and β-actin PCR products synthesized from pooled deoxyheads were used to establish standard curves, and the data were analyzed using Rotor-Gene Software (Corbett, Experla). The relative amount of Clq/C in each sample was normalized with β-actin and expressed as arbitrary units (AU) considering 1 AU obtained in PBDMs used as a positive control (42).

**Immunoenzymatic assays**

The level of Clq secreted by CTBs in the culture supernatant and the presence of gClqR in immunoprecipitated samples were measured by a sandwich ELISA as previously described (43).

ECM proteins (25 μg/ml) bound to wells of 96-well ELISA plates (Corning Costar, Acton, MA) in 0.1 mM sodium bicarbonate buffer (pH 9.6) by overnight incubation at 4°C were tested for their interaction with Clq. Hyaluronan and laminin-5 were used at the concentration of 50 μg/ml and 150 μg/ml, respectively. After washing the wells with PBS containing CaCl2 and MgCl2 (0.7 mM) and 0.05% Tween-20 and 0.7 mM Ca2+ and Mg2+ and allowed to bind to ECMs for 90 min at 37°C. Bound C1q was revealed using mAb anti-C1q (1:1000) and β integrin (Beckman Coulter, Fullerton, CA) previously bound to protein G-Sepharose beads. After washing with Tris buffer (50 mM, pH 7.4) containing 150 mM NaCl, 5 mM EDTA-Na, 0.25% Triton X-100 (Sigma-Aldrich), and 0.1% SDS, the immune complexes were resuspended in sample buffer with 2-ME, boiled for 3 min, resolved on 9% SDS-PAGE, and transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was then sequentially immunoblotted with rabbit IgG against the β1 integrin subunit and mAb against gClqR (mAb 60.11). Immunoreactivity was revealed using an ECL kit (Amersham International, Amersham, U.K.).

**Confocal analysis**

CTB cells were plated at 37°C on eight-chamber culture slides (BD Biosciences Discovery Labware, Milan, Italy) coated with Clq (10 μg/ml) or FN (10 μg/ml) and left to adhere for various lengths of time between 15 min and 2 h. Postfixation, the cells were permeabilized and stained with N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-conjugated phallacidin (NBD-phallacidin) (Molecular Probes, Invitrogen) and mAb antipaxillin followed by Cy3-conjugated F(ab’)2 goat anti-mouse IgG (1:300) as previously described (19). In other experiments, the Clq-bound CTBs were fixed with 3% paraformaldehyde and incubated with Ultra V Block (Lab Vision, Bio Optica Milan, Milan, Italy) for 10 min at room temperature. The cells were then incubated with mAb P1H4 anti-human β1 integrin (2 μg/ml) for 1 h at room temperature followed by Cy3-conjugated F(ab’)2 goat anti-mouse IgG. After extensive washing and blocking with mouse serum (1:100) in Ultra V Block (Lab Vision) for 10 min, the cells were incubated with biotin-labeled mAb anti-gClqR 74.5.2 for a further 1 h at room temperature followed by FITC-conjugated streptavidin (1:100) (DakoCytomation).

Images were acquired with a Leica TCS SP2 confocal system (Leica Microsystems) using the Leica Conofocal Software and a 63× fluorescence objective on a Leica DM IRE2 microscope (Leica Microsystems) or with a Nikon C1Si confocal system, using the Nikon EZ-C1 Conofocal Software and a 63× fluorescence objective on a Nikon TE2000-U inverted microscope (Nikon, Melville, NY).

**Table I. Primers used for PCR analysis in accord to the method of Kaul and Loos (41)**

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<th>Sample</th>
<th>Primers</th>
<th>Sequence 5'→3'</th>
<th>Annealing Temperature (°C)</th>
<th>Size of PCR Product (bp)</th>
<th>EMBL Database Accession No.</th>
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<td>528</td>
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<tr>
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<td>Reverse</td>
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<td></td>
<td>Reverse</td>
<td>TTTCTTGCGTGTTAATGCCT</td>
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</table>

EMBL, European Molecular Biology Laboratory.
Pregnancy studies in mice

Eight- to 10-wk-old virgin female C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) and C1q-deficient mice (provided by Dr. Marina Botto, Imperial College, London, U.K.) were mated with 8–14-wk-old C57BL/6 or C1q-deficient mice, respectively. A group of pregnant females was killed at day 15, and the frequency of fetal resorption was calculated as previously described (44). Fetal weights were also determined. Some mice were studied until delivery, and the litter sizes were recorded at birth. Procedures that involved mice were approved by the Institutional Animal Care and Use Committee of the Hospital for Special Surgery and were conducted in strict accordance with guidelines for the care and use of laboratory research animals promulgated by the National Institutes of Health.

Immunohistochemical studies were performed on implantation sites from 6–8-wk-old pregnant mice kept at the Animal House of Istituto Clinico Humanitatis (Rozzano, Italy) and collected at days 9.5, 10.5, and 12.5 of pregnancy. The experimental procedures were performed in accordance with the institutional guidelines and in compliance with the European (86/609/EEC) and Italian (D.L.116/92) laws.

Results

C1q is localized in areas of invading trophoblasts

Immunohistochemical analysis of paraffin-embedded first-trimester placenta with rabbit IgG to human C1q showed a wide distribution of this complement component in the decidual stroma in the vessel wall. A strong signal was also seen in the Hofbauer cells (Fig. 1B, arrow) and in limited surface areas of the villi, whereas the overall staining of syncytiotrophoblasts and the underlying CTB layer of the villi was fainter (Fig. 1B). This finding was confirmed using goat IgG anti-C1q. Double staining of tissue sections for C1q and cytokeratin 7 to identify trophoblast cells documented the presence of C1q on trophoblasts localized in the trophoblast shell (Fig. 1C) and at the level of the CTB columns on their way to invade the decidua (Fig. 1D). Staining for C1q was particularly evident in the EVT spread in the decidua (Fig. 1E) and surrounding the spiral arteries (Fig. 1F), and a closer analysis of these cells revealed marked cytoplasmic C1q localization. We failed to detect C4 and C3 in the decidual tissue at sites of C1q deposition (Supplemental Fig. 1).

CTB cells express C1q at mRNA and protein level

The finding of intracellular staining for C1q in EVTs led us to investigate these cells for their ability to synthesize and secrete this complement component. Initially, we performed RT-PCR for the three chains of C1q (A, B, and C) on mRNA extracted from the whole placenta, CTBs, DSCs, the most abundant cells present in decidua, and PBDMs examined as a positive control. The results presented in Fig. 2 show that mRNA for the three chains was expressed in PBDMs and in whole decidual tissue, suggesting local production of C1q. Analysis of decidual cells revealed the presence of the three transcripts in CTBs, whereas DSCs express only the mRNA for the A chain (Fig. 2A).

Because C1q was observed predominately in invasive trophoblasts, we hypothesized that the expression of C1q in CTBs was correlated with the acquisition of the invasive phenotype by these cells. To confirm this possibility, we analyzed CTBs expressing HLA-G, a marker of invasive trophoblasts, and HLA-negative cells, including villous trophoblasts, by RQ-PCR for the presence of mRNA for C1qC that discriminates C1q-secreting from nonsecret- ing cells (29). As shown in Fig. 2B, HLA-G+ cells expressed amounts of mRNA C1qC essentially similar to those detected in PBDMs, whereas HLA− CTBs failed to express the C1qC transcript.

Next, we investigated the ability of CTBs to synthesize and secrete C1q. For this purpose, 10 6 invading CTBs were plated in multiwell chambers, and the cell supernatants were collected after 48 h of culture and stored at −80°C. The amount of C1q measured by ELISA in these samples varied between 50 and 100 ng/10 6 cells and was approximately half of the amount of C1q secreted by PBDMs (Fig. 2C).

C1q secreted by trophoblasts is used by these cells to invade the decidua

Having found that C1q is secreted by the invading CTBs and widely distributed in decidua, we sought to investigate the role played by this complement component in trophoblast motility. To evaluate cell binding to C1q, freshly isolated CTBs were labeled with the fluorescent probe FAST Dil and allowed to adhere to solid phase-bound C1q or purified gC1q in 96-well plates for 30 min at 37°C, using BSA and FN as negative and positive controls, respectively. Approximately 40% of CTBs adhered to C1q as opposed to 20% of cells adhering to BSA and 60% to FN. Interestingly, the percentage of CTBs binding to gC1q was essentially similar to that observed with the whole C1q molecule, suggesting that trophoblast cells interact with the globular domain of the molecule (Fig. 3A).

CTBs attached to C1q exhibited a morphologic appearance different from that of FN-bound cells, being much smaller and displaying wide ruffles that extended in multiple directions, in contrast with the marked spreading of CTBs adherent to FN (Fig. 3B). Staining of FN-bound cells with NBD-phallacidin revealed a high number of actin-containing stress fibers and localization of paxillin at the tips of stress fibers already 30 min after cell adhesion. In contrast, the cells attached to C1q showed diffuse...
positivity for paxillin and subcortical organization of actin at the same time point (Fig. 3B), but some focal plaques were seen after 2 h of CTB adhesion (data not shown).

Next, we examined CIq and FN for their ability to promote CTB migration. The upper side of the transwell inserts was coated with CIq or FN or both, and CTBs were allowed to migrate in the presence or in the absence of DCCMs added to the lower chamber as a chemotactic stimulus. CIq and FN caused migration of ∼10% of CTBs in the absence of DCCMs (data not shown). This value raised to 24% when the cells were allowed to migrate through FN in the presence of DCCMs and further increased to 41% through the insert coated with CIq (Fig. 3C), suggesting that this complement component was more efficient than FN in promoting CTB migration.

To mimic more closely the situation encountered by CTBs that interact with the ECM while invading the decidua, the insert of the transwell was first coated with FN and then with CIq (Supplemental Fig. 2). The results presented in Fig. 3C clearly show that CIq bound to FN maintains the ability to support migration of 40% CTBs.

gCIqR and β1 integrin are involved in trophoblast–CIq interaction

We have previously shown that gCIqR/p33 (gCIqR) on endovascular trophoblasts is involved in the adhesion of these cells to DECs expressing CIq on their surface (29). To evaluate the contribution of gCIqR to CTB–CIq interaction, the adhesion assay was performed using CTBs preincubated with two different Abs to gCIqR, mAb 60.11 or mAb 74.5.2, that recognize distinct epitopes on the molecule, or an Ab to cCIqR for 30 min at 37˚C. As shown in Fig. 4A, the adhesion of CTBs to CIq was inhibited almost completely by mAb 60.11, whereas the other mAb 74.5.2 to gCIqR and the rabbit IgG to cCIqR were ineffective. Based on our previous findings that trophoblasts cells use α4β1 integrin to interact with EMILIN1, a member of CIq family proteins sharing a CIq signature domain (19, 45), we examined the effect of neutralizing Abs to α4 and β1 integrin in the adhesion assay. Our results (Fig. 4A) suggest that both α4 and β1 integrin are also involved in CTB adhesion to CIq, and the same molecules appear to play a role in trophoblast migration through CIq (Fig. 4B).
Activation of ERK1/2 MAPKs that was already evident at 5 min and noblotting. As shown in Fig. 6, binding of CTBs to C1q resulted in the phosphorylation status of ERK1/2 MAPKs on total cell lysates was evaluated by immunoblot analysis. Binding of trophoblast cells to C1q enhances ERK1/2 MAPK phosphorylation.

Defective invasion of CTBs in C1q−/− mice associated with embryo growth restriction

To examine the actual contribution of C1q to pregnancy outcomes, we performed a gross and microscopic time-course analysis of implantation sites from C1q−/− mice and wild-type (WT) controls.

Histopathologic analysis of implantation sites from C1q−/− mice at different time points of gestation from 7.5–14.5 d revealed signs of impaired development as compared with WT controls matched for gestational age. Changes were mostly noticed starting from day 9.5 and were characterized by impaired labyrinth development (Fig. 8, upper panels), lower degree of vascular remodeling (Fig. 8, lower panels), and marked edema in the interstitium of decidual stroma. In addition, staining of decidua from C1q−/− mice for cytokeratin revealed marked reduction in trophoblast invasion and nearly complete absence of periarteriolar clusters of trophoblasts compared with the decidua obtained from gestational age-matched WT mice.

Discussion

Trophoblast invasion of decidua is a critical process for placentation and embryo implantation and is regulated by a series of integrated adhesion and signaling events that include the cross talk between trophoblast and decidual cells requiring interaction between cell-surface adhesion molecules and ECM proteins. The data presented in this study suggest that C1q synthesized and secreted by EVTs is part of this complex network and is used by EVTs to migrate inside the decidua.

While searching for complement involvement in tissue remodeling that occurs in maternal decidua following embryo implantation, we observed deposits of C1q on decidual stroma in the absence of C4 and C3. This finding was unexpected because C1q is usually detected on cells and tissues in pathological conditions associated with complement activation through the classical pathway. As a result, biologically active products that are primarily involved in the removal of immune complexes, apoptotic cells, and infectious and other harmful agents are released. Even more surprising was the observation that the deposits of C1q in maternal decidua were mainly seen in areas of trophoblast invasion, suggesting that C1q may have been synthesized locally and used for some special functions unrelated to complement activation. Staining for C1q was also observed in the villi, particularly in the Hofbauer cells that displayed a strong signal. This is not surprising because they belong to the monocyte/macrophage lineage known to be a major source of C1q (49–51). A low level of staining was also apparent in villous trophoblast cells, most likely due to C1q deposits from maternal blood, as these cells were found to lack the C1qC message required for the assembly of C1q.
The extracellular deposition of C1q in close proximity to the surface of trophoblasts invading the decidua and, more importantly, the cytoplasmic staining of EVTs for this complement component, suggest that EVT may be a good candidate for the local production of C1q. Evidence supporting this conclusion was obtained by the finding that EVTs expressed the messages for the three chains of C1q and secreted a detectable amount of the molecule in the culture supernatant. C1q is one of the few complement components synthesized at the extrahepatic sites, as suggested by the observation that bone marrow transplants from complement-sufficient mice restore C1q levels in C1q-deficient animals (52). Besides cells of the monocyte/macrophage lineage, other cell types contribute to the production of C1q, including follicular dendritic cells and interdigitating cells (53), fibroblasts (54), and epithelial cells of the intestinal and urogenital tracts (55). Our data suggest that decidual tissue is an important source of C1q in pregnancy and contributes to its local synthesis both with EVTs and, as recently reported, with the ECs of spiral arteries (29). C1q secreted by DECs binds avidly to the cell surface and acts as a physical link between endovascular trophoblasts and DECs (29).

The strong staining of EVTs for C1q explains the preferential localization of this complement component at sites of trophoblast invasion. The association of C1q with the invasive phenotype of trophoblast cells is further supported by the finding that this protein is synthesized exclusively by trophoblasts expressing HLA-G, a well-

**FIGURE 5.** Colocalization gC1qR/β1 integrin. A, Western blot analysis of cell lysates from CTBs left to adhere to C1q (20 μg/ml) or FN (20 μg/ml) for 30 min at 37°C and immunoprecipitated first with normal mouse IgG and then either with mAb 60.11 anti-gC1qR or mAb 4B4 anti-β1 integrin subunit. The presence of β1 integrin subunit (130 kDa) and gC1qR (33 kDa) was revealed using rabbit polyclonal Abs. These results are representative of one out of three independent experiments. B, Surface distribution of gC1qR and β1 integrin molecules on CTB cells allowed to adhere to C1q for 1 h at 37°C. Postfixation, the cells were immunostained for gC1qR (green) and for β1 integrin (red), as detailed in Materials and Methods and then examined by confocal microscopy. Ba, A representative field of a z-step (xy) analysis and the corresponding z projections (yz and xz). A representative colocalization analysis of gC1qR (Bb) and β1 integrin (Bc). Bd, Merged image for gC1qR and β1 integrin showing several spots (pseudocolored in gray by the software) of evidence of the presence of both signals in the plasma membrane of several cells. Be, The relative colocalization graph indicating the pixels of the colocalized signals (yellow ellipse, ROI) calculated by colocalization function of Leica Confocal Software (Leica Microsystems). Original magnification ×200. ROI, region of interest.

**FIGURE 6.** Binding of trophoblast cells to C1q enhances ERK1/2 MAPK phosphorylation. CTBs were allowed to adhere to C1q-, FN-, or BSA-coated wells for different lengths of time, and the phosphorylation status of ERK1/2 MAPKs (42 and 44 kDa) was evaluated on total cell lysates by immunoblotting. The amount of ERK and p-ERK1/2 MAPK was quantified by densitometric analysis using the ImageJ 1.37v program (National Institutes of Health) and expressed as absolute value as well as percentage of p-ERK.
known marker of EVT. Thus, C1q can be considered as an additional marker of EVT, and its synthesis can be added to the list of phenotypic changes of decidual trophoblasts, including the expression pattern of adhesion molecules (56) and the secretion of hyperglycosylated human chorionic gonadotropin (57).

The recognition that C1q secreted by EVT as a soluble molecule is deposited on decidual stroma in the absence of C4 and C3 led us to hypothesize that trophoblast cells may use C1q to invade the decidua. This process requires interaction of C1q with proteins of the ECM present in the decidua with a characteristic distribution in areas of trophoblast invasion and around the spiral arteries. Binding of C1q to several of these proteins was documented by the results of the immunoenzymatic assay and is in line with previous reports showing interaction of C1q with FN (58), laminin (59), small leucine-rich repeat proteins, and mucopolysaccharides including heparin sulfate and dermatan sulfate (60).

Several pieces of evidence argue for gC1q as the portion of C1q molecule involved in the promotion of trophoblast adhesion and migration, and the most compelling one is that the effect of C1q was reproduced with purified gC1q. Strictly related to this point is the finding that EMILIN1, an important component of decidual stroma and a member of C1q and TNF superfamily characterized by a common gC1q signature domain (45), is also implicated in trophoblast invasion of decidua (19). Equally important in this context is our previous observation that CTBs only express gC1qR, which selectively recognizes gC1q while failing to express the receptor for the collagen portion of C1q (29). The role played by gC1qR–gC1q interaction in trophoblast adhesion and migration is further supported by the inhibition obtained with mAb 60.11 to gC1qR, which recognizes a C1q binding site on this receptor, whereas mAb 74.5.2, which reacts with an epitope of gC1qR involved in the reaction with the high m.w. kininogen (61), was ineffective.

Interaction of C1q with CTBs results in cell activation, as suggested by the enhanced phosphorylation of ERK1/2 MAPK to a level essentially similar to that observed in CTBs bound to FN. This finding extends previous observation by Liu et al. (62), who showed that activation of PI3K/akt, ERK, and JNK pathways is required for the chemotaxis of immature dendritic cells to C1q, whereas activation of AKT and p38 pathways is needed for C1q-mediated enhancement of mature dendritic cell chemotaxis to CCL19. However, because gC1qR lacks direct access to the intracellular compartment, the sole engagement of this receptor by bound C1q is unlikely to deliver the activation signal to CTBs for which the contribution of β1 integrin is required. The implication of αvβ3 integrin expressed on the surface of invasive trophoblasts (19, 20, 63) in C1q-mediated cell adhesion and migration is suggested by the inhibition obtained with mAbs to αv and β3 integrins, the colocalization of this integrin with gC1qR on CTBs adhering to bound C1q, and precipitation with the same receptor in extract of these cells. Similar results were obtained by Feng and colleagues (46), who showed that EC adhesion and spreading on C1q-coated microtiter wells was prevented both by mAb 60.11 to gC1qR and mAb to β3 integrins and that gC1qR and β3 integrin coprecipitated on C1q-bound ECs. Involvement of integrins in cell activation by C1q has also been established on murine mast cells that have been shown to express αvβ3 integrin as a novel receptor for the collagen-like sequence of C1q (64–66).

The best way to prove the in vivo relevance of our observations is to evaluate pregnancy outcome in C1q-deficient patients. Unfortunately, this is difficult to ascertain because C1q deficiency is a rare genetic defect and is often associated with a severe form of systemic lupus erythematosus. However, judging from the reduced litter size, the decreased fetal weight, and, more importantly, the increased number of fetal losses observed in C1q knockout mice, one can confidently conclude that the absence of C1q may impair pregnancy outcome. This is in line with the deficient trophoblast invasion and reduced labyrinth development and remodeling of spiral arteries observed in pregnant C1q−/− mice compared with WT animals of the same gestational age.

In summary, we have shown that C1q secreted by migrating CTB cells acts on the same cells through an autocrine mechanism to promote decidual invasion and more generally regular pregnancy outcome.

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References


