Plasmodium falciparum–Infected Erythrocyte Adhesion Induces Caspase Activation and Apoptosis in Human Endothelial Cells

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During Plasmodium falciparum infection leading to cerebral malaria, cytokine production and cytoadherence of parasitized erythrocytes (PRBCs) to postcapillary venules are involved. We demonstrate that PRBC adhesion induces apoptosis in human endothelial cells (HLECs). PRBC adhesion modulated HLEC gene expression in tumor necrosis factor–α superfamily genes (Fas, Fas L, and DR-6) and apoptosis-related genes (Bad, Bax, caspase-3, SARP 2, DFF45/ICAD, IFN-γ receptor 2, Bel-w, Bik, and iNOS). Apoptosis was confirmed by (1) morphological modifications by electron microscopy, (2) annexin V binding, (3) DNA degradation, by measuring intracytoplasmic nucleosomes, and (4) caspase activity. The apoptotic stimulus was physical contact between HLECs and PRBCs and not parasite-secreted molecules. In addition, it was found that cytoplasmic (caspase 8) and mitochondrial (caspase 9) pathways were involved in this process. These data not only describe the direct apoptotic effect of PRBC adhesion on endothelial cells but also provide new useful tools that allow an evaluation of potential pharmaceuticals.

Cerebral malaria (CM), coupled with anemia, is one of the most tragic complications associated with infection by Plasmodium falciparum. Although its physiopathology has been extensively investigated, the cellular and molecular bases of the neurological pathology are still unclear, particularly the intricacy of the different factors described as being involved in its pathogenesis: sequestration of parasitized red blood cells (PRBCs) leading to a mechanical blockade of microvessels, secretion of cytokines, modifications of the T cell repertoire, the immune status and genetic background of the host, and parasite factors [1]. The sequestration of PRBCs to the surface of the microvasculature of various organs, including brain and lungs, is mediated by different endothelial cell-surface receptors, including thrombospondin, CD36, intercellular adhesion molecule–1 (ICAM-1), E-selectin, vascular cellular adhesion molecule–1 (VCAM-1), CD31, α,β, integrin, and hyaluronic acid [2]. The expression of part of these adhesion molecules has been described to be modulated by cytokines [3], such as tumor necrosis factor (TNF)–α, a molecule that is known to be involved in the pathogenesis of CM [4–6]. Indeed, TNF-α induces profound alterations in vascular endothelial cells, including morphological reorganization from an epithelioid to a fibroblastoid morphology [7], with redistribution of cell junction protein [8], production of inflammatory cytokines, and apoptosis [9, 10]. In addition, it is known that the cross-linking of adhesion molecules on the cell surface is a sufficient stimulus to induce the same phenomena [11, 12]. Of interest, sev-
eral adhesion molecules able to transduce such signals—CD36, ICAM-1, αβ integrin, and CD23—are involved in PRBC sequestration [2].

In the case of CM, endothelial cells are known to be stimulated by both PRBC adhesion and plasmatic inflammatory cytokines, but so far no link has been described between blood-brain barrier dysfunction and hemorrhagic lesions, which are the essential pathologic features of the severe pathology [8, 13]. The present article shows that the functional consequence of PRBC adhesion to endothelial cells is an induction of apoptosis through a caspase-dependent pathway, opening a new field of pharmacological investigations in CM.

MATERIALS AND METHODS

Culture of Human Endothelial Cells (HLECs)

Endothelial cells were isolated from human lung and derived from one batch, as described elsewhere [14]. HLECs were cultured in collagen I-coated flasks in Medium 199 with 10% (vol/vol) fetal calf serum (Life Technologies) and 10 μg/mL endothelial cell growth supplement (Upstate Biotechnology) at 37°C with 5% CO₂. The cells were used at passages 7 or 8 and were characterized for their expression of Von Willebrand factor, ICAM-1, VCAM-1, CD31, CD36, E/P-selectin, and chondroitin sulfate A (CSA), as published elsewhere [14]. The HLECs were seeded at a density of 0.5–1 × 10⁵ cells/cm² and grown until confluence.

Plasmodium falciparum Parasites

P. falciparum 3D7 and F12 clones were used for our experiments. The PRBCs were maintained in culture according to the method of Trager [15] in RPMI 1640 medium supplemented with 8.3 g/L HEPES, 2.1 g/L sodium bicarbonate, 0.1 mg/mL gentamycin, 2 g/L of d-glucose (all from Gibco BRL), and 0.4% Albumax II (Life Technologies). For each experiment, parasite cultures were enriched in mature forms by Plasmagel floating [16]. Parasitemia was then adjusted to 50% and hematocrit to 5%, except where specified.

In Vitro Drug Susceptibility Assay on Blood Stage of P. falciparum

The drug susceptibility was evaluated by using a modification of the proliferation test described by Desjardins et al. [17], which is based on the level of hypoxanthine incorporation. A suspension of PRBCs (ring stage) was distributed in 96-well plates predosed with 40 μL of drug in RPMI 1640 medium (200 μL/well, 1.8% hematocrit, and 0.5% parasitemia). The plates were incubated at 37°C for 96 h. [³H]-hypoxanthine (Amersham) was added (2 μCi/well) 24 h before the end of the test. At the end of incubation period, the plates were frozen and thawed, and cell lysates were collected from each well onto filter papers with a cell harvester (Skatron Instruments). The amount of radioactivity incorporated by the parasite was measured in a liquid scintillation counter (Beckman). All drug concentrations were tested in triplicate. The parasite growth inhibition for each concentration was determined by comparison of the radioactivity incorporated in treated cultures with that of control culture maintained without drug and processed in the same way. The 50% inhibition concentration was determined by a linear-regression model used to summarize the concentration-effect relationship.

Incubation of PRBCs with HLECs

Contact incubation. Endothelial cells were subcultured in 96- or 24-well plates (Costar) for inhibition assays or in 25-cm² culture flasks (Costar) for RNA preparations and were allowed to reach confluence. The infected erythrocytes were added and incubated with the cells for various times in parasite culture medium. In controls, the HLECs were incubated with uninfected erythrocytes, as described above. At the end of the coculture, PRBCs or red blood cells (RBCs) were removed, and the remaining HLECs were analyzed. The adherence of PRBCs was examined on LabTek (Nunc) stained with Giemsa, as described elsewhere [18].

Noncontact incubation. HLECs were subcultured in the wells of transwell culture plates (Costar). At cell confluence, culture chambers with 0.4-μm pore polycarbonate or polyester with or without collagen membranes (Costar) containing PRBCs were added. There appeared to be no difference between using polycarbonate or polyester with or without collagen membrane material.

Analysis of the Gene Expression of HLECs

Macroarray analysis. Gene expression in HLECs was evaluated using human cytokines and apoptosis arrays (R&D Systems), as recommended by the manufacturer. In brief, total RNA was extracted from HLECs stimulated by PRBCs or RBCs for 6 h. PRBCs or control RBCs were added to the HLECs at a hematocrit level of 5% and a parasitemia level of 50% for the tests. RNeasy columns (QIAGEN) were used for RNA extractions, as recommended by the manufacturer. RNA samples were treated with RNase-free DNase (QIAGEN), to avoid any contamination with genomic DNA. Radiolabeled cDNA was generated by reverse transcription (RT) with [α-³²P]dCTP at 42°C. Unincorporated radioactive nucleotides were removed by using Sephadex G-25 columns. The cDNA arrays were hybridized overnight at 65°C. After washes, the arrays were exposed for 24 h to a Phospholmager screen (Fuji BAS-IP-PM2040). The image files were analyzed using FLA-2000 and Aida 2.1 software. The average signal of a pair of duplicated spots representing each gene was normalized with the signals from a series of housekeeping genes. Corresponding normalized signals were compared on different arrays, to determine the fold in-
duction or reduction in the expression of gene-specific RNA between samples.

**Real-time RT–polymerase chain reaction (PCR).** The cDNA Cycle kit (Invitrogen) was used to produce cDNA from total RNA, prepared as described above. Real-time PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma), as recommended by the manufacturer, and a Mx4000 (Stratagene) apparatus and associated software. The PCR pro-

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**Figure 1.** Analysis of gene expression of human endothelial cells (HLECs). Results are expressed as the modulation ratio, obtained by comparing HLECs cocultivated with parasitized erythrocytes or with red blood cells as controls. TNF, tumor necrosis factor.

**Figure 2.** Ultrastructure of parasitized erythrocyte (PRBC)–activated and control human endothelial cells (HLECs). Ultrastructure of HLECs cocultivated with PRBCs (A), compared with HLECs cocultivated with red blood cells (RBCs) (B). An accumulation of Golgi vesicles (thin arrow, B1) and then of endoplasmic reticulum (thick arrow, B2) in PRBC-activated HLECs, compared with HLECs cocultivated with control RBCs, showing the alteration of the cell surface. PRBC-stimulated endothelial cells are highly vesiculated (V) and show the condensed electron-dense cytoplasm typical of apoptosis, compared with HLECs cocultivated with RBCs. Bar, 1 μm.
Figure 3. Annexin V binding assays. Human endothelial cells (HLECs) were cocultivated with parasitized erythrocytes (PRBCs; left) or red blood cells (RBCs; right) for 4 h and stained with annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI).

gram used was 1 min at 95°C, 40 cycles of 30 s at 95°C, 1 min at 65°C, and a temperature ramp from 95°C to 65°C for 30 min. The sequences of the used primers were, from 5′ to 3′: Bad, CCGGAGGATGAGTGAGTT and GGGTGGAGTTTCC-GGATGT; Bax, CATGGAGCTGAGAGGATGAT and CCAGTTGAAGTTCCGTCAGA; caspase-3, ATACAGGTTGAG-GGTTC and TTTTCTCCCTGCTTTCCAG; DR-6, GCTACGTTGCCCCCA and CCGGAGCAGACTGTTACA; Fas, GCCTGTCCCTCAGGAAA and TGCTGGTGAGT-GCATTCT; Fas ligand, GCTGAGGAAAGTGGCCCATT and GGCAGGTTGAGCATGTTA; and iNOS, ACCAGG-CTCGTGAGTAC and CACGGAGCGGTATTAC-TCT. The results were normalized and analyzed using GAPDH expression and Mx4000 Stratagene software.

Apoptosis Assays

Transmission electron microscopy. Morphological changes were assessed by examination of the cells in an inverted phase-contrast microscope and by electron microscopy. HLECs were incubated with PRBCs or control RBCs for 18 h, washed with PBS and fixed in 1% glutaraldehyde in 0.1 M Na cacodylate.

Figure 4. Apoptosis induction in human endothelial cells (HLECs) is time and dose dependent. In these representations, HLECs cocultivated with parasitized erythrocytes are compared with HLECs cocultivated with control red blood cells. The resulting apoptosis ratios are plotted against (A) time, (B) parasitemia, or (C) hematocrit. The coculture time was 4 h.
concentrations of 50–200 (Bachem), Z-IETD-fmk, and Z-LEHD-fmk (R&D Systems) were used in the pretreatment of HLEC for 2 h at room temperature. The F12 strain was used as a nonadherent control parasite strain. The induction of apoptosis in HLEC was assessed by measuring intracytoplasmic nucleosome release.

buffer (pH 7.2) for 1 h, rinsed in buffer, and postfixed in ferriosmium [1% wt/vol OsO₄/K₃Fe(CN)₆ in cacodylate buffer] for 1 h at room temperature. After ethanolic dehydration, the samples were embedded in Spurr resin. The sections were stained with uranyl acetate and lead citrate and then examined with the transmission electron microscope JEOL JEM at 100× magnification.

Annexin V assays. HLEC were cocultivated with PRBCs at 5% hematocrit and 50% parasitemia for 4 h or with RBCs, for controls. HLEC were then washed with binding medium, detached from culture flasks with diluted trypsin (Invitrogen), and stained with annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (Oncogene), as recommended by the manufacturer.

Nucleosome release assays. The proportion of apoptotic cells was estimated by measuring DNA fragmentation in the cultures. An ELISA technique dosing the liberation of mono- and oligonucleosomes in the cytoplasm of apoptotic cells (Roche) [19] was adapted to our model for this purpose.

The HLEC were cocultivated with PRBCs or control RBCs, as described above. For the inhibition of apoptosis experiments, Z-VAD-fmk (Bachem), Z-IETD-fmk, and Z-LEHD-fmk (R&D Systems) were used in the pretreatment of HLEC for 2 h at concentrations of 50–200 μM alone or in combination.

Caspase activity assays. Caspase 3 and 8 activities were assessed by using the Biomol QuantiZyme assay system. Cryptic extracts of HLEC were prepared from 4 × 10⁶ cells after 4 h of coculture with PRBCs (3D7 clone) at 50% parasite and 5% hematocrit, RBCs at hematocrit 5%, and untreated cells. The total protein amount in cytosolic extracts was quantified, and 10 μg of protein was used per caspase assay, as recommended by the manufacturer. The degradation of colorimetric substrates, Z-DEVD-pNA, or Z-IETD-pNA was recorded every 10 min during a 130-min period at 405 nm in a microtiter plate reader. Results are expressed as caspase activity (pmol of pNA/min).

RESULTS

The endothelial phenotype of HLEC was evaluated by immunohistochemistry; these cells express Von Willebrand factor, ICAM-1, CD31, VCAM-1, E/P-selectin, CSA, and CD36 (data not shown). To assess the biological impact of the cytoadherence phenomenon on endothelial cell functions, we used macroarrays to screen the gene-specific expression of endothelial cells cocultivated with the 3D7 clone of *P. falciparum*.

In these experiments, the expression of cytokines and apoptosis-related mRNA in endothelial cells after incubation with PRBCs was examined. As is shown in figure 1A, proinflammatory and proapoptotic genes were up-regulated in the HLEC cocultivated with PRBCs, compared with control cells cocultivated with RBCs. Members of the TNF superfamily, VEGI and TRAIL R2, had their expression increased 4.0 and 2.7 times, respectively, whereas expression of death receptor (DR)–6 was increased 4.3 times at 6 h of coculture. In addition, genes encoding Fas and Fas ligand appeared to be up-regulated in HLEC after PRBC adhesion, 3.5 and 3.4 times, respectively. Related to the endothelial stress, other different genes encoding molecules that are directly or indirectly involved in the regulation of apoptosis or inflammation were differentially expressed in parasite-activated HLEC: eNOS, SARP 2, DFF45/ICAD, IFN-γ receptor 2, Bel-w, and Bik. These results were confirmed by real-time RT-PCR (figure 1B), and additional genes were investigated—iNOS, Bad, Bak, and caspase 3—that appeared to be up-regulated in HLEC cocultivated with PRBCs. Different tools were used to confirm endothelial apoptosis observed at the RNA level, ultrastructural studies, annexin-V binding, internucleosomal DNA cleavage, and caspase activity.

HLECs stimulated by PRBCs showed alterations leading to apoptosis (figure 2B). These alterations were not observed in HLEC cocultivated with control RBCs (figure 2A). Morphological changes included (1) the formation of cytoplasmic protruberances and the development of Golgi vesicles (figure 2B1), (2) a rearrangement of the endoplasmic reticulum apparently correlated with the apparition of vacuoles at the cell surface (figure 2B2), and (3) the final condensation of the cytoplasm typical of apoptosis associated with the extensive vacuolization of the cell surface (figure 2B3).

Double-labeling annexin V and propidium iodide showed that 15.3% of annexin V–FITC–positive cells and 5.0% of double annexin V and propidium iodide–positive cells in HLEC cocultivated with PRBCs, whereas only 0.5% and 1.0% of control HLEC cocultivated with RBCs were stained (figure 3). These data were confirmed using an ELISA assay that allowed...
Figure 6. Enrichment of nucleosomes in the cytoplasm of human endothelial cells (HLECs) treated with caspase inhibitors. HLECs were pretreated with different concentrations of inhibitors for 2 h at 37°C, before the addition of parasitized erythrocytes (PRBCs), red blood cells (RBCs), or were treated as the same inhibitor concentration at PRBC addition. The coculture duration was 4 h. Black diamonds, inhibitor treatment. The inhibitor was added to the HLECs at the same time as PRBCs. HLECs cocultivated with PRBCs were compared with HLECs cocultivated with control RBCs. The resulting apoptosis ratio is represented. White squares, inhibitor pretreatment. The HLECs were pretreated with the caspase inhibitor for 2 h and washed with medium before the addition of PRBCs. The same apoptosis ratio is plotted. A, HLECs were treated or pretreated with the general caspase inhibitor Z-VAD. The caspase 8 inhibitors Z-IETD (B) and Z-LEHD (C) were also used. D, Z-IETD or Z-LEHD were used in combination in HLEC pretreatment. All the inhibitors tested were assessed for any modulation of apoptosis on control HLECs cocultivated with RBCs, and no activity was found (data not shown).

for the quantification of the internucleosomal DNA cleavage. As shown in figure 4A, an increase of intracytoplasmic nucleosomes in PRBC-activated HLECs was observed (up to a factor of 6.0 ± 1.2, compared with RBC controls). This effect was time dependent, from a factor of 3.1 ± 0.5 at 4 h of coculture to a factor of 6.0 ± 1.2 at 24 h (figure 4A). In addition, apoptosis appears to depend on the parasitemia and hematocrit levels, as shown in figure 3B, 3C.

We used transwell culture plates, which allow the coculture of different cell types without physical contact, through a 0.4-μm porous membrane. A noncontact incubation of HLECs with PRBCs did not induce HLEC apoptosis (figure 5). In addition, the use of the F12 clone, which is poorly adherent (1 PRBC/20 HLECs at maximum, compared with the 3D7 clone, for which >1 PRBC/HLEC is usually observed), did not induce apoptosis in HLECs.

Caspase 3 and 8 activities were induced in HLECs cocultivated with PRBCs, whereas culture with RBCs or untreated HLECs did not reveal such activity. As shown in figure 6, pretreatment of the HLECs with the general caspase inhibitor Z-VAD prior to the addition of PRBCs reduced the enrichment in cytoplasmic nucleosomes. When added to HLECs at the same time as PRBCs, Z-VAD’s anti-apoptotic effect was significantly increased. To eliminate a direct effect of Z-VAD on P. falciparum development, Z-VAD was assessed by using a method described by Desjardins et al. [17]. No inhibition of development was observed (data not shown), which demonstrates that Z-VAD only affected the consequence of the PRBC/endothelial cell interactions and that it does not have any antimalarial activity per se.

More-specific caspase inhibitors, Z-IETD and Z-LEHD, were then used in apoptosis inhibition assays. Z-IETD or Z-LEHD pretreatments of HLECs reduced apoptosis induction by PRBC adhesion (figure 6).
DISCUSSION

The adhesion of *P. falciparum*–infected erythrocytes to the vascular endothelium is the major biological mechanism that mediates the sequestration phenomenon of PRBCs [20, 21]. In the present article, we clearly demonstrate that the adhesion of PRBCs to endothelial cells through their interactions with cellular adhesion molecules (from the immunoglobulin and integrin families) induces a deleterious endothelial response. This mechanism could be directly involved in the disruption of the blood-brain barrier [8].

The expression of cytokines and apoptosis-related mRNA in endothelial cells after incubation with PRBCs was examined. Proinflammatory and proapoptotic genes from the TNF superfamily were up-regulated in the HLECs cocultivated with PRBCs, compared with control cells cocultivated with RBCs. Taken together, the overexpression of these genes suggested an induction of endothelial apoptosis. The induction of apoptosis was confirmed on the basis of morphology. The development of Golgi vesicles and endoplasmic reticulum reorganization indicate an important cell membrane activation.

It is well established that the cross-linking of adhesion molecules on the endothelial cell membrane can induce intracellular signaling and lead to various cellular responses such as apoptosis [22, 23]. We verified the possible role of this mechanism in PRBC-induced HLEC apoptosis. By using transwell culture plates, we showed that physical interactions between HLECs and PRBCs are necessary to induce apoptosis in HLECs. The F12 clone, which is poorly adherent, did not induce apoptosis in HLECs, which supports the idea that the apoptosis induction was dependent on PRBC adhesion. The requirement of direct contact of PRBCs with HLECs for apoptosis induction suggests a transmembrane receptor–mediated activation. We showed, at the transcriptional level, the up-regulation of Fas, DR-6, and TRAIL receptor–2, 3 death-domain–containing receptors able to induce apoptosis, when stimulated by their ligands. In the same way, Jimenez et al. [24] demonstrated that the binding of the CD36 ligand thrombospondin-1 on endothelial cells is sufficient to transduce a signal through p59/fyn and caspases that leads to endothelial cell apoptosis. In our model, we also observed an up-regulation of the caspase 3 gene. Similarly, the cross-linking of integrin αβ, by antagonist antibodies can induce the same phenomenon [25].

Apoptosis is a tightly regulated form of physiological cell death that depends on the expression of intrinsic cell suicide machinery [26]. The terminal stage of apoptosis involves the activation of a related family of proteases, the caspases [27]. The cellular mechanism of action of this PRBC-induced apoptosis appeared to be related to the induction of caspase activity (figure 7). Indeed, caspase 3 and 8 activities were induced in HLECs cocultivated with PRBCs, whereas culture with RBCs or untreated HLECs did not reveal such activity.

Because caspases appeared to be the effectors of apoptosis in our model, we evaluated the pharmacological effect of different caspase inhibitors, Z-VAD-fmk, Z-IETD-fmk, and Z-LEHD-fmk. The general caspase inhibitor Z-VAD inhibited apoptosis induction—a consequence of the PRBC/endothelial cell interaction.

The use of Z-IETD-FMK and Z-LEHD-FMK, which are specific inhibitors of caspases 8 and 9, respectively, helped us to sort out the cellular pathways involved. Two major pathways have been described to transduce signal after a proapoptotic treatment: the cytoplasmic pathway from a death receptor (DR-6, Fas, or TNF-R1) through caspase 8 [28] and the mitochondrial pathway through caspase 9 activation [27–29]. Z-IETD and Z-LEHD treatments protected HLECs against PRBC-induced apoptosis. Both caspases 8 and 9 are involved in the process.

In conclusion, the present study demonstrates that PRBC adhesion to HLECs induces caspase-8– and 9–dependent apoptosis in endothelial cells. These data enlarge on those of other studies that described lesions in the endothelial bed associated with PRBC adhesion [8, 30]. Indeed, the apoptosis of endothelial cells as a consequence of PRBC adhesion might result in blood-brain barrier dysfunctions and lesions. In addition, it was demonstrated that apoptotic endothelial cells up-regulate the expression of cellular adhesion molecules on normal endothelial cells, resulting in hyperadhesiveness [31], which might contribute to and amplify the pathological process. Even though the fine mechanisms and signal pathways involved in this process remain to be further defined, these data provide a new approach toward investigations...
into the mechanisms involved in CM and an appreciation of the potential effect of pharmaceuticals.

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