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Trophoblast Contact Deactivates Human Neutrophils

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Trophoblasts are fetal epithelial cells that form an interface between mother and offspring. To evaluate their anti-inflammatory capacity, we tested the hypothesis that trophoblasts deactivate neutrophils using single-cell assays. Several biophysical (Ca^{2+} and NAD(P)H oscillation frequency) and physiological (oxidant production) markers of activated neutrophils revert to a nonactivated phenotype as activated cells make contact with trophoblasts. Indistinguishable results were obtained using syncytiotrophoblasts and in experiments using trophoblasts and neutrophils from the same mother to recapitulate the semiallogeneic system. These changes suggest reduced hexose monophosphate shunt (HMS) activity. We discovered that two metabolic regulatory points, glucose transport and HMS enzyme trafficking, are affected by trophoblasts. This restriction in HMS activity deactivates neutrophils, thereby limiting oxidative DNA damage within trophoblasts. The Journal of Immunology, 2006, 176: 3205–3214.

Successful pregnancy remains one of the great paradoxes of immunology: the fetus survives despite its semiallograft status. One likely site of immunoregulation is the placenta, which forms an interface such that the maternal immune system interacts directly with the placenta, not the fetus. The principal placental cell type interacting with the maternal immune system is the trophoblast. Although the mechanisms allowing the immune system to spare the fetus are not understood, it is becoming clear that trophoblasts are important participants. Trophoblasts exhibit some characteristics in common with immune cells, such as their ability to migrate, phagocytose, and invade tissues (1–3). These cells also express complement regulatory proteins, cytokine receptors, TLRs, FcRn, and proteases (4–9). The expression of complement-receptor-1-related protein y is important in the maintenance of rodent pregnancy due to its ability to inhibit the deposition of complement at the materno-fetal interface (9). Trophoblasts do not express conventional HLA class I Ags, but express HLA-G, which promotes tolerance (10, 11). In addition to these rather passive defense strategies to deflect immune responses, trophoblasts may also actively modify the mother’s immune response. For example, it is widely known that trophoblasts produce and secrete cytokines, largely Th2 cytokines, that promote the Th2-biased phenotype observed in pregnant women (12). The Th1 to Th2 shift during pregnancy is thought to be crucial for normal pregnancy, because proinflammatory responses associated with Th1 cytokines have been associated with spontaneous abortion and preterm labor (12). Other active mechanisms of maternal leukocyte regulation are suggested by studies showing that the conceptus is protected from destruction by activated macrophages (13) and that neutrophil activation is depressed during pregnancy (14).

To better understand neutrophil activation in pregnancy, we have analyzed the phenotypic properties of peripheral blood cells from pregnant women. We have recently shown that neutrophils from pregnant women decrease their production of oxidants by translocating enzymes of the hexose monophosphate shunt (HMS) from the plasma membrane to the centrosome, thereby limiting the availability of glucose-6-phosphate, which is produced at the cell periphery, and decreasing HMS activity and oxidant production (14–16). In this study we use the opposite approach to characterize neutrophil regulation in pregnancy; we activate cells from nonpregnant individuals in vitro, then watch as they interact with trophoblasts. Using highly sensitive single-cell assays to follow these intercellular interactions, we show that neutrophils rapidly deactivate upon trophoblast contact. Our results suggest that trophoblasts accomplish this by influencing both glucose transport and the intracellular location of HMS enzyme complexes, which reduces the neutrophil’s ability to make reactive oxygen metabolites (ROMs) and damage neighboring cells.

Materials and Methods

Patients

Peripheral blood was collected from nonpregnant and pregnant women after written informed consent was provided. Placental samples were obtained after childbirth, with written informed consent. These studies were approved by the Institutional Review Board of the participating institutions. The nonpregnant group consisted of women in the secretory phase of the menstrual cycle who were not taking oral contraceptives and who had no history of acute or chronic inflammatory conditions. The pregnant group was made up of women with normal pregnancies. Eligible patients were approached at the Detroit Medical Center (Detroit, MI).

Materials

FMLP, LPS (0111:B4), and hydroethidine (HE) were obtained from Sigma-Aldrich. IL-8 and TNF-α were obtained from Chemicon International. Indo-1 AM, dihydrotetramethylrosamine, (H_TMRos) fluorescein-conjugated N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (Fl-FNLNPNTL), H_2-7 dichlorofluorescein (DCF), and 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminoo)-2-deoxyglucose (2-NBDG) were obtained from Molecular Probes. Rabbit anti-
glucose-6-phosphate dehydrogenase (G-6-PDase) and goat anti-lactate dehydrogenase polyclonal Ab were obtained from Chemicon International. Anti-transaldolase was obtained from Nordic Immunological Laboratories.

**Leukocyte isolation**

Neutrophils were obtained using two Ficoll-Hypaque solutions of different buoyant densities (Histopaque 1077 and 1119; Sigma-Aldrich) and centrifugation. Cells were washed twice by centrifugation, then resuspended in HBSS (Invitrogen Life Technologies). Trypan blue staining indicated that 95–99% of the cells were viable.

**Cell culture**

JEG-3, JAR, and HTR8/SVneo trophoblasts were grown in RPMI 1640 containing 10% FCS and 1% penicillin G/streptomycin/amphotericin B (Invitrogen Life Technologies). Before microscopy experiments, cells were grown for 24 h attached to glass coverslips.

**Isolation of placental tissue and cells**

Placental villi, which are covered with syncytiotrophoblasts, were dissected from placental tissue, washed thoroughly with HBSS, then used immediately for experimentation. Primary trophoblast cultures were obtained as previously described (4). Cells were scraped from the membranes, then treated with trypsin-ENDTA (Invitrogen Life Technologies) for 10 min at 37°C. DMEM containing 10% FBS was added to inhibit further proteolytic digestion. The extract was vortexed for 20 s, then tissue fragments were allowed to sediment, and the supernatants were collected. After this protocol was repeated, the sample was centrifuged to pellet the trophoblasts, then suspended and centrifuged again using a layer of Lymphocyte Separation Medium (ICN Biomedicals). The trophoblasts were collected, then grown in DMEM containing FBS. To verify trophoblast purity, cells were routinely stained with anti-cytokeratin-7 (Sigma-Aldrich), which was positive for >98% of the cells.

**Immunofluorescence staining**

Neutrophils were placed on glass coverslips, incubated with reagents as described below, then fixed as previously described (14). Briefly, cells were fixed with 2% paraformaldehyde, permeabilized with 1% Brij-58, then fixed a second time with 2% paraformaldehyde at room temperature for 20 min. Cells were washed with HBSS, labeled with 1 μg of FITC-conjugated Abs at 4°C for 30 min., then washed again with HBSS at room temperature.

**Fluorescence microscopy**

Cells were observed using an Axiosvert fluorescence microscope (Zeiss) with mercury illumination interfaced to a computer using Scion image processing software (17). A narrow bandpass-discriminating filter set (Omega Optical) was used with excitation at 485/22 nm and emission at 530/30 nm for FITC, and excitation at 540/20 nm and emission at 590/30 nm for tetramethylrhodamine isothiocyanate. Long-pass dichroic mirrors of 510 and 560 nm were used for FITC and tetramethylrhodamine isothiocyanate, respectively. The fluorescence images were collected with intensified, charge-coupled device cameras (Princeton Instruments and Q Imaging).

**Ca\(^{2+}\) and NAD(P)H oscillations**

Ca\(^{2+}\) and NAD(P)H oscillations were monitored at 37°C as previously described (18–20). Indo 1 was used to detect Ca\(^{2+}\) transients, whereas unlabeled cells were used to measure NAD(P)H oscillations. For NAD(P)H autofluorescence, a 365WB50 excitation filter, a 400-nm long-pass dichroic mirror, and a 450AF58 emission filter were used. In the case of indo 1 detection, an emission filter at 405DF43 was used. A cooled, high sensitivity photomultiplier tube in a D104 detection system (Photon Technology International) attached to a Zeiss microscope was used. Data were analyzed using Felix software (Photon Technologies). Calcium data were smoothed using a Savitzky-Golay function with a buffer size of seven.

**Detection of ROMs**

Pericellular ROM release from single cells was monitored as previously described (21). Briefly, adherent neutrophils were surrounded in 2% gelatin containing 100 ng/ml H\(_2\)TMRos (Molecular Probes). ROMs, especially H\(_2\)O\(_2\), released by the cells entered the gelatin matrix, where they oxidized H\(_2\)TMRos to TMRos, which was detected by fluorescence microscopy. Experiments to detect intracellular ROMs were performed as previously described (22).

**Detection of DNA damage**

Trophoblasts were grown overnight on glass coverslips. For ROM detection, trophoblasts were labeled with H\(_2\)TMRos as described above. Neutrophils in suspension were mixed with 50 nM FMLP, then placed on subconfluent trophoblast cultures for 1.5 h at 37°C. Coverslips were gently washed, followed by collection of differential interference contrast (DIC) and TMRos images. Coverslips were removed, then fixed with 3.7% paraformaldehyde. After cells were permeabilized with 70% ethanol for 30 min, TUNEL staining was performed as recommended by the manufacturer (Upstate Biotechnology). Coverslips were then remounted on slides, and the same regions were photographed. Images of TMRos and TUNEL staining were pseudocolored and overlaid using Image-Pro Plus (Media Cybernetics). For quantitation of DNA damage, 1.2 × 10⁴ neutrophils were added to subconfluent trophoblasts at a 2:1 ratio.

**Results**

**Single-cell features of activated neutrophils**

Two important features of neutrophil activation are changes in Ca\(^{2+}\) signaling (23–26) and metabolism, especially activation of the HMS and the accompanying respiratory burst (15, 18). Fig. 1 illustrates the relationships between neutrophil physiologic and metabolic dynamics. Unstimulated spherical neutrophils displayed low-amplitude oscillations, with a period of ~3 min. (Fig. 1, A and B), which probably reflects the dynamic properties of phosphofructokinase. When cells were morphologically polarized, which is generally associated with cell migration, metabolic oscillations with a period of ~20 s were observed (Fig. 1, C and D). After activation with 100 nM FMLP, oscillations with a period of ~10 s were observed (Fig. 1, E and F), which may be due to the diversion of carbon away from the glycolytic pathway to the shunt. Parallel changes in the Ca\(^{2+}\) signaling pathway have also been observed (18). These noninvasive readouts were used to probe the physiologic status of neutrophils in the presence of trophoblasts.
**Trophoblast contact alters Ca²⁺ signaling in activated neutrophils**

In view of the trophoblast’s potential immunoregulatory capacity, we have tested the hypothesis that trophoblasts directly regulate the activation status of neutrophils. We therefore examined the trophoblast’s ability to affect the neutrophil’s metabolic and signal transduction apparatus. We studied Ca²⁺ signaling using microfluorometry of single activated neutrophils (100 nM FMLP at 37°C) before and after contact with JEG-3 trophoblasts (Fig. 2, A–C). Previous studies from this group have shown that unstimulated, adherent neutrophils display Ca²⁺ spikes at ~20-s intervals, whereas FMLP-activated neutrophils exhibit Ca²⁺ spikes at 10-s intervals (18, 19), which parallels the metabolic changes noted in Fig. 1. Morphologically polarized and activated neutrophils also displayed Ca²⁺ spikes at ~10-s intervals when mixed with trophoblasts (Fig. 2). At the time scale used in Fig. 2C, the spikes appeared featureless, because the number of data points per spike was small; when data were collected over a short time scale (a large number of pixels per spike), there was a shoulder at short times and a decay after the peak at longer time periods. However, the important point is that the high-frequency oscillations returned to normal (~20-s period) after neutrophil-trophoblast contact. Fig. 2C illustrates the shift in Ca²⁺ spike frequency, whereas A and B document the contact of the neutrophil with the trophoblast. Similar results were obtained for both JEG-3 and JAR (data not shown) trophoblast cell lines. However, the HTR-8/SVneo trophoblasts, a model of invasive trophoblasts, did not demonstrate neutrophil deactivation (data not shown). Furthermore, activated neutrophils did not undergo deactivation when contact was made with HT1080 fibrosarcoma cells (data not shown). Thus, Ca²⁺ oscillations return to a nonactivated profile after contact with trophoblasts.

Because FMLP is known to promote Ca²⁺ signals and neutrophil activation, we reasoned that trophoblast-mediated neutrophil deactivation could be accomplished by dissociation of the activating stimulus from the cell. To test this idea, we used the fluorescent FMLP analog Fl-FNLPNTL to stimulate neutrophils. Although Fl-FNLPNTL-labeled neutrophils underwent deactivation after contact with trophoblasts (data not shown), the fluorescence intensity did not noticeably change in intensity or distribution. Therefore, the immunoregulatory effects of trophoblasts on neutrophils cannot be explained by ligand dissociation.

**Trophoblasts deactivate neutrophil metabolic oscillations**

The change in Ca²⁺ signaling suggests that trophoblast-neutrophil contact may affect neutrophil functions. Because previous studies have shown that Ca²⁺ signals and metabolic oscillations correlate with one another (18), we examined metabolic oscillations during interactions between activated neutrophils and trophoblasts. Metabolic oscillations were monitored using the autofluorescence of NAD(P)H, which provides a noninvasive tool to monitor cells. Neutrophils were first activated with FMLP. Activated cells were then added to subconfluent trophoblasts. Subconfluence was chosen so that some neutrophils would be in contact with the trophoblasts, whereas other neutrophils (internal controls) would not be in contact with trophoblasts. This allowed the study of metabolic oscillations of trophoblast-attached and nonattached neutrophils within a small area of a microscope slide. Fig. 2D shows a non-attached activated neutrophil, and the left side of Fig. 2F shows its metabolic oscillations; high frequency NAD(P)H oscillations indicated that the HMS is activated (14, 18, 20). However, when this activated neutrophil made contact with a trophoblast (Fig. 2E), the metabolic oscillations decreased in frequency and returned to the normal, nonactivated profile (Fig. 2F). Thus, it is not sufficient to have trophoblasts near an activated neutrophil (Fig. 2D); the activated neutrophil appears to require physical contact with a trophoblast to deactivate. As negative controls, we cocultured neutrophils with tumor cells (HT1080 fibrosarcoma). Importantly, tumor cell contact switched NAD(P)H from the low to the high frequency (activated) mode (data not shown), just the opposite of the trophoblast’s effects. Hence, trophoblast contact regulates certain aspects of neutrophil metabolism.

The neutrophil deactivation events were not specific for FMLP-activated neutrophils. Several neutrophil-activating agents, including LPS, TNF-α, and IL-8, activate neutrophil metabolism, as judged by an increase in oscillation frequency (15, 18). When LPS-, TNF-α-, and IL-8-activated neutrophils were incubated with subconfluent JEG-3 cells, their metabolic oscillations were found to shift from ~10 to ~20 s (data not shown). Hence, multiple neutrophil-activating stimuli may be regulated by trophoblasts.

**Trophoblasts diminish ROM production by activated neutrophils**

We next sought to determine the physiological consequences of trophoblast contact with neutrophils. We chose to monitor ROM production because of its role in inflammatory processes and its
connection with NAD(P)H oscillations (14, 20). We again evaluated single cells before and during neutrophil contact, because test tube assays of ROM production would not be informative, as many nonattached cells would produce ROMs. Using subconfluent trophoblast cultures, we labeled neutrophils with the nonfluorescent molecules HE, CD CF, or dihydrorosamine using procedures established previously (20). These molecules were converted into the highly fluorescent molecules ethidium, fluorescein, and TMROS, respectively, upon exposure to ROMs. Labeled, but nonfluorescent, neutrophils were then cocultured with subconfluent trophoblasts. FMLP (100 nM) was added to activate the neutrophils. Fig. 3 shows two representative experiments using two probes for ROM detection, HE and H₂-CDCF. The addition of FMLP to these cocultures caused extensive conversion of HE and H₂-CDCF to their fluorescent products in neutrophils not adherent to trophoblasts. This was true for neutrophils only 1 μm away from a trophoblast cell. However, neutrophils bound to trophoblasts were dim in these experiments. The presence of unbound/bright and bound/dim neutrophils indicated that this was not a nonspecific artifact of the assay, such as slow oxidation of the labels by air. Similar results were obtained with two different trophoblast cell lines (JEG-3 and JAR).

Because the experiments shown in Fig. 3 measure the amount of probe oxidation inside neutrophils, we next tested ROM deposition outside neutrophils using other methods that detect ROM release (21). Neutrophils were FMLP activated before addition to trophoblasts. Including H₂-TMROS in the gelatin matrix surrounding trophoblasts and neutrophils monitored ROM release. In this experiment, a neutrophil approaching a clump of trophoblasts was identified (Fig. 2G). The kinetics of ROM release were measured by microfluorometry, which showed a substantial rate reduction during the experiment (Fig. 2I). When the sample was imaged after acquiring microfluorometry data, the neutrophil was found to be in contact with a trophoblast (Fig. 2H). Our studies indicate that contact with a trophoblast down-regulates neutrophil inflammatory activity by a mechanism involving a reduction in Ca²⁺ and NAD(P)H oscillations frequencies, which is consistent with HMS inhibition and reduced ROM production.

**Neutrophil deactivation is observed for trophoblasts and neutrophils from the same patients**

The above experiments have focused on neutrophil interactions with a trophoblast cell line. The interpretations of this work may be limited by the facts that 1) this is a transformed cell line and may not be representative of normal trophoblasts; and 2) the trophoblasts and neutrophils are from dissimilar genetic backgrounds. We therefore performed a series of experiments using trophoblasts and neutrophils from the same patient. After delivery by patients undergoing cesarean sections, trophoblasts were obtained from placenta. Before discharge on day 3, blood was drawn from the patient. Experiments indicated that at least 95% of the patients’ neutrophils could be activated on day 3 with 100 nM FMLP, as observed for cells from nonpregnant individuals. Fig. 4 shows a series of experiments with trophoblasts and neutrophils derived from the same mother that parallel those shown in Fig. 2. Fig. 4 shows Ca²⁺ responses, NAD(P)H oscillations, and ROM production by neutrophils in response to binding semiallogeneic trophoblasts from the same individual. The left sides of Fig. 4, C, F, and I, indicate that the neutrophils were activated by FMLP treatment. Fig. 4, A, B, D, E, G, and H, shows images of cells before and after neutrophil binding to trophoblasts. In all cases, the frequencies of Ca²⁺ spikes and NAD(P)H oscillations and the amount of ROMs produced were reduced immediately after trophoblast binding. Thus, the normal semiallogeneic biological system displays the same properties as those described above for the trophoblast cell line.

**Villous syncytiotrophoblasts deactivate neutrophils**

The experiments described above have focused on cytrophoblasts. To determine whether syncytiotrophoblasts exhibit these same properties, placental villi were studied. Because placental villi must be studied immediately, it was not possible to study villi and leukocytes from the same normal patient, as her leukocytes would have been refractory to FMLP activation. Fig. 5 shows representative studies of neutrophil interactions with this tissue. Again, the left sides of Fig. 5, C, F, and I, clearly show that the cells were activated before contact with syncytiotrophoblasts. Fig. 5, A, B, D, E, G, and H, confirms that neutrophils made contact with the tissue under study. After contact, all three cell parameters changed, as described above for cell lines and the natural semiallogeneic systems. This suggests that the ability to deactivate neutrophils is shared among multiple types of trophoblasts.

**Trophoblasts influence metabolic control points of neutrophils**

As mentioned above, enhanced Ca²⁺ signaling can lead to activation of the neutrophil’s HMS (23, 24, 26). Importantly, inhibition of the HMS of activated neutrophils or other cell types using 6-aminonicotinamide can lead to changes in Ca²⁺ signaling (27) (our unpublished observations). Given the parallel reduction in Ca²⁺ signaling, NAD(P)H oscillation frequency, and ROM release.
noted above for several different types of trophoblasts and the correspondence of these parameters with HMS activity, we hypothesized that trophoblasts influence one or more metabolic control points preceding the HMS. It is known that glucose transport is a rate-controlling step in metabolism (28) and that enhanced glucose transport is required for neutrophil activation (29–32). To test glucose transport at the single-cell level, we used the fluorescent glucose analog 2-NBDG (33). To illustrate the properties of neutrophil Ca\(^{2+}\) spikes (34), the frequency of NAD(P)H oscillations, and the ROM production rate were reduced by trophoblast contact. The vertical bar = 2 × 10^4 counts. Magnification, ×790. n = 4.

FIGURE 4. Single-cell studies of neutrophil-trophoblast interactions. Neutrophils and trophoblasts were derived from the same patient. DIC micrographs are shown in A, B, D, E, G, and H, which were acquired immediately before (A, D, and G) or after (B, E, and H) collection of the kinetic fluorescence data shown in C, F, and I. Studies of Ca\(^{2+}\) spikes (A–C), NAD(P)H oscillations (D–F), and ROM release (G–I) are shown. In all cases, the neutrophils were activated with 100 nM FMLP. Quantitative microfluorometry experiments of these samples showed that the frequency of neutrophil Ca\(^{2+}\) spikes (C), the frequency of NAD(P)H oscillations, and the ROM production rate were reduced after trophoblast contact. The vertical bar = 2 × 10^4 counts. Magnification, ×790. n = 4.

FIGURE 5. Single-cell studies of neutrophil-syncytiotrophoblast interactions. Neutrophils were studied during interactions with freshly isolated placental villi. DIC micrographs are shown in A, B, D, E, G, and H, which were acquired immediately before (A, D, and G) or after (B, E, and H) collection of the kinetic fluorescence data shown in C, F, and I. Separate studies of Ca\(^{2+}\) spikes (A–C), NAD(P)H oscillations (D–F), and ROM release (G–I) are shown. In all cases, the neutrophils were activated with 100 nM FMLP. Quantitative microfluorometry showed that the frequency of neutrophil Ca\(^{2+}\) spikes (C), the frequency of NAD(P)H oscillations, and the ROM production rate were reduced after contact with trophoblasts. The vertical bar = 2 × 10^4 counts. Magnification: A, B, D, and E, ×740; G and H, ×820. n = 4.

uptake with trophoblast-mediated glucose uptake, trophoblast membrane fragments, prepared by osmotic lysis, were used. FMLP-activated neutrophils were added to adherent lysed trophoblast fragments for 20 min at 37°C to allow cell-cell contact to take place. 2-NBDG at 25 μg/ml in HBSS was then added for 35 min at 37°C. This time was chosen because 2-NBDG cellular fluorescence is linear at this incubation time. Coverslips were washed four times with HBSS, then examined microscopically. A representative experiment is shown in Fig. 6, F–H. Fig. 6F shows that some FMLP-activated neutrophils, prepared as described above, were in contact with the glass substrate, whereas others were touching cell fragments derived from trophoblasts. The fluorescence of 2-NBDG is shown in Fig. 6G. Unbound neutrophils were brightly labeled with 2-NBDG (intensity, 141 ± 18), whereas neutrophils in contact with trophoblast fragments were comparatively dim (intensity, 48 ± 16), as indicated by the arrows. To control for possible nonspecific effects, we evaluated the ability of HT1080
Neutrophil deactivation protects trophoblasts

The preceding paragraphs have identified a unique anti-inflammatory mechanism of trophoblasts as well as its underlying biochemical regulatory circuits. To explore the physiological ramifications of this new pathway, we examined the ability of FMLP-treated neutrophils to deposit ROMs and elicit DNA damage in neighboring trophoblasts. Trophoblasts were labeled with 
H₂TMRos, as described above, then incubated with FMLP-treated neutrophils at 37°C for 1.5 h. Although little TMRos fluorescence could be found in JEG-3 cells, considerable fluorescence could be found in HTR8/SVneo cells (Fig. 8). Because ROMs are known to damage DNA, we next evaluated DNA damage using the TUNEL protocol for labeling strand breaks. TUNEL staining was not observed for JEG-3 cells, indicating that FMLP-treated neutrophils did not affect these cells, although they contacted these trophoblasts. However, TUNEL staining was observed when HTR8/SVneo cells were cocultured with FMLP-treated neutrophils (Fig. 8D). Although both JEG-3 and HTR8/SVneo cells had associated neutrophils (69 ± 9 vs 74 ± 8%, respectively), only 3.8 ± 2.9% of JEG-3 cells were stained by the TUNEL method compared with 60 ± 12% of HTR8/SVneo cells (p < 0.001). However, it remains possible that these differences might be accounted for by something other than the neutrophil’s activation state. JEG-3 and HTR8/SVneo cells did not display TUNEL labeling in the absence of ROMs (Fig. 9, A–D), but did display TUNEL staining after incubation with 0.005% H₂O₂ for 2 h at 37°C (Fig. 9, E–H). Thus, both cell types displayed DNA damage after exposure to ROMs. As similarly noted above (Fig. 8), DNA damage was detected in HTR8/SVneo cells after incubation with FMLP-activated neutrophils for 80 min at 37°C (Fig. 9L), but not in parallel experiments with JEG-3 cells (Fig. 9J). This effect, however, appeared to be specific for activation of the neutrophil’s HMS, because it was inhibited by 6-aminonicotinamide. Fig. 9P shows that pretreatment of neutrophils with 10 mM 6-aminonicotinamide for 30
min, followed by coincubation with HTR8/SVneo in the presence of 
5 mM 6-aminonicotinamide for 2 h at 37°C blocked DNA damage in 
HTR8/SVneo cells. We next sought to confirm that H2O2 is capable 
of entering JEG-3 and HTR8/SVneo cells with equal efficacy. Fig. 9, 
Q and R, shows JEG-3 and HTR8/SVneo cells, respectively, treated 
with various amounts of H2O2. Individual cells were exposed to 0 
(trace 1), 0.001% (trace 2), 0.005% (trace 3), and 0.01% (trace 4) 
H2O2, then followed for several minutes to observe the kinetics of 
TMRos formation. As this figure shows, no significant differences in 
the rate of TMRos formation in these cells were observed. Hence, 
differences in trophoblast DNA damage are not likely to be explained 
by differences in ROM entry or ROM-mediated DNA damage, but 
appear to be closely linked with the ability of trophoblasts to affect 
the neutrophil’s HMS, inasmuch as 6-aminonicotinamide affects the abil-
ity of neutrophils to damage HTR8/SVneo cells, and HMS activity is 
depressed by JEG-3 cells. We therefore suggest that neutrophil deac-
tivation protects JEG-3 cells from neutrophil-mediated DNA damage 
during cell-cell contact.

Discussion

Our goal in these experiments was to examine the possibility that 
trophoblasts directly regulate the activation status of nearby neu-
trophils. To unravel these cell-cell interactions, it was necessary to 
use single-cell assays to investigate the properties of each cell. Single-cell microscope-based technologies allow us to identify tro-
phoblast-neutrophil events and characterize their chemical proper-
ties over time. To model trophoblast-mediated cellular immuno-
regulation in vitro, we used trophoblasts grown on microscope 
slides combined with neutrophils stimulated with FMLP. Multiple 
single-cell parameters of activation reveal that trophoblast contact deacti-
vates activated neutrophils. This finding is consistent with previ-
ous studies suggesting that crude cell fractions, plasma membrane 
vesicles, and glycosylated components of trophoblasts inhibit the allo-
geneic reactivity of lymphocytes (34–36). A previous study has
suggested that the trophectoderm deflects the injurious potential of LPS-activated macrophages (13). Unfortunately, the mechanisms responsible for these immunoregulatory processes have remained obscure. In this study we show that trophoblasts are capable of a contact-dependent deactivation of neutrophils via a mechanism involving at least two metabolic “choke” points preceding the HMS: glucose transport and HMS enzyme trafficking, which lessen the ROM burden and DNA damage of neighboring trophoblasts.

There is broad agreement that Ca\(^{2+}\) signaling plays a central role in neutrophil activation (19, 23–26). It seemed likely, therefore, that trophoblast-mediated immunomodulation of neutrophils would be accompanied by alterations in Ca\(^{2+}\) signaling. We have shown that the rapid periodic Ca\(^{2+}\) spikes of activated adherent neutrophils are normalized to the profiles seen for unstimulated neutrophils after trophoblast contact. It is not surprising that perturbation of an activating signal causes downstream effects on cell activation. Because other neutrophil functions, such as degranulation, are also regulated by Ca\(^{2+}\) signaling, it is likely that additional cell functions are depressed by trophoblasts.

Neutrophil activation leads to metabolic changes, such as NADPH production, that are required for ROM production by NADPH oxidase. Trophoblast contact alters the dynamics of NAD(P)H production by neutrophils and, in parallel, ROM production. Furthermore, kinetic studies show that the rate of ROM release by activated neutrophils is dramatically reduced by contact with trophoblasts. Our results have shown that fewer ROMs accumulate in and around neutrophils associated with trophoblasts. Not surprisingly, fewer ROMs accumulate within trophoblasts capable of deactivating neutrophils. Moreover, trophoblast DNA damage was not detected in trophoblasts capable of deactivating neutrophils. Hence, one important consequence of trophoblast contact is depression of neutrophil-mediated ROM production and DNA damage in neighboring cells, which would minimize potential harm to the placenta and conceptus by inflammatory cells. Although most placental immunobiology studies are restricted to cell lines and/or cytotrophoblasts, we have also examined syncytiotrophoblasts, cytotrophoblasts, and neutrophils from the same mother. As identical regulatory events and ROM production

**FIGURE 9.** Accumulation of DNA damage as judged by the TUNEL method (A–P) and ROM delivery as measured by TMRos fluorescence (Q and R) in clumps of JEG-3 (left) and HTR8/SVneo (right) cells. Untreated cell lines do not show fluorescence (A–D). However, when exposed to 0.005% H\(_2\)O\(_2\) for 2 h at 37°C, both cell lines display fluorescence after staining using the TUNEL assay (E–H). Neutrophils (black arrows) were first activated using 100 nM FMLP, then added to JEG-3 or HTR8/SVneo cells (I–P). When activated neutrophils were added to JEG-3 cells, no significant increase in fluorescence was noted (J). However, when activated neutrophils were incubated with HTR8/SVneo cells, fluorescence was observed to increase in HTR8/SVneo cells (L), indicating DNA damage under these conditions. However, in the presence of 6-aminonicotinamide at 5 mM, fluorescence was not observed in either cell line in the presence of activated neutrophils (M–P), suggesting that cellular responses require activation of the HMS. To provide quantitative data on ROM delivery, the fluorescence intensity of TMRos was monitored in single cells to measure the rate of oxidation. Q and R, JEG-3 and HTR8/SVneo cells, respectively, exposed to 0 (trace 1), 0.001% (trace 2), 0.005% (trace 3), and 0.01% (trace 4) H\(_2\)O\(_2\). No differences in the rates of TMRos reaction were noted in these cell lines, suggesting that the differences in ROM delivery to JEG-3 and HTR8/SVneo are accounted for by differences in hexose monophosphate shunt activity of the neutrophil.
changes were observed using multiple trophoblast types, the findings are likely to be of physiological relevance.

An immunoregulatory model based on metabolic control

Our studies suggest the model of trophoblast-mediated inflammatory regulation shown in Fig. 10. Under normal conditions, neutrophil stimuli enhance Ca\(^{2+}\) signaling events (23, 24, 26) that accompany an increase in glucose uptake (32), which, in turn, is required for ROM production (Fig. 10). Inhibitors of G-6-PDase (37) or glucose transport (U. Kummer, J. Zobeley, K. Naxerova, J. C. Brasen, R. Fahmy, A. L. Kindzelskii, A. R. Petty, and H. R. Petty, manuscript in preparation) block HMS activation despite the presence of activating signals. In the absence of glucose or enhanced glucose uptake, there is no HMS-driven respiratory burst (29–32). During neutrophil activation, Ca\(^{2+}\) signals contribute to an increase in the affinity of the glucose transporter and an increase in glucose flux. This and perhaps other chemical events lead to HMS activation. Once activated, the HMS draws down G-6-P levels, causing the increase in glycolytic oscillation frequency noted above (Kummer et al., manuscript in preparation). Enhanced NADPH levels support ROM production. When neutrophils are attached to trophoblast membrane fragments, glucose uptake is significantly lower than that observed for neighboring activated neutrophils, thus limiting G-6-P, NADPH, and ROM production. The reduction in glucose transport is sufficient to shut down the HMS, thus causing normalization of the oscillatory frequency due to the return of normal carbon flux through glycolysis. Hence, trophoblast-mediated alterations in glucose transport affect downstream biochemical pathways, such as ROM production. Moreover, these trophoblast-mediated changes in neutrophil activation do not appear to be restricted to FMLP, because other neutrophil activators, such as LPS and TNF-\(\alpha\), can be deactivated by trophoblasts.

In addition to this acute regulatory pathway, trophoblasts are capable of promoting the retrograde transport of the neutrophil’s HMS enzymes on microtubules. HMS enzymes form a macromolecular complex that includes G-6-PDase, 6-PGDase, and transaldolase (14–16). Because HMS complex trafficking to the centrosome requires microtubule-based motility, it requires hours instead of seconds to be accomplished. The metabolic microcompartmentalization of the HMS at the centrosome also limits the availability of G-6-P to the HMS by allowing G-6-P to be irreversibly metabolized at the cell periphery by glycolytic enzymes (14). Although the regulation of glucose transport may account for the rapid changes in cell activation at the materno-fetal interface, the systemic changes in peripheral blood neutrophil activation may be more closely related to HMS metabolic microcompartmentalization. Thus, we have identified two new mechanisms that allow trophoblasts to actively regulate the inflammatory potential of neutrophils.

Clinical implications

The present study of trophoblasts offers insight into neutrophil properties during pregnancy. It is widely known that ROM production by peripheral blood neutrophils from pregnant women cannot be fully activated (14–16, 38–40). Because diminished neutrophil activation lasts for ~2 h after trophoblast contact, and the placental surface area allows large volumes of maternal blood to pass villous syncytiotrophoblasts, it is possible that the placental interface, through contact with circulating neutrophils, is capable of regulating their propensity to activate. Hence, trophoblast to neutrophil interactions may contribute to the systemic properties previously observed for normal maternal neutrophils, including HMS enzyme trafficking (14).

Placental oxidative damage plays a central role in preeclampsia, diabetic pregnancy, and miscarriage (41). Neutrophils from preeclamptic patients produce enhanced levels of ROMs (42), whichprobably contribute to oxidative stress (43). Although syncytiotrophoblasts derived from normal pregnant women deactivate neutrophil ROM production in this study, syncytiotrophoblasts from patients with severe preeclampsia stimulate neutrophil superoxide production (44). This suggests that there is a fundamental biological difference between trophoblasts from normal and pre-eclamptic pregnancies. Enhanced placental oxidative stress may account for the DNA damage found in cytrophoblasts from pre-eclamptic patients (45, 46). Because large numbers of leukocytes come into contact with trophoblasts, the aberrant regulation of ROM production may contribute to disease mechanisms.

Although we have discovered a novel cellular mechanism regulating neutrophil activation, some details have not yet been established. It seems likely that a component of the trophoblast’s plasma membrane participates in neutrophil deactivation, because both intact and lysed cells possess this activity. Although the ligand(s) and receptor(s) mediating this process are unknown, it has not escaped our attention that they will probably form important new lead compounds in drug discovery to manage inflammatory diseases.

Disclosures

The authors have no financial conflict of interest.
References


