ORIGINAL ARTICLE

Bi-Directional Calcium Signaling Between Adjacent Leukocytes and Trophoblast-Like Cells

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Problem
Trophoblasts are believed to play an important role in mitigating immunological responses against the fetus. To better understand the nature of trophoblast–leukocyte interactions, we have studied signal transduction during intercellular interactions.

Method of study
Using a highly sensitive microfluorometric ratioing method and Ca^{2+}-sensitive dyes, we measured Ca^{2+} signals in trophoblast-like cell lines (JEG-3 and JAR) or in leukocytes (neutrophils and monocytes) during intercellular contact.

Results
Trophoblast cell lines exhibit Ca^{2+} signals during leukocyte contact. In contrast, leukocytes cannot elicit Ca^{2+} signals in non-opsonized tumour cells, suggesting that Ca^{2+} signaling is not a general feature of cell–cell encounters. Similarly, leukocytes demonstrate Ca^{2+} signals during contact with trophoblast cell lines. Ca^{2+} signals were confirmed using three dyes and with the Ca^{2+} buffer BAPTA.

Conclusion
We suggest that leukocyte-to-trophoblast interactions lead to mutual Ca^{2+} signaling events in both cell types, which may contribute to immunoregulation at the materno–fetal interface.

Introduction

In hemochorial placentas, which are found in catarrhine primates, trophoblasts of fetal origin are immersed in maternal blood. As maternal leukocytes and trophoblasts come into contact, some immunological compromise must be reached between the mother and fetus to insure the latter's survival.[1,2] This immunological compromise is not only reflected locally at the placenta, but is apparent systemically as well, because many maternal autoimmune diseases remit during pregnancy and relapse after delivery.[2–4] Although our understanding of immunoregulation during pregnancy is incomplete, it seems likely that it is multifactorial in nature.

The factors affecting maternal immunoregulation may be mediated by soluble mechanisms or contact-dependent phenomena. For example, soluble products of indoleamine-2,3-dioxygenase may affect maternal immunoregulation.[5] Cell surface molecules likely influence contact-dependent processes. For example, trophoblasts express human leukocyte antigen-G (HLA-G) molecules, which promote tolerance, instead of immunostimulatory HLA class I antigens.[6,7] Trophoblasts also express B7-H2 and B7-H3 molecules, which interact with members of the

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CD28 family of molecules on lymphocytes, that are prone to stimulate Th2-type responses.[8] These and other contact-dependent immunoregulatory processes are necessarily accompanied by heterologous cell-to-cell interactions. Indeed, maternal leukocyte-to-trophoblast interactions have been observed, which lead to physiological changes, such as adherence.[9] Importantly, macrophages activated by multiple mechanisms display cytotoxicity against tumour cell targets, but not trophoblasts.[10] Moreover, trophoblasts have been shown to protect the conceptus from destruction by neighboring activated macrophages.[11] Hence, multiple mechanisms likely participate in maternal immunoregulation.

Although some of the molecules participating in contact-dependent immunoregulation during pregnancy have been identified, as well as certain physiological outcomes, the underlying cellular processes are unclear. For example, the nature of intercellular signaling within trophoblasts during leukocyte binding has not been thoroughly studied. In a recent study, Jiang et al.[12] have reported activation of mitogen-activated protein kinase (MAPK) in co-cultures of trophoblasts and PBMC. In this study, we test the hypothesis that leukocyte-to-trophoblast interactions stimulate intracellular Ca^{2+} signaling events. Our work indicates that leukocyte-to-trophoblast-like cell line interactions trigger reciprocal Ca^{2+} signaling events in both leukocytes and trophoblasts, suggesting complex intracellular signaling between maternal and fetal cells.

Materials and methods

Materials

Fura Red-AM, Fluo-4-AM, Indo-1-AM, 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester-AM (BAPTA-AM), Pluronic-127, cell culture media, and phosphate buffered saline (PBS) were obtained from Invitrogen Corp. (Carlsbad, CA, USA). Cover-glass-bottom dishes were purchased from MatTek Corporation (Ashland, MA, USA). Ficoll-Histopaque (10771 and 11191) and heparin were obtained from Sigma Chemical Company (St Louis, MO, USA).

Neutrophil and Monocyte Preparation

Peripheral blood was collected from healthy human donors in compliance with the guidelines of the University of Michigan Institutional Review Board for Human Subject Research. Neutrophils and monocytes were collected from different density interfaces following Ficoll-Histopaque density gradient centrifugation then re-suspended and washed in PBS by centrifugation.

Cell Lines

Cell lines (JEG-3, line #HTB-36; JAR, line #HTB-144; and HT-1080, line #CCL-121) were obtained from the American Type Culture Collection (Manassas, VA, USA). Trophoblast lines were maintained in RPMI-1640 medium (Invitrogen). HT1080 cells were maintained in DMEM medium (Invitrogen). Both media types contained 10% fetal bovine serum and 1% antibiotic/antimycotic (Invitrogen). Cell lines were occasionally replaced with the frozen samples of the original cell line as a quality control measure. Prior to use, JEG-3, JAR, and HT-1080 cell cultures were grown on uncoated glass-bottom microwell dishes for 2–3 days.

Cell Labeling for Ca^{2+} Experiments and Incubation Conditions

Leukocytes were incubated with 5.5 μM Fura Red-AM, 2.6 μM Fluo-4-AM, and 2% Pluronic-127 in PBS for 1 hr at 37°C.[13] For Indo-1 experiments, cells were incubated for 1 hr at 37°C with 8 μM Indo-1-AM and 2% Pluronic-127. After incubation, cells were washed with PBS, re-suspended in Ca^{2+} imaging buffer (150 mM NaCl, 4 mM KCl, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3 mM $CaCl_2$, 5 mM pyruvate, 10 mM glucose, and 1 mg/mL bovine serum albumin, pH7.9) and incubated for a further 30 min at 37°C, to purge excess dye. Cells were washed with imaging buffer before use.

Cells lines were labeled with Ca^{2+} indicators as described earlier, with two modifications. First, labeling was performed for 1.5–2 hr. Second, cell lines were labeled then purged in media (RPMI1640 or DMEM).

After the addition of monocytes or neutrophils to cell lines, plates were allowed to incubate for 10–15 min at 37°C to allow the cells to settle before transfer to the microscope stage. During experiments, the stage was scanned to identify leukocytes associated with trophoblasts or HT1080 cells; cell binding was confirmed by gentle movement of the plate or media.

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Super-Quiet Microfluorometry

Calcium signaling was detected with super-quiet microfluorometry using a previously described instrument.[14] Briefly, the illumination system utilizes a super-quiet mercury–xenon light source (Hamamatsu, Bridgewater, NJ, USA) powered by a model 69907 arc lamp power supply (Newport Corp., Irvine, CA, USA) and regulated by an Oriel light intensity controller (model 68950, Newport). Light was delivered to a Zeiss microscope via a liquid light guide attached to a custom Flash-Cube (Rapp OptoElectronic, Hamburg, Germany). The Flash-Cube diverted a fraction of the light to a cooled photodiode to provide a feedback signal for the light controller. For Indo-1 Red studies, light was delivered to the sample using an HQ475/40x excitation filter and a 510-nm dichroic long-pass mirror (Chroma Technology Corp., Rockingham, VT, USA). A D-104 microscope photometer (Photon Technology International (PTI), Birmingham, NJ, USA) was connected to two refrigerated PMT housings (Products for Research, Danvers, MA, USA), each containing a Hamamatsu R1527P photon-counting photomultiplier tube. For Fluo-4/Fura Red experiments, the photometer contained a 600-nm dichroic reflector, an HQ530/30m emission filter, and an HQ670/50m emission filter. To bring the intensity ratios closer to one, an XB16-ND1 neutral density filter was used with the D485/25m filter. For Indo-1 experiments, excitation light was selected using a D365/10x filter and a 380-nm dichroic beamsplitter. Indo-1's two emission wavelengths were detected using D405/30m and D485/25m filters in conjunction with a 440-nm dichroic reflector within the photometer. An adjustable diaphragm was used to exclude other cells from measurements. All experiments were performed in a dark room within an aluminum enclosure with the microscope stage set to 37°C.

Data Analysis

Data were acquired and analyzed using Felix software (PTI). Data files were subsequently exported as Excel files for further analysis and figure preparation. Data are reported as the measured ratios (not renormalized). Experiments were performed with the Fluo-4/Fura Red dye combination and the Indo-1 ratiometric dye. These ratiometric probes have slightly different Ca^{2+}-sensitive behaviors, but their intensity scales as the Ca^{2+} concentration.

Results

In this study, we test the hypothesis that leukocyte interactions with trophoblast-like cells elicit intracellular Ca^{2+} signals within these cells. Leukocytes were added to adherent trophoblast-like cells. Fig. 1 shows a differential interference contrast (DIC) image of a neutrophil-to-JEG-3 cell conjugate. The region of intercellular contact is indicated by the arrow. In addition to the area of cell–cell contact, it should be noted that the JEG-3 cell is substantially larger than the neutrophil.

Trophoblast Cell Lines Exhibit Signaling in Response to Leukocytes

The first series of experiments tested the ability of neutrophils and monocytes to promote Ca^{2+} signaling within trophoblast cell lines. Trophoblast cell lines were grown on cover glass-bottom culture dishes. After a period of at least 2 days of growth, cells were labeled with the Ca^{2+} ratiometric dye indo-1, as described in the Materials and methods. Unlabeled neutrophils were then added to labeled trophoblast cultures. Samples were scanned to identify leukocyte–trophoblast conjugates, as illustrated by Fig. 1; leukocyte–trophoblast cell binding was then further confirmed by gentle agitation. Trophoblast cell line signaling was evaluated using super-quiet microfluorometric recording. In this method,

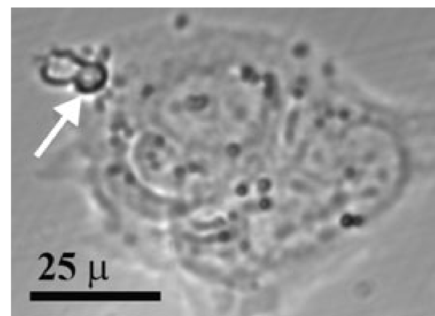

Fig. 1 Differential interference contrast micrograph of a neutrophil-to-JEG-3 trophoblast-like conjugate. Neutrophils were added to a sub-confluent culture of JEG-3 cells. After 15 min. at 37°C, the plates were gently washed to remove unbound neutrophils. The site of cell–cell interaction is indicated by an arrow. (Bar = 25 μm.)

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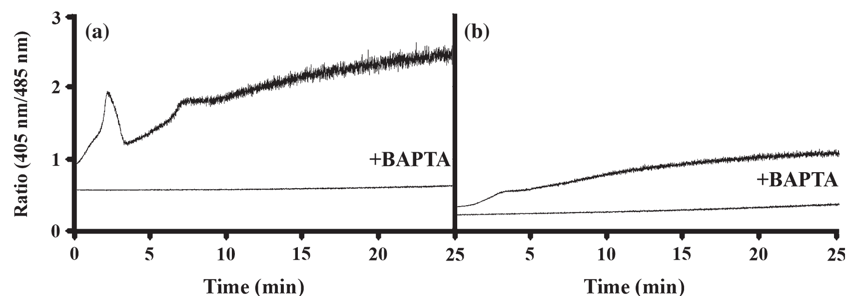

Fig. 2 Super-quiet microfluorometry of Ca^{2+} signaling in JEG-3 trophoblast-like cells during contact with leukocytes. The Indo-1 ratio is plotted at the ordinate, whereas time is given at the abscissa. All experiments were performed at 37°C using a highly stabilized microfluorometry system. Panel A shows a recording of Indo-1-labeled JEG-3 cells during neutrophil binding. An early spike followed by a gradual increase in the ratio was observed. Inclusion of the Ca^{2+} buffer BAPTA abrogated these changes, as indicated in the lower trace. Experiments with the JAR cell line are shown in panel B. Indo-1-labeled JAR cells also exhibited an increase in the fluorescence ratio during incubation with neutrophils, indicating that the observed changes were not restricted to JEG-3 cells. Signaling in JAR cells was similarly blocked by inclusion of BAPTA (lower trace). Although increases in the fluorescence ratio were noted for JAR cells, these changes were not as pronounced as those noted for JEG-3 cells. (The relative noise in the measured ratio increases at longer times owing to mechanisms of dye loss, such as photobleaching, during the observation period.)

illumination and detector noise are substantially eliminated from the measurement, making a sample's quantum noise the major source of error. Fig. 2 shows kinetic microfluorometric studies of two trophoblast-like cell lines, JEG-3 (panel a) and JAR (panel b), during interactions with neutrophils. As these data illustrate, both cell lines exhibit increases in the 405 nm/485 nm fluorescence ratio when associated with neutrophils. Although trophoblast-like cells exhibiting bound neutrophils displayed changes in the Ca^{2+} ratio, trophoblasts that in the presence of unbound neutrophils did not (data not shown). These changes were associated with Ca^{2+}, as the Ca^{2+} buffer BAPTA blocked the changes in the fluorescence ratio measurements. Although the shapes of the curves are similar, the JEG-3 cell response was significantly larger. Consequently, we focused on the JEG-3 cells in subsequent studies.

We further tested the specificity of these responses using another Ca^{2+} ratio dye system: Fluo-4/Fura Red. In addition to providing a means of further substantiating the findings with the indo-1 dye, this dye combination exhibits a distinct dynamic range in Ca^{2+} sensitivity, thus providing further insight into signaling.[13] Fig. 3a (upper trace) shows a fluorescence recording of the Fluo-4/Fura Red ratio of labeled JEG-3 cells while bound to neutrophils. As these data show, the fluorescence ratio increases over several minutes followed by stabilization at a high level, which we interpret as high intracellular Ca^{2+} concentrations. To confirm that the increase in the Fluo-4/Fura Red ratio was attributable to Ca^{2+}, JEG-3 cells were treated with the Ca^{2+} buffer BAPTA at 10 μM in imaging buffer. BAPTA-labeled JEG-3 cells did not exhibit an enhanced fluorescence ratio (Fig. 3a, lower trace).

We next assessed the cellular specificity of these signaling events. To ascertain whether this type of cell-to-cell communication is specific for neutrophils, we examined the effect of monocytes on the Fluo-4 and Fura Red ratio of labeled JEG-3 cells. Fig. 3b (upper trace) shows the Fluo-4 and Fura Red ratio during incubation with monocytes. As noted for neutrophil–trophoblast interactions earlier, monocytes also stimulated an increase in the Fluo-4 and Fura Red ratio of JEG-3 cells, thus indicating a rise in the intracellular level of Ca^{2+}. This change in fluorescence ratio was specific for Ca^{2+}, because cell treatment with BAPTA blocked these changes (Fig. 3b, lower trace). To test whether the elicitation of Ca^{2+} signals is a general property of cell-to-cell interactions, we examined the response of tumour cells to neutrophil binding. To parallel the trophoblast cell line experiments described earlier, HT1080 tumour cells were grown on glass surfaces, labeled with Fluo-4 and Fura Red, and were not opsonized. As shown in Fig. 3c, upper trace, monocytes did not affect the Fluo-4 and Fura Red ratio of HT-1080 cells. Treatment with BAPTA slightly reduced the measured Fluo-4 and Fura Red ratio (Fig. 3c, lower trace). Hence, both neutrophils and monocytes induce Ca^{2+} signals in adjacent trophoblast-like cell lines, but this is not a general feature of cells interacting with leukocytes.

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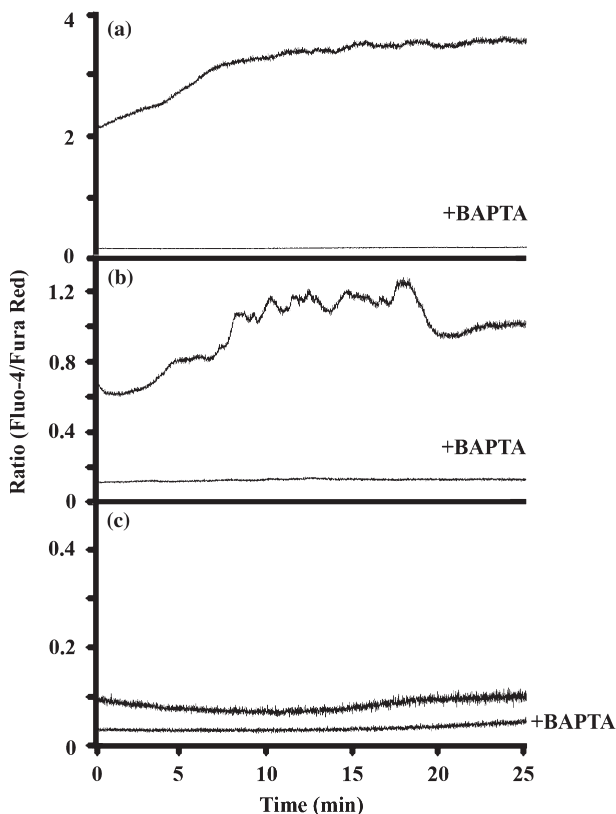

Fig. 3 Quantitative microfluorometry of Ca^{2+} signaling in JEG-3 cells during contact with leukocytes. The Fluo-4/Fura Red ratio is plotted at the ordinate, whereas the time is given at the abscissa. All experiments were performed at 37°C. Panel A: The upper trace shows Fluo-4/Fura Red-labeled JEG-3 cells during incubation with neutrophils. The Fluo-4/Fura Red ratio increases, thus indicating a rise in intracellular Ca^{2+} concentration. Negative controls using the Ca^{2+} buffer BAPTA are shown. Panel B: Fluo-4/Fura Red-labeled JEG-3 cells were incubated with monocytes. Again, the Fluo-4/Fura Red ratio increases over time (upper trace) but is BAPTA-inhibitable (lower trace). Panel C: Although leukocytes elicit Ca^{2+} signals in JEG-3 cells, they do not promote Ca^{2+} signals in other cells. HT-1080 fibrosarcoma cells were labeled with Fluo-4 and Fura Red then incubated with unlabeled monocytes. No significant changes in the Fluo-4/Fura Red ratio were observed.

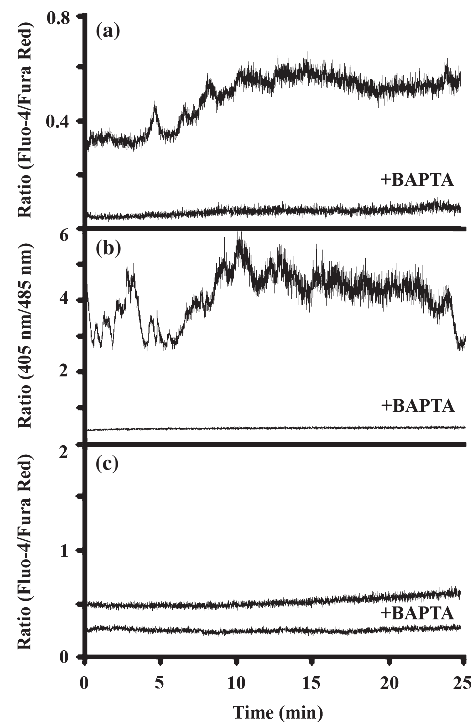

Fig. 4 Microfluorometry studies of Ca^{2+} signaling in neutrophils during contact with JEG-3 trophoblasts. The Fluo-4/Fura Red ratio is plotted at the ordinate, whereas time is given at the abscissa. All experiments were performed at 37°C, as described earlier. Panel A: The upper trace shows Fluo-4/Fura Red-labeled neutrophils during incubation with JEG-3 cells. The increase in the Fluo-4/Fura Red ratio indicates a rise in intracellular Ca^{2+} concentration. Inclusion of the Ca^{2+} buffer BAPTA abrogated these changes, as shown in the lower trace. Panel B: Indo-1-labeled neutrophils were incubated with JEG-3 cells. As described for Fluo-4/Fura Red, the Indo-1 Red ratio increases in a BAPTA-inhibitable fashion. Panel C: The upper trace shows Fluo-4/Fura Red-labeled neutrophils during incubation with HT-1080 cells. No significant change in the Fluo-4/Fura Red ratio is observed. A control using the Ca^{2+} buffer BAPTA is shown in the lower trace.

Leukocyte Signaling in Response to Trophoblast Cell Lines

As neutrophils were found to trigger Ca^{2+} signals in adjacent trophoblast-like cells, we examined the possibility that trophoblast-like cells induce Ca^{2+} signals in neutrophils. To test this hypothesis, neutrophils were labeled with Fluo-4 and Fura Red. Labeled neutrophils were then added to JEG-3 cells in cover glass-bottom culture dishes. Samples were examined as described earlier. Fig. 4a (upper trace) shows a fluorescence recording of the Fluo-4/Fura Red ratio of a neutrophil bound to a JEG-3 trophoblast. As these measurements indicate, the fluorescence ratio increases over time. To confirm that the increase in the Fluo-4/Fura Red ratio was attributable to Ca^{2+}, neutrophils were treated with BAPTA. When neutrophils were labeled with BAPTA, no enhanced fluorescence ratio could be detected in neutrophil-JEG-3 cell conjugates (Fig. 4a, lower trace). We went on to confirm that these changes in Ca^{2+} dye ratios could be obtained using Indo-1. Fig. 4b (upper trace) shows a fluorescence recording of the Indo-1 ratio of a labeled neutrophil bound to a JEG-3 cell. As these findings show, the Indo-1 ratio of

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neutrophils increases during incubation with these cells. In contrast to the signals within trophoblast-like cells, the neutrophil signals are more oscillatory in nature. As noted for Fluo-4/Fura Red earlier, treatment with BAPTA eliminated the increase in the Indo-1 ratio (Fig. 4b, lower trace). Consequently, trophoblast-induced changes in neutrophil dye ratios are dependent upon Ca^{2+}, but they can be detected using different dyes. To ascertain whether these changes in neutrophil signaling are observed using non-trophoblast cell lines, Fluo-4/Fura Red-labeled neutrophils were incubated with HT-1080 cells. As shown in Fig. 4c, upper trace, HT-1080 cells had little effect on the Ca^{2+} signaling apparatus of neutrophils. The lower trace shows a control using the Ca^{2+} chelator BAPTA. Hence, neutrophil signaling is not dependent upon the Ca^{2+} dye used for analysis, but it is dependent upon the presence of JEG-3 cells.

We next tested the ability of another trophoblast cell line, JAR, to elicit Ca^{2+} signals within leukocytes. Adherent JAR cells were grown on glass-bottom culture dishes, as described earlier. When Fluo-4/Fura Red-labeled neutrophils were added to unlabeled JAR cells, the neutrophil's dye emission ratio was found to increase (Fig. 5a, upper trace), but not in the presence of BAPTA (lower trace). In panels b and c, Indo-1-labeled neutrophils were added to the culture plates. Fig. 5b shows a fluorescence recording of the Indo-1 ratio of a neutrophil bound to a JAR trophoblast. Again, these data indicate that JAR cells, like JEG-3 trophoblasts but unlike HT1080 cells, elicit Ca^{2+} signaling events in bound neutrophils. For comparison, HT1080 cells incubated with Indo-1-labeled neutrophils are shown in Fig. 5c. Matched controls using BAPTA-treated Indo-1-labeled neutrophils associated with JAR and HT1080 cells are shown in the lower traces of panels b and c, respectively. These experiments demonstrate that the JAR trophoblast cell line also stimulates Ca^{2+} signaling within bound neutrophils.

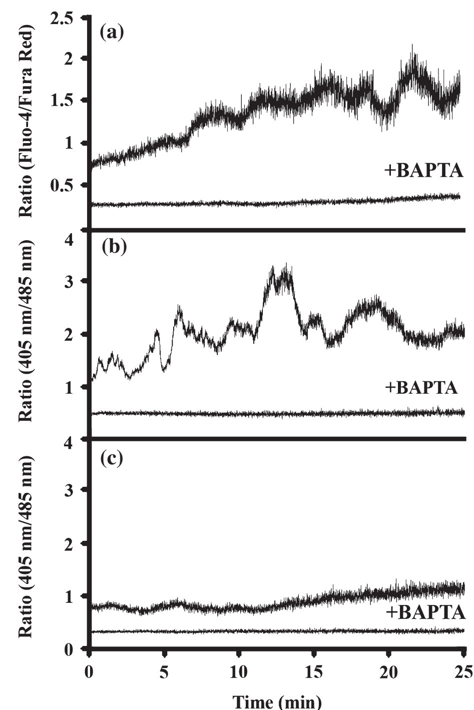

Fig. 5 Microfluorometry studies of Ca^{2+} signaling in neutrophils during contact with JAR trophoblasts. The fluorescence ratio is plotted versus time in minutes. All experiments were performed at 37°C. Panel A: In these experiments, Fluo-4 and Fura Red were used as Ca^{2+} dyes to label neutrophils. The dye ratio increases during contact with JAR cells (upper trace), but not when cell were treated with BAPTA (lower trace). Panel B: The upper trace shows the Indo-1 ratio of neutrophils during contact with JAR trophoblasts. The ratio increases and oscillates over time, indicating increases in cytoplasmic Ca^{2+} concentrations. The matched control experiment using BAPTA is given in the lower trace. Panel C: The upper trace shows an Indo-1-labeled neutrophil adherent to an HT-1080 tumour cell. Only relatively small changes in the Indo-1 ratio are observed in comparison to the large changes noted for trophoblasts. The BAPTA control is given in the lower trace.

Discussion

Changes in cytoplasmic Ca^{2+} concentrations play crucial roles in many cell functions. Included among these functions are: electrical activity, secretion, muscle contraction, membrane permeability, enzyme activity, apoptosis, proliferation, protein synthesis, and gene expression.[15] In addition to its intracellular roles, Ca^{2+} also participates in cell-to-cell communication. Contact-dependent intercellular communication takes several forms, such as communication via gap junctions and receptor pathways.[16,17] As mentioned earlier, cell contact-dependent immunoregulatory events participate in maternal immunomodulation during pregnancy. However, the existence of Ca^{2+} signaling responses during intercellular contact between maternal and fetal cells has not been reported, although it is known that trophoblasts may exhibit Ca^{2+} signals in certain circumstances.[18,19] In this study, we have tested the hypothesis that leukocytes and trophoblast cell lines elicit Ca^{2+} signals in one another during their inter-

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action. To study Ca^{2+} signals, it was necessary to use an *in vitro* model, in this case trophoblast cell lines and peripheral blood leukocytes. We have found that both neutrophils and monocytes stimulate Ca^{2+} signals in adherent trophoblast cell lines. The converse is also true; trophoblast cell lines induce Ca^{2+} signals in bound neutrophils and monocytes. These signals were dependent upon Ca^{2+} and could not be elicited within HT1080 tumour cells. To the best of our knowledge, this is the first study to report Ca^{2+} signal transduction events during leukocyte-to-trophoblast cell line contact.

To provide the most reliable data possible, we employed several technically advantageous approaches. To detect Ca^{2+} changes, we used single cell microfluorometry incorporating super-quiet illumination and photon-counting detection; this instrumentation provides very low noise excitation and fluorescence detection. The major source of noise in these experiments is the quantum (or shot) noise associated with photon emission by the dyes. Consequently, small changes in intensity are of a biological nature. In addition, we used ratiometric analysis of the Ca^{2+} dyes. Ratiometric methods have many advantages such as increased dynamic range, correction for variations in cell thickness, dye artifacts such as compartmentalization and leakage, and reduction of illumination noise. This approach was useful because the Ca^{2+} changes observed were not as large as those seen during other biological processes, such as antibody-dependent cellular cytotoxicity (data not shown). Furthermore, to insure that these biophysical analyses of signal transduction were biologically important, we tested our hypothesis using: two different fluorescent ratioing dyes, the Ca^{2+} -buffering molecule BAPTA, two trophoblast-like cell lines, two types of leukocytes, and a non-trophoblast-like fibrosarcoma cell line. Our careful experimentation has shown clearly that trophoblast-like cells and leukocytes elicit Ca^{2+} signals in one another during physical contact.

Our work strongly supports the concept of bi-directional communication between cells of the mother and fetus. Bi-directional communication between the mother and fetus has been previously proposed by several laboratories.[12,20–22] For example, this may take place in the form of cytokine cross-talk. In an important study, Jiang et al.[12] demonstrated signaling in co-cultures of leukocytes and trophoblasts that could be transferred by incubation of JAR cells with leukocyte-conditioned media suggesting that the effect is not contact-dependent. In this study, we have shown intercellular contact-dependent stimulation of Ca^{2+} signaling, as judged by microscopic observation and microfluorometry. It would seem likely that both contact-dependent and contact-independent mechanisms participate in immunoregulatory signaling at the maternal–fetal interface.

The Ca^{2+} signals reported in this article are reasonable from the standpoint of signal transduction. As mentioned earlier, Jiang et al.[12] noted MAPK signaling in co-cultures of leukocytes and trophoblasts. This observation is consistent with our work inasmuch as both the MAPK cascade and Ca^{2+} changes can be affected by the same signaling stimulus. For example, Syk participates in tyrosine phosphorylation of proteins that lead to both MAPK and Ca^{2+} pathways.[23] Therefore, stimulation of both pathways is not unreasonable.

The Ca^{2+} signals noted earlier may contribute at several levels. For example, they may be directly related to the adherence events observed microscopically, which have also been observed by others.[9] These findings may constitute a model for early events in syncytiotrophoblast damage.[24,25] Alternatively, these Ca^{2+} signals may play an important role in the down-regulation of leukocyte function, which have been previously observed to protect trophoblasts.[10,11] It may seem paradoxical that Ca^{2+} signals might play a role in both the prevention and promotion of trophoblast damage, because it is widely known that Ca^{2+} signals broadly promote activation of immune cells. Included among the activation events are degranulation, production of reactive oxygen metabolites, cytokine release, migration, and many others. However, in certain circumstances, Ca^{2+} signals may be inhibitory in nature (e.g. rod outer segments and pinealocytes).[26,27] It seems possible that the inhibitory or stimulatory nature of the cell–cell interaction may depend upon its physiological context as well as the activation of additional signaling pathways. We are currently studying the physical and physiological attributes of these signals to further decode the nature of maternal-to-fetal cell signaling.

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