Lymphocytes of human term decidua decrease cell adhesion to a plastic substrate

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Although human decidual lymphocytes have been widely studied, their function and possible interaction with trophoblast are still unclear. Here we show that whereas human early (EDL) and term (TDL) decidual lymphocytes were unable to kill human trophoblast by necrosis (assessed by the ⁵¹Cr-release assay) or apoptosis (DNA fragment assay), TDL but not EDL decreased trophoblast adhesion to a plastic substrate as determined by a [³H]thymidine assay. This effect, however, was not selective for trophoblast, as TDL also decreased the adhesion to plastic of human decidual stromal cells and HeLa cells. Our results suggest that TDL may play a role in placental detachment during parturition by decreasing trophoblast or decidual stromal cell adhesion.

Key words: cell adhesion/decidual lymphocytes/parturition/plastic substrate/trophoblast

Introduction

In early human decidua most lymphocytes are CD56⁺CD16⁻natural killer (NK) cells (Starkey et al., 1988; King et al., 1989b; Bulmer et al., 1991) and a smaller proportion are T cells (both TCRαβ⁺ and TCRγδ⁺ cells) (Mincheva-Nilsson et al., 1992; Chernyshov et al., 1993). In term decidua, the largest proportion of lymphocytes consists of TCRαβ⁺ T cells, with a small proportion of CD16⁺CD56⁺ lymphocytes and an even smaller percentage of TCRγδ⁺ T cells (Abadía-Molina et al., 1996). In both early and term decidua, a significant percentage of lymphocytes express activation markers, and very few B cells are present (Vargas et al., 1993; Abadía-Molina et al., 1996). The functions of these cell subpopulations are unknown. It has been suggested that these cells exert spontaneous cytotoxic control over trophoblast growth or trophoblast invasion during pregnancy; however, decidual lymphocytes were found to be unable to spontaneously lyse normal or malignant trophoblast (King et al., 1989a; Abadía-Molina et al., 1996). Some investigators have speculated that activated decidual lymphocytes may play an immunosuppressive role (Mincheva-Nilsson et al., 1992), while others (Saito et al., 1992), in accordance with Wegmann’s immunotrophic theory (Athanassakis et al., 1987), have proposed that these cells may synthesize cytokines which could promote trophoblast growth. Here we demonstrate that decidual lymphocytes collected at term decrease cell adhesion, and suggest that this effect may play a role in placental detachment during parturition.

Materials and methods

Extraction of decidual lymphocytes

First trimester placental tissue was obtained from vaginal termination of pregnancy at the Clínica el Sur (Málaga, Spain) or Gineclínica (Granada, Spain). Normal term placentas were collected from healthy pregnancies at the Departamento de Obstetricia y Ginecología, Hospital Clínico Universitario de Granada, Spain. The method of extraction and the characterization of decidual lymphocytes have been described elsewhere (King et al., 1989a; Abadía-Molina et al., 1996). Briefly, samples of early or term decidua were dissected from the maternal-facing surface, identified macroscopically and washed extensively in phosphate-buffered saline (PBS) solution. Decidual fragments were minced finely between two scalpels in a small volume of Roswell Park Memorial Institute 1640 (RPMI 1640) (Sigma, St Louis, MO, USA) and then pushed through a 53 μm sieve (Gallenkamp, Loughborough, UK). The resultant cell suspension was washed with RPMI, layered on an equivalent volume of Lymphoprep (Flow Laboratories, Irvine, UK) at room temperature and centrifuged for 20 min at 600 g. The cells were collected from the interface, suspended in RPMI and washed. They were then resuspended in complete culture medium (RPMI 1640, 10% fetal calf serum (FCS; Flow Laboratories), 100 IU/ml penicillin and 50 μg/ml gentamicin) and incubated for 2 h at 37°C in an atmosphere of 5% CO₂ to allow adherent cells to attach to the plastic. Supernatant containing early decidual lymphocytes (EDL) or term decidual lymphocytes (TDL) was then collected and centrifuged, and the cells were suspended in complete culture medium.

To obtain peripheral blood lymphocytes (PBL), blood samples were taken from healthy volunteers. Samples were diluted with an equal volume of 0.25% phosphate-buffered saline–ethylenediaminetetraacetic acid (PBS–EDTA), and centrifuged on Lymphoprep. Thereafter the procedure for decidual lymphocytes was followed.

Trophoblast

Chorionic villi from first trimester placental tissue were minced into small pieces and incubated for 10 min at 37°C in 0.25% trypsin (Difco, East Molesey, UK) and 0.02% EDTA (Sigma). The resultant cell suspension was filtered through muslin and centrifuged at 400 g for 5 min. The pellet was suspended in PBS, layered onto Lymphoprep and centrifuged at 800 g for 15 min. The band of cells was resuspended in Ham’s F-12 medium (Flow Laboratories) supplemented with L-
glutamine, antibiotics and 20% FCS, adjusted to $10^6$ cells/ml and plated onto 35 mm culture dishes precoated for 45 min with 20 μg/ml laminin (Sigma). The dishes were incubated at 37°C in an atmosphere of 5% CO₂ for 2 days. This method selects extracellular cytotoxic effector cells (IEC), the type of effector that invades decidua (Loke and Burland, 1988).

**Decidual stromal cells (DSC)**

The method of extraction of human DSC has been described elsewhere (Montes et al., 1996). Briefly, samples of first trimester decidua were washed extensively in RPMI 1640 medium containing 100 IU/ml penicillin and 50 μg/ml gentamicin, and the decidua was freed carefully from the trophoblast. It was then washed in Ca²⁺, Mg²⁺-free PBS, minced and incubated in a solution of 0.25% trypsin (Sigma) and 0.02% EDTA (Merck, Darmstadt, Germany) for 15 min at 37°C. The reaction was stopped by adding cold RPMI containing 20% FCS; the suspension was filtered through gauze and centrifuged at 425 g for 7 min. The supernatant was discarded and the cell pellet resuspended in RPMI and centrifuged on Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) for 20 min at 600 g. Cells were collected from the interface, suspended in RPMI and washed. This suspension, containing mainly DSC and leukocytes, was incubated in culture flasks for 1 h in complete RPMI containing 10% FCS to allow macrophages and granulocytes to adhere to the flask. The supernatant, which contained DSC, was decanted from another flask and incubated overnight to allow the DSC to adhere. Lymphocytes in the supernatant were then discarded, leaving a highly purified population of DSC free of granulocytes and macrophages. Proliferating DSC were cultured for several passages; the medium was changed twice a week.

**Cell lines**

The following human cell lines were used: JEG, an EVT choriocarcinoma cell line; JAR, a villous trophoblast choriocarcinoma; and HeLa cells, derived from cervical carcinoma. All three cell lines, which adhere to plastic, were maintained in complete culture RPMI 1640 medium containing 5% FCS.

**51Cr-release assay**

JEG cells were removed from the flask with 0.05% trypsin/0.02% EDTA, washed and suspended in complete culture medium. Cells ($2 \times 10^4$) were added to each well of a 96-well flat-bottomed plate (Becton-Dickinson, San Jose, CA, USA) and incubated overnight at 37°C with 5% CO₂. The supernatants were discarded and the cell pellet resuspended in RPMI and centrifuged on Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) for 20 min at 600 g. Cells were collected from the interface, washed and suspended in RPMI and washed. This suspension, containing mainly DSC and leukocytes, was incubated in culture flasks for 1 h in complete RPMI containing 10% FCS to allow macrophages and granulocytes to adhere to the flask. The supernatant, which contained DSC, was decanted into another flask and incubated overnight to allow the DSC to adhere. Lymphocytes in the supernatant were then discarded, leaving a highly purified population of DSC free of granulocytes and macrophages. Proliferating DSC were cultured for several passages; the medium was changed twice a week.

**MTT assay**

This assay was performed as described above for the $^{51}$Cr-release assay, except that the target labelling was omitted. After 5 h of incubation of target and effector, supernatants were removed and the wells washed three to five times with PBS, 100 μl complete medium and 25 μl 5 mg/ml MTT [(3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma] solution in PBS were added per well, and plates were incubated for 2 h at 37°C in 5% CO₂. After incubation, 100 μl of a solution containing 20% sodium dodecyl sulphate (SDS) in 50% N,N-dimethyl formamide (Fluka, Buchs, Switzerland) were added per well. The plates were shaken for 10 min and the optical density at 570 nm was determined. Wells with target cells and no effectors were used as controls. The results were expressed as percentage activity according to the formula: % activity = [(control absorbance - test absorbance)/control absorbance] × 100.

**$[^{3}H]$thymidine pre-labelling assay**

Complete medium (100 μl) containing $2 \times 10^4$ cells of the appropriate target cell suspension was added to each well of a 96-well flat-bottomed plate. Then 0.5 μCi $[^{3}H]$thymidine (Amersham) were added per well, and the plates incubated overnight at 37°C under 5% CO₂. The supernatants were discarded and 100 μl complete culture medium added. Different amounts of effector cells from decidua or PBL were added in a volume of 100 μl culture medium to obtain different effector/target ratios, as shown in the figures. After 5 h of incubation at 37°C under 5% CO₂, the supernatants were removed and the plate was washed three to five times with PBS, 100 μl per well of a 0.05% trypsin/0.02% EDTA solution were added to the target cells that remained adherent to the plate. Cells were collected by a cell harvester. Radioactivity was counted in a β-scintillation counter. Wells with target cells and no effectors were used as controls. The results were calculated according to the formula: % activity = [(control c.p.m. - test c.p.m.)/control c.p.m.] × 100.

Alternatively, after incubation with the effectors, supernatants from each well were transferred to an independent plate, collected by cell harvesting and counted in a β-scintillation counter. In this case the results were expressed as: activity = test c.p.m./control c.p.m.

In experiments in which normal EVT was used, trophoblast cells were added to microplates that had been precoated for 45 min with 20 μg/ml laminin (Sigma). In this case trophoblast cells were pre-labelled with $[^{3}H]$uridine (Amersham).

In some experiments we compared the detachability of live and dead TDL. Lymphocytes were killed by three freeze-thaw cycles.

In all cases parallel experiments were performed to test cell viability (Trypan blue exclusion).

**DNA fragment assay**

To investigate the induction of apoptosis in target cells DNA fragmentation was studied as an early sign of this phenomenon (Wyllie et al., 1984). This assay followed the same procedure as in the $[^{3}H]$thymidine pre-labelling assay until incubation with the effectors. The plates were then centrifuged and supernatants replaced with 200 μl hypotonic lysing buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing 0.2% Triton X-100. 25 μl supernatant were carefully removed from each well and counted in a β-scintillation counter. Wells with target cells and no effectors were used to determine spontaneous release. A solution of 0.1% SDS was used as a positive control for DNA fragmentation. The results were expressed according to the formula: % fragmented DNA = [(test c.p.m. - spontaneous c.p.m.)/(maximum c.p.m. - spontaneous c.p.m.)] × 100.

**Results**

**TDL but not EDL decrease the adhesion of JEG cells to plastic**

In contrast to the negative results of the $^{51}$Cr-release assay, TDL, but not EDL nor control PBL, were active on JEG
Term decidual lymphocytes decrease cell adhesion

cells in the MTT and [3H]thymidine assays (Figure 1). The lack of activity in the 51Cr-release assay clearly showed that there was no cell death, at least by necrosis. On the other hand, the positive results with MTT and the [3H]thymidine pre-labelling method showed that there was a ‘loss’ of target cells in the wells to which TDL were added. Cell viability was confirmed by vital cell staining with Trypan blue solution. Light microscopic examinations showed that JEG cells—which are easily distinguished from lymphocytes by their larger size—remained viable after

Figure 1. Activity of early (▲) and term (●) decidual lymphocytes and peripheral blood lymphocytes (□) on JEG cells studied using four different methods: 51Cr-release (cytotoxicity), MTT (detaching activity), [3H]thymidine pre-labelling (detaching activity) and DNA fragment (apoptosis) assays. A solution of 0.1% sodium dodecyl sulphate was used as a positive control for DNA fragmentation and yielded ~100% maximal radioactivity. Values are expressed as mean ± SD (n = 3).

adhered cells

supernatant cells

Figure 2. Detaching activity of term decidual lymphocytes (●) and peripheral blood lymphocytes (□) on JEG cells studied using the [3H]thymidine pre-labelling method. After incubation with the effectors, activity was determined by measuring the radioactivity of target cells that remained adhered to the plastic (left) or of target cells in the supernatant (right). Values are expressed as mean ± SD (n = 3).
incubation with TDL, EDL or PBL. Therefore target cells were indeed not killed. However, in wells in which target cells were incubated with TDL, but not in those incubated with EDL or PBL, many JEG cells were seen in the supernatant, having become detached from the plastic wells (results not shown). As in the MTT and \[^{3}\text{H}\]thymidine assays, the supernatants were washed off after incubation (see Materials and methods). Those target cells detached by the effect of TDL were 'lost', which may account for the lower absorbance or radioactivity detected in these wells. In the \[^{3}\text{H}\]thymidine pre-labelling assay the effect of TDL on cell adhesion could alternatively be determined by counting the radioactivity of the detached target cells in the supernatants (Figure 2).

**Decidual lymphocytes do not induce apoptosis of JEG cells**

To find out whether decidual lymphocytes were able to induce apoptotic death of JEG cells, we studied the release of DNA fragments by these cells after incubation with TDL, EDL or PBL. Although a solution of SDS, used as a positive control, induced between 80 and 100% DNA fragmentation, we found no activity with any of the three effectors (Figure 1), even after 24 h of incubation (results not shown).

**Dead TDL are unable to detach JEG cells**

To confirm that the detachment of JEG cells was actually caused by functional TDL and not by the presence of contaminants with proteolytic activity, we repeated the \[^{3}\text{H}\]thymidine pre-labelling assay with TDL which had been previously disrupted by freeze-thawing. Dead TDL had no

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**Figure 3.** Detaching activity of live (●) and dead (○) term decidual lymphocytes on JEG cells, studied using the \[^{3}\text{H}\]thymidine pre-labelling method. Values are expressed as mean ± SD (n = 3).

**Figure 4.** Detaching activity of term decidual lymphocytes (●) and peripheral blood lymphocytes (□) on normal extravillous trophoblast (EVT), normal decidual stromal cells (DSC), JAR cells and HeLa cells, studied using the \[^{3}\text{H}\]thymidine pre-labelling method. Values are expressed as mean ± SD (n = 3).
Effect of TDL on the adhesion of normal extravillous trophoblast and other cell types to plastic

The effect of TDL on the adhesion of different cell types was studied using the [3H]thymidine pre-labelling assay (Figure 4). Normal EVT that were attached to plastic through laminin were also detached by TDL but not by control PBL. Normal human DSC, another cell in the maternal–fetal interface, also became less adherent to plastic after exposure to TDL. The human villous trophoblast cell line JAR was also detached by TDL but not by PBL. That this effect was not selective for tissue or cell type was confirmed by the observation that TDL also decreased the adhesion of HeLa cells, an epithelial cell line that is not derived from the maternal–fetal interface. In all cases the lack of cytotoxicity was confirmed by Trypan blue staining.

Discussion

A significant number of lymphocytes remain in the human decidua throughout pregnancy to term (Vargas et al., 1993), whereas other components of the decidua begin to regress as pregnancy progresses (McCombs and Craig, 1964). This suggests that decidual lymphocytes are involved in important, albeit poorly understood, functions. Although the existence of subpopulations of these lymphocytes with an NK phenotype suggest that they might be involved in the cytotoxic control of trophoblast invasion of the decidua, these cells were unable to lyse trophoblast spontaneously in vitro (King et al., 1989a; Abadía-Molina et al., 1996). In our study we found that TDL affected the adhesion of JEG and trophoblast cells; however, this effect was not due to cytotoxicity or to induction of apoptosis. Nevertheless, unlike TDL, EDL did not decrease trophoblast adhesion (Figures 1 and 4). This is probably because the subpopulations of cells detected in EDL are different from those of TDL (Starkey et al., 1988; King et al., 1989b; Bulmer et al., 1991; Abadía-Molina et al., 1996). Both subpopulations of TDL (CD3+ T cells and CD16+CD56+ NK cells) appeared to have in common the detaching activity, as after eliminating either T cells or NK cells with anti-CD3 or anti-CD16 cytotoxic monoclonal antibodies and complement, the surviving subpopulation still retained the ability to detach JEG cells from the substrate (unpublished data).

The effect on cell adhesion was, however, not confined to trophoblast: DSC and HeLa cells were also detached by TDL (Figure 4). This may have been because different cell types all share a common mechanism for cellular attachment (Newham and Humphries, 1996). Cell adhesion to plastic is mediated by adhesion molecules (integrins) that bind to the extracellular matrix, so that in culture, cell spreading is preceded by the secretion of extracellular matrix proteins. It is the matrix that adheres to the plastic, and the cells then bind to the matrix via the adhesion molecules; therefore cell adhesion to plastic mirrors the physiological adhesion of different types of cell to the extracellular matrix in normal tissues (Yamada, 1991). TDL may hinder the binding of target cell integrins to extracellular matrix proteins, inhibit the expression of integrins or exert a local proteolytic effect on the molecules involved in binding. The fact that TDL must be viable to exhibit their activity on cell adhesion (Figure 3) suggests that a molecule secreted by lymphocytes may be responsible for the detaching effect. A possible candidate is tumour necrosis factor (TNF; Todt et al., 1996): the mRNA of this cytokine is detected in decidual lymphocytes (Jokhi et al., 1994), and trophoblast expression of TNF receptor mRNA is elevated at term in comparison with first trimester placental tissue (Yelavarthi and Hunt, 1993). Further molecular research is, however, necessary to study the mechanisms involved in the detaching effect. The adhesion of human trophoblast to extracellular matrix proteins is dependent on the expression of integrins αβ1, αβ3 and αβ13, which bind laminin, and of integrin α5β1 which binds fibronectin (Burrows et al., 1993). It would be interesting to examine whether TDL-treated trophoblast down-modulates the expression of these integrins or the integrin-mediated signal transduction (Burrows et al., 1995).

Nevertheless, the fact that this effect is exerted by lymphocytes of term decidua, but not of early decidua, suggests that TDL may be involved in some end-of-pregnancy processes such as placental detachment, which occurs during parturition. The finding that cytokine production is increased in term labour and parturition (Steinborn et al., 1996), together with our results, supports the existence of immune processes involved in the mechanisms of normal delivery.

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