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Modulation of mouse sperm-egg interaction, early embryonic development and trophoblastic outgrowth by activated and unactivated macrophages

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Exposure of mouse spermatozoa and oocytes during *in vitro* fertilization (IVF) to lipopolysaccharide (LPS) and phorbol myristate acetate (PMA) activated macrophages (U937 cell line), but not unactivated macrophages culture-conditioned medium or control medium (RMPI + DMEM with 0.5% FBS) resulted in inhibition of IVF (87.2%), first cleavage (90.8%) and total blastocyst formation 97.5%). The direct coculture of the activated macrophages with 2-cell stage embryos resulted in arrested development (91.2%), an effect that was significantly diminished in the presence of monolayer of human endometrial stromal cells in the coculture (58.3%). In contrast, the majority of 2-cell embryos developed to blastocysts when exposed to unactivated macrophages, or macrophage-stromal cell cocultures (94.1%). The majority of 2-cell embryos cultured in control medium (DMEM/Ham's F12 with 2% FBS) developed to morulae (96.2%), then underwent growth arrest and degeneration. Furthermore, culturing blastocyst stage embryos in the above groups resulted in a significant enhancement of trophoblast outgrowth, particularly in coculture with activated macrophages as compared to any other group ($P < 0.005$). There was a significant increase in the levels of TGF-β, GM-CSF, IL-1α, IL-1β, TNF-α, PGE_2, TXB_2 and LTB_4 released into the culture conditioned medium of activated macrophages compared to unactivated macrophages ($P < 0.001$). These results suggest that the secretory products of activated macrophages, among them those determined in this study, in a stage-specific manner can directly effect sperm-egg interaction, early embryonic development and trophoblastic outgrowth. This data provides further support for the hypothesis that in endometriosis-associated infertility, continuous exposure of spermatozoa, oocytes and early embryos to activated macrophage-derived factors may play a vital role in their survival during transportation and fertilization as well as development during early embryonic stage.

Keywords *In vitro* fertilization; early embryo; endometrial cell; macrophages; coculture; implantation, *in vitro*

Introduction

Spermatozoa, oocytes and the embryos during transportation, fertilization and development interact with the epithelial lining of female reproductive tract tissues and encounter a variety of their secretory products. During these periods they also become exposed to the secretory products of inflammatory cells, namely the macrophages, which are normally present in the ovarian, oviductal and the uterine tissues (Tabibzadeh, 1991; Rappolee & Werb, 1992; Brannstrom & Norman, 1993; Honda *et al.*, 1994). In ovarian tissue, macrophages have been demonstrated to be present among stromal, theca/granulosa and luteal cells, as well as in pre-ovulatory follicular fluid in human as well as in other species (Brannstrom & Norman, 1993). The secretory products (cytokines and growth factors) of these macrophages and those produced by various ovarian cell types have been demonstrated to be involved in the ovulatory process, as well as, other ovarian biological activities (Brannstrom & Norman, 1993; Schomberg, 1989). In the oviduct, to which peritoneal macrophages have access through peritoneal fluid, macrophages are also present in the tubal subepithelial region (Honda *et al.*, 1994). In addition to macrophage-derived growth factors and cytokines, the oviductal epithelial cells have recently been shown to express mRNA and protein for several growth factors and cytokines (Chegini *et al.*, 1994a; Pfeifer & Chegini, 1994; Watson *et al.*, 1994; Zhao *et al.*, 1994; Zhao & Chegini, 1994). In the uterus, macrophages are present among the endometrial and decidual cells during the menstrual cycle as well as throughout pregnancy, and are abundant at the site of implantation in the human (Kabawat *et al.*, 1985; Lea & Clark, 1991; Tabibzadeh, 1991; Hunt & Pollard, 1992). Uterine macrophages, as well as various uterine cell types express several cytokines and growth factors (Tabibzadeh, 1991; Chegini *et al.*, 1992; Hunt & Pollard, 1992; Chegini *et al.*, 1994b; Guidice, 1994; Tang *et al.*, 1994a). The local expression of growth factors and cytokines have been implicated in several uterine biological activities during the menstrual cycle, as well as peri and post-implantation processes (Simmen & Simmen, 1991; Tabibzadeh, 1991; Hunt & Pollard 1992; Schultz & Heyner, 1993; Chegini *et al.*, 1994b; Tang *et al.*, 1994a; Guidice, 1994).

Some of the macrophage-derived secretory products have been demonstrated to be potent chemotactic factors for other macrophages, neutrophils and fibroblasts. They have been shown to stimulate cell proliferation, differentiation, angiogenesis and extracellular

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matrix formation, all of which are among the parallel events occurring not only in the reproductive tract tissues, but also during embryonic development and implantation (Simmen & Simmen, 1991; Tabibzadeh, 1991; Hunt & Pollard, 1992; Rappolee & Werb, 1992; Brannstrom & Norman, 1993; Schultz & Heyner, 1993; Chegini *et al.*, 1994b; Guidice, 1994; Tang *et al.*, 1994a; Watson *et al.*, 1994). It has recently been demonstrated that co-culturing of unactivated peritoneal macrophages with mouse spermatozoa and oocytes have an adverse effect on the rate of fertilization, whereas, these unactivated peritoneal macrophages significantly improve embryonic development *in vitro* (Honda *et al.*, 1994). Furthermore, human oviductal cells and their conditioned medium maintained the motility, hyperactivation and capacitation of human spermatozoa (Kervancioglu *et al.*, 1994; Yeung *et al.*, 1994). At the site of implantation these secretory products may modulate the biological activity of endometrial and decidual cells, leading to cellular transformations thought to be important in embryo implantation (Lea & Clark, 1991; Hunt & Pollard, 1992). Macrophages are thought to be important in maternal acceptance of the fetal allograft at implantation and the establishment of the feto-placental unit during early pregnancy (Hunt & Pollard, 1992).

In pathological conditions such as endometriosis, macrophages and their secretory products have been postulated to modulate the endometrial implants and participate either directly or indirectly in endometriosis-associated infertility (Ramey & Archer, 1993, Chegini *et al.*, 1994c). Conversely, macrophage activation by bacterial endotoxins, which result in increased macrophage secretory products, has been shown to have adverse effects on the developing embryo (Storeng & Johne, 1987). In the present study we determine the effect of activated and unactivated macrophage-derived secretory products on sperm-egg interaction (*in vitro* fertilization), as well as the direct action of macrophages either alone, or in a coculture with monolayer of human endometrial stromal cells on early embryonic development and trophoblastic outgrowth.

Results

The U937 cells require RPMI 1640 medium supplemented with 10% FBS for their optimal growth, whereas, ideal culture media for endometrial stromal cells are MEM, DMEM or DMEM/Ham's F12 supplemented with 10% FBS. Due to the coculture of stromal cells with U937 cells, in the initial experiments the macrophages were grown in RPMI as well as RPMI/MEM or RPMI/DMEM at a 1:1 ratio to determine their growth characteristics in these media. The U937 cells grew exponentially in RPMI supplemented with 10% FBS with a doubling time of 24.1 h (Figure 1). The growth of stromal cells cultured in RPMI/MEM was not significantly different from those grown in MEM or RPMI alone, with a doubling time of 41.8 h (in RPMI or MEM) and 41.9 h in RPMI/MEM and RPMI/DMEM, which is similar to that reported for stromal cells grown in DMEM/Ham's F12 (Chegini *et al.*, 1992). The U937 cells grown in RPMI/MEM showed a greater growth rate compared to RPMI alone, with a doubling time of 20.3 h after 8 days of incubation (Figure 1).

In the second experiments culture-conditioned medium from LPS/PMA activated macrophages (AΦ-CM) caused the inhibition of *in vitro* fertilization IVF (87.2%), first cleavage (89.8%) and total blastocyst formation (94.4%). Vacuole formation was also observed in fertilized ova exposed to the AΦ-CM treated group (Figure 2, Table I). In contrast, culture-conditioned medium from unactivated macrophages (Φ-CM) or the control medium (C-CM, RPMI+ DMEM with 0.5% FBS) resulted in approximately 92% of fertilization, with 82% first cleavage, and 32% and 41% blastocyst formation, respectively (Figure 2, Table I). No vacuole was formed in fertilized ova either exposed to unactivated macrophage-culture conditioned medium or control medium treated groups (Figure 2).

The activated macrophage culture-conditioned medium had a significantly higher level of IL-1α, IL-1β, GM-CSF, TNF-α, PGE_2, TXB_2 and LTB_4 compared to the unactivated macrophage culture conditioned medium (Table 2). The level of TGF-β1 in unactivated macrophage conditioned medium was 160 ± 15 pg/ml and 700 ± 22.4 pg/ml before and after acidification, respectively, which was significantly increased in culture-conditioned medium from activated macrophages (4.2 ± 0.1ng/ml and 10.7 ± 0.1 ng/ml before and after acidification, respectively). This indicates that a significant level of TGF-β secreted into the activated and unactivated macrophages culture conditioned medium is in a latent or biologically unactive form. Control medium (RPMI/DMEM containing 0.5% FBS) unexposed to macrophages did not have detectable amounts of any of the cytokines or eicosanoids evaluated with the exception of TGF-β1 which was 80 ± 9 pg/ml and 850 ± 34 pg/ml before and after acidification, respectively. The level of endotoxin in the macrophage culture conditioned medium after LPS and PMA activation was negligible to induce any embryo toxicity as previously reported (Storeng & Johne, 1987).

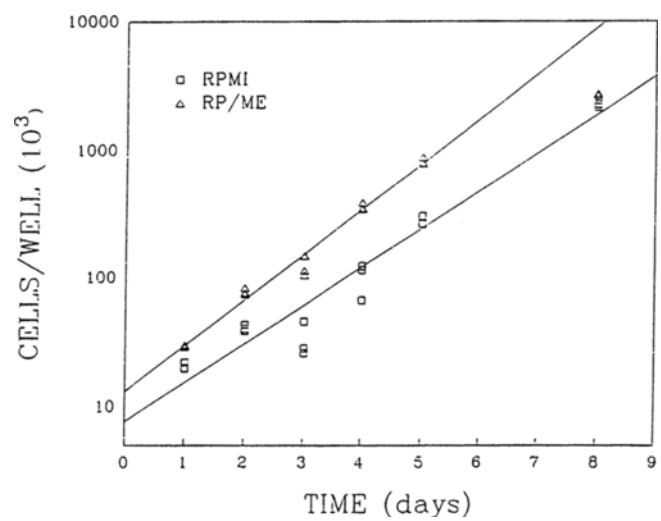

Figure 1 U937 cells incubated in RPMI (□) or RPMI/MEM (△) containing 10% FBS indicating the log growth of these cells with a doubling time of 24.1 and 20.3 h respectively

In the third experiments, 2-cell embryos cultured in control medium (DMEM/Ham's F12 with 2% FBS) had poor blastocyst formation (1/27, 3.7%), with growth arrest and/or degeneration occurring after reaching morula stage (Figure 3). Coculturing of 2-cell embryos with unactivated macrophages resulted in a significant improvement in development (32/34, 94.1% blastocyst formation, Figure 3). In contrast, development was arrested in the presence of activated macrophages, with degeneration occurring mainly at the 2-cell stage (3/34, 8.8% blastocyst formation, Figure 3). All 2-cell embryos placed in Ham's F10/BSA medium

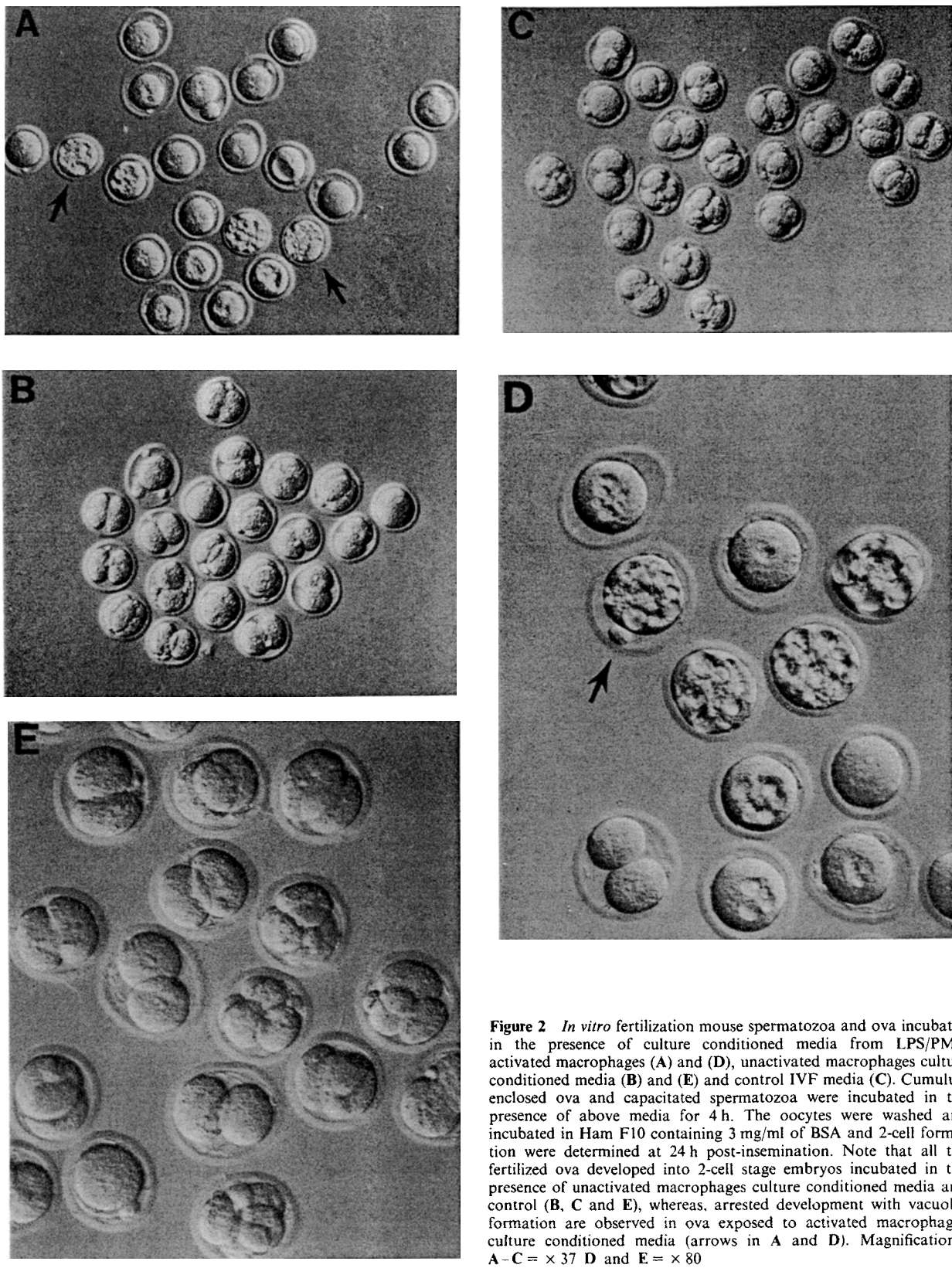

Figure 2 *In vitro* fertilization mouse spermatozoa and ova incubated in the presence of culture conditioned media from LPS/PMA activated macrophages (**A**) and (**D**), unactivated macrophages culture conditioned media (**B**) and (**E**) and control IVF media (**C**). Cumulus-enclosed ova and capacitated spermatozoa were incubated in the presence of above media for 4 h. The oocytes were washed and incubated in Ham F10 containing 3 mg/ml of BSA and 2-cell formation were determined at 24 h post-insemination. Note that all the fertilized ova developed into 2-cell stage embryos incubated in the presence of unactivated macrophages culture conditioned media and control (**B**, **C** and **E**), whereas, arrested development with vacuoles formation are observed in ova exposed to activated macrophages culture conditioned media (arrows in **A** and **D**). Magnifications. **A**–**C** = × 37 **D** and **E** = × 80

Table 1 The effect of culture conditioned media from LPS/PMA activated and unactivated macrophages on mouse sperm-egg interaction

Medium	*% IVF(N)*	*% Cleavage*	*% Blastocyst*	*% Eggs (with Vacuoles)*
C-CM	92.8 (84)	82.1	41.6	0.0
Φ-CM	91.9 (87)	85.0	32.1	0.0
AΦ-CM	12.8 (78)**	10.2**	2.56*	37.1**

N = number of ova, C-CM = Control culture media (RMPI + DMEM with 0.5% FBS), Φ-CM = Unactivated macrophages culture conditioned medium, AΦ-CM = LPS/PMA activated macrophage culture conditioned medium. $*P<0.10^{-5}$ and $**P<0.10^{-15}$ differs from their respective control value

Table 2 Concentration of cytokines and eicosanoids in activated and unactivated macrophages conditioned media

Products	*Activated*[a]	*Unactivated*[b]
TNFα	1108.5 ± 44.5	<5.0
IL-1α	7.5 ± 0.3	<0.2
IL-1β	171.9 ± 39.4	4.84 ± 1.4
GM-CSF	262.6 ± 8.2	<5.4
PGE_2	102.1 ± 11.5	29.6 ± 1.2
TXB_2	2169.5 ± 306	65.5 ± 17
LTB_4	16.03 ± 4.3	<8.9

Values are pg/ml and expressed as mean ± SEM. [a]differs from [b]$P<0.001$. The level of cytokines and eicosanoids was undetectable in control media containing 0.5% FBS

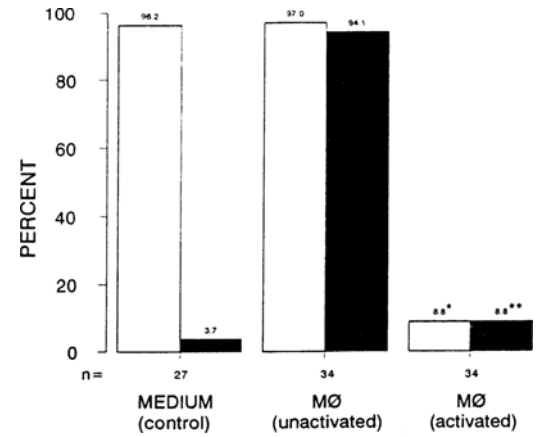

Figure 3 Development of 2-cell embryos after 80 h of culture in control media (DMEM/Ham's F12 with 2% FBS), and cocultured with activated, or unactivated macrophages (MΦ). □ = Morula formation, ■ = blastocyst formation, n = number of embryos; *morula formation differs from control and unactivated MΦ ($P<0.001$); **blastocyst formation differs from control and activated MΦ ($P<0.001$). In control 26/27 of 2-cell embryos developed to morula stage, 1/26 morulae developed to blastocyst stage and the rest remained arrested and degenerated at morula stage, indicating the sub-optimal nature of the control media. In the presence of activated MΦ 31 out of 34 2-cell embryos remained arrested at 2-cell stage and underwent degeneration and only 3 out of 34 2-cell embryos developed upto blastocysts

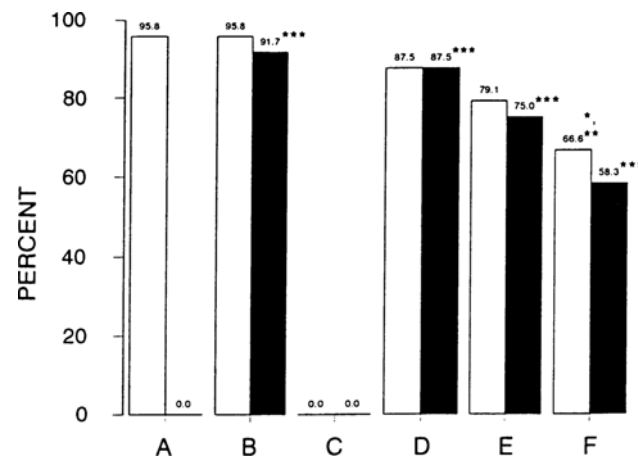

Figure 4 Development of 2-cell embryos after 80 h of culture in control media DMEM/Ham's F12 with 2% FBS (A), and coculture with unactivated MΦ (B), activated MΦ (C), endometrial stromal cell monolayer, ESC (D), ESC + unactivated MΦ (E) and ESC + activated MΦ (F). □ = morula formation, ■ = blastocyst formation. *$P<0.05$, morula formation differs from control; **$P<0.001$, morula formation differs from activated MΦ; ***$P<0.001$, blastocyst formation differs from control and activated MΦ; n = 24 embryos in each group

developed to blastocyst stage (25/25, 100%), indicating good quality embryos.

In the fourth experiments, 2-cell embryos cultured in control medium (DMEM/Ham's F12 with 2% FBS), and cocultured with unactivated or activated macrophages showed a similar pattern of development to that of their respective counterparts in experiment 3 (Figure 4 and Figure 5A–C). Coculturing of the 2-cell embryos with a monolayer of endometrial stromal cells showed a higher rate of blastocyst formation as compared to the control ($P<0.001$), and was similar to that seen with unactivated macrophages (21/24, 87.5%, Figures 4 and 5D). Coculturing of unactivated macrophages and stromal cells improved the embryonic development compared to control medium, but to a lesser extent than that induced by either unactivated macrophages or stromal cells (18/24, 75%, Figures 4 and 5E). Coculturing of 2-cell embryos with stromal cells and activated macrophages substantially improved their development (14/24, 58.3% blastocysts) as compared with control medium ($P<0.001$) or activated macrophages alone ($P<0.001$, Figures 4 and 5F).

The majority of blastocysts cultured in control medium alone had poor trophoblastic outgrowth (Figure 6 and Figure 7A). Trophoblast outgrowth was similar in embryos cultured with stromal cells, unactivated macrophages, stromal-unactivated macrophages and stromal-activated macrophages (Figure 6), but, with a larger area of outgrowth than that induced by medium alone ($P<0.05$). Blastocysts exposed to activated macrophages alone had the greatest area of outgrowth compared to control medium ($P<0.005$) and all other groups ($P<0.05$). Figure 7 shows the morphological appearance and trophoblast outgrowth of embryos cultured in medium alone (Figure 7A), unactivated macrophages (Figure 7B) and activated macrophages (Figure 7C), as well as those cocultured with stromal cells (Figure 7D), stromal cell-unactivated macrophages (Figure 7E) and stromal cell-activated macrophages (Figure 7F).

Discussion

During transportation, fertilization, as well as pre- and postimplantation, the oocyte, spermatozoa and embryo interact with the epithelial lining of the female rep-

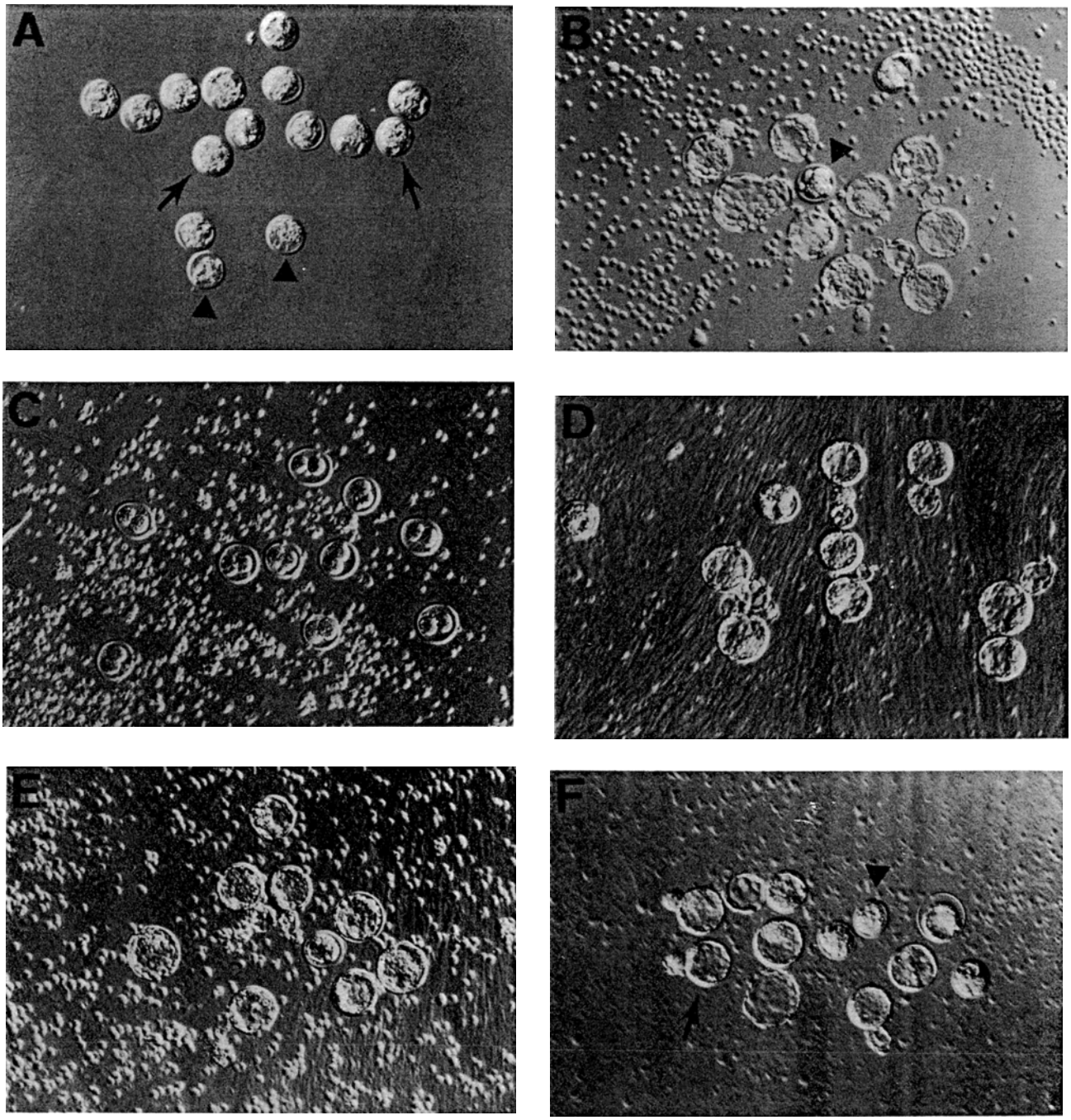

Figure 5 Photomicrographs of the embryos developed from 2-cell embryos after 80 h of culture in control media, DMEM/Ham's F12 with 2% FBS (**A**), and cocultured with unactivated MΦ (**B**), activated MΦ (**C**), endometrial stromal cells, ESC (**D**), ESC + unactivated MΦ (**E**) and ESC + activated MΦ (**F**). Embryos cultured in control media with arrest of development (arrows) and degeneration (arrow heads) at morula stage embryos are shown in A. C indicates degeneration at 2-cell stage in all the embryos. **B**, **D–F** show majority of embryos developed to blastocyst stage (arrow in **F**) and few developed to morula stage (arrow heads in **B** and **F**). Activated macrophages are attached to the culture dish (**C**) and stromal cells (**F**) and unactivated macrophages are not attached in **B** and **E** and they appear as small cells in the background in **B**, **C**, **E** and **F**. Magnification: × 68

roductive tract and its secretory products. While these secretory products have beneficial and regulatory effects on a variety of biological activities of sperm, oocytes and their fertilization, as well as pre- and postimplantation embryonic development, the presence of an unfavorable environment in these tissues may ultimately lead to a failed pregnancy. One cause of such detrimental environment may be the existence of an inflammatory condition in either ovarian, oviductal or uterine tissues, as well as in the peritoneal cavity. In patients with endometriosis, peritoneal macrophages and peritoneal fluids have been shown to influence fertilization by phagocytosing spermatozoa and having an adverse influence on sperm mobility, sperm-egg interaction and embryonic development (for review see Ramey & Archer, 1993). The direct role of inflammatory cells and their interaction with spermatozoa, oocytes and the early embryo during fertilization and development is not yet fully understood, although release of cytotoxic factors by activated mac-

rophages have been suggested as a major contributor to this disorder (Ramey & Archer, 1993).

In the present study we have developed a coculture system consisting of human endometrial stromal cells and a well characterized human monocyte/macrophage cell line, U937 and examined their direct influence in both activated and unactivated conditions on fertilization, early embryonic development and trophoblastic outgrowth using mouse spermatozoa, oocytes and embryos. The advantage of using U937 cells instead of peripheral, peritoneal or tissue macrophages (ovarian, oviductal or uterine) is due to several reasons. The isolation of sufficient numbers of tissue macrophages from reproductive tract tissues is technically difficult and was found to not be ideal. Furthermore, the use of either peripheral or peritoneal macrophages cocultured with endometrial stromal cells resulted in inconsistent proliferation of stromal cells, possibly due to their variable status related to exposure to various antigens (personal observations). In contrast, U937 cells are well characterized regarding their secretory products, can be passaged without changes in their growth and secretory products, and can be used both in an activated and unactivated state.

Our data indicated that in contrast to culture-conditioned medium from unactivated macrophages, exposure of cumulus-enclosed ova and capacitated spermatozoa to activated macrophage culture-conditioned medium caused the inhibition of *in vitro* fertilization, first cleavage and total blastocyst formation. These observations are consistent with a recent study which indicated that the direct coculturing of mouse sperm-egg with unactivated peritoneal macrophages reduced the rate of fertilization by approximately 20%, compared to human tubal fluid (Honda *et al.*, 1994). Although the rate of fertilization in our study using unactivated macrophage culture-conditioned medium was similar to that observed with human tubal fluid, noticeable differences were evident when compared to direct incubation with unactivated macrophages with a lower fertilization rate (Honda *et al.*, 1994). The experimental approach may account for the differences observed in our study and that reported by Honda *et al.*, 1994. In our study spermatozoa and oocytes were exposed for 4 h during fertilization to macrophage culture-conditioned medium collected after 24 h of macrophage culturing, whereas spermatozoa and oocytes were concurrently exposed to mouse peritoneal macrophages for 24 h in Honda's *et al* experiments (Honda *et al.*, 1994).

The development of 2-cell mouse embryos was either enhanced or inhibited when cocultured with U937 cells depending on the activation state of the macrophages, and the presence or absence of a monolayer of endometrial stromal cells. In a medium suboptimal for embryo development, co-culturing with unactivated macrophages resulted in a high rate of blastocyst formation. In contrast, activation of macrophages resulted in degeneration of embryos, with most remaining at the 2-cell stage. This effect was different from that seen in control medium, where 2-cell embryo development progressed to morula stage and remained arrested and/or underwent degeneration. An improvement in the embryonic development occurred in coculture with a monolayer of endometrial stromal cells. The presence of stromal cells actually appeared to reverse some of the inhibitory effect of activated macrophages, with blastocyst formation occurring in over 50% of the cultured embryos.

It has been suggested that one of the beneficial effect of coculturing mouse embryos with somatic monolayer cells may be due to the removal of inhibitory or toxic products from the culture by the monolayer (Bongso *et al.*, 1991). However, this is unlikely as many of the secretory components that are toxic to embryos (Hill *et al.*, 1987; Fukuda *et al.*, 1989; Schneider *et al.*, 1989; Chaouat *et al.*, 1990; Clark *et al.*, 1991; Kanzaki *et al.*, 1991; Mori *et al.*, 1991; Pampfer *et al.*, 1991; Robertson & Seamark, 1992; Haimovici & Anderson, 1993; Lea & Clark, 1993) may also be cytotoxic for the monolayer cells. We postulate that the monolayer cells, uterine stromal cells in this case, can directly interact with the macrophages, and via specific biochemical signal(s) generated by monolayer cells, alter the macrophages secretory pattern. This in turn results in the formation of a more favorable environment for early embryonic development. In contrast to their detrimental effects on fertilization and early embryonic development, coculturing with activated macrophages either alone, or in combination with endometrial stromal cells, enhanced the trophoblasts outgrowth. This suggests that activated macrophages secretory products may influence trophoblast outgrowth during implantation period. In support of our observations is the recent report indicating that the development of blastocysts and trophoblast outgrowth were undisturbed and nonadhesive to unactivated and LPS activated macrophages and thus prevented them from reaching the inner cell mass (Sionov *et al.*, 1993). However, the removal of the trophoectoderm increased the macrophages adhesiveness and they became destructive to the inner cell mass when activated (Sionov *et al.*, 1993).

From these observations we postulate that one of the causes of unsuccessful pregnancy may be due to exposure of spermatozoa, oocytes or early embryos to an unfavorable microenvironment, possibly an inflammatory condition in the ovary, oviduct, uterus or in the peritoneal cavity, resulting in inhibition of fertilization and early embryonic development prior to

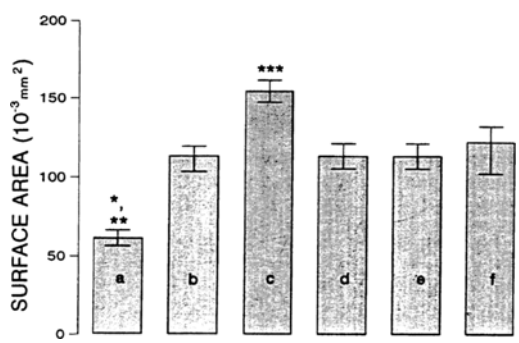

Figure 6 Surface area (mm^2) of trophoblast cells outgrowth determined by point counting after 108 h of incubation of blastocysts in control media DMEM/Ham's F12 with 2% FBS (a), and cocultured with unactivated MΦ (b), activated MΦ (c), endometrial stromal cell monolayer ESC (d) ESC + unactivated MΦ (e), and ESC + activated MΦ (f). Values are expressed as mean ± SEM. *differ from b, d, e and f at $P = 0.05$; **differs from c at $P < 0.005$; ***differ from b, d, e and f at $P < 0.05$

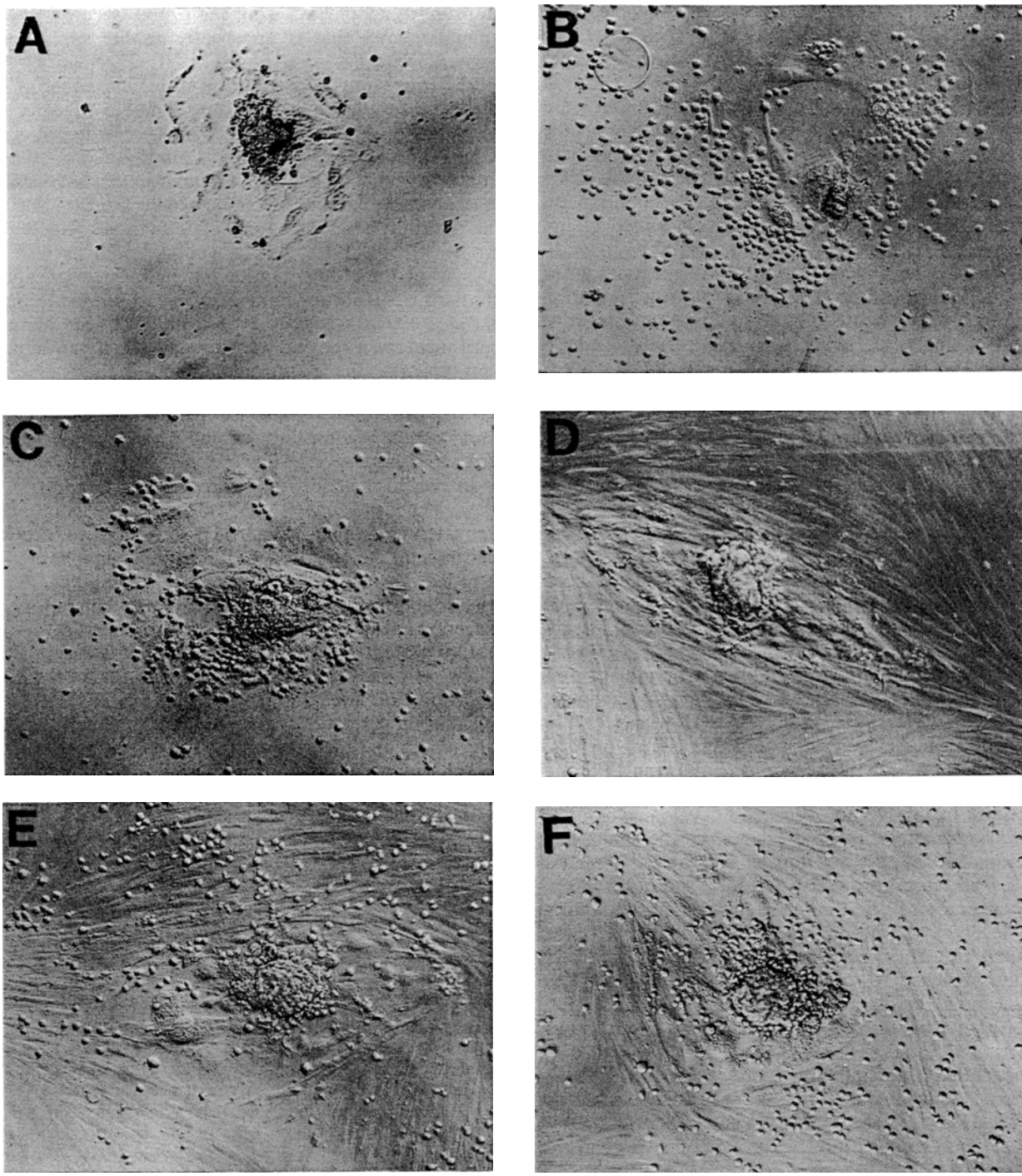

Figure 7 Photomicrographs of trophoblasts outgrowth after 108 h of culture of blastocysts in control media DMEM/Ham's F12 with 2% FBS (**A**), and cocultured with unactivated MΦ (**B**), activated MΦ (**C**), endometrial stromal cell monolayer, ESC (**D**), ESC + unactivated MΦ (**E**) and ESC + activated MΦ (**F**). Small cells in background of B–F are macrophages and the activated macrophages are all adhered to the surface. Magnification: × 68 MΦ = Macrophage

implantation. Once a preimplantation embryo, either unexposed or protected from such hostile environment(s) arrives at the implantation site, an inflammatory microenvironment at the implantation site may contribute to a successful pregnancy. Differences in the microenvironment at the implantation site compared to other sites within the reproductive tract may be due to elaboration or modulation of the local growth factors, cytokines and eicosanoids milieu initiated by maternally-derived biochemical signals through cell-cell interaction. Whether there are any differences in the type of secretory products released by inflammatory cells or other cell types at the site of implantation, compared with other sites in the reproductive tract, is unknown. However, coculturing with either endometrial stromal, unactivated macrophages or the combination of the two, effectively provided a more favorable environment for trophoblast outgrowth.

Analysis of the activated macrophages culture-

conditioned medium revealed a significant increase in the level of IL-1α, IL-1β, GM-CSF, TNF-α, TGF-β and eicosanoids compared to unactivated macrophages culture-conditioned medium. The effects of some of these secretory products have been investigated on murine embryonic development. An example of the stage-specific influence of these factors is seen with GM-CSF. While exposure of 2-cell embryos to GM-CSF causes arrested development, it's addition to morula stage embryos has a beneficial effect on development (Robertson & Seamark, 1992). In addition, GM-CSF has been shown to be expressed at the feto-placental interface and also to be effective in preventing spontaneous abortions in abortion-prone mice (Chaouat *et al.*, 1990; Clark *et al.*, 1991; Kanzaki *et al.*, 1991). However, GM-CSF has been shown not to have any growth stimulatory effect of ectoplacental cone trophoblasts (Lea & Clark, 1993), whereas in high concentrations it was found to inhibit mouse blastocyst outgrowth either alone (Hill *et al.*, 1987) or in coculture with endometrial epithelial cells (Robertson & Seamark, 1992). On the other hand EGF and PDGF have been reported to significantly stimulate DNA synthesis by ectoplacental cone trophoblasts (Lea & Clark, 1993). Furthermore, IL-1α and IL-1β have been reported not to be embryo toxic *in vitro* (Schneider *et al.*, 1989), whereas, IL-1β inhibited blastocyst attachment, but significantly enhanced trophoblasts outgrowth, gamma interferon inhibited trophoblast outgrowth and GM-CSF inhibited blastocysts attachment (Haimovici *et al.*, 1991). PGE_2, IL-1, IL-6 and TNF-α are found in higher concentrations in cases of preterm delivery associated with infections. Following LPS and PMA activation, macrophages became toxic to early embryos, resulting in arrested development. This cytotoxic activity of macrophages in the embryonic development is possibly due to some of the macrophages secretory products that are produced in increased amounts resulting from endotoxin activation. The level of endotoxin remained in the conditioned medium after the macrophages activation, measured by limulus amebocyte lysate assay, was negligible to induce any cytotoxicity, although LPS has been reported to be embryo toxic at high concentrations (Storeng & Johne, 1987).

In addition to inflammatory cell-derived factors, maternal derived factors are thought to be important in preparing a microenvironment favorable for embryo implantation and continued development (Fukuda *et al.*, 1989; Healy, 1991; Kanzaki *et al.*, 1991; Mori *et al.*, 1991). In this regard, the supernatant from cultures of a human decidual cell line has been shown to have a growth promoting effect on cultured mouse embryos (Mori *et al.*, 1991). The addition of medium from mixed lymphocyte cultures to this system results in blastocyst attachment and outgrowth (Fukuda *et al.*, 1989). Furthermore, EGF, TGF-α, PDGF, FGF, CSF-1 and IL-1β as well as their combinations were effective in the enhancement of trophoblast outgrowth following incubation of mouse blastocysts with these growth factors (Haimovici *et al.*, 1991; Haimovici & Anderson, 1993). These data suggest that synergism between factors derived from maternal decidual and immune cells may enhance embryonic development and implantation. In accordance with others, we have shown that coculturing with stromal cells results in enhanced early embryo development, a phenomenon seen in many animal models that is apparently neither species nor tissue specific (Bongso *et al.*, 1991; Freeman *et al.*, 1993). Interestingly, coculturing with unactivated macrophages resulted in the greatest number of embryos forming blastocysts compared to those cultured with either endometrial stromal cells alone, or a combination of stromal cells and macrophages. Paradoxically, macrophages have been implicated in contributing to the infertility associated with endometriosis (Ramey & Archer, 1993). Both normal endometrium as well as ectopic endometrium in the rat endometriosis model contains a significant number of macrophages (Simms *et al.*, 1991; Chegini *et al.*, 1994c). In human, peritoneal macrophages are thought to play an important role in the pathogenesis and maintenance of endometrial implants, a hypothesis supported by the growth-promoting effects of peritoneal macrophage-conditioned medium on cultured fibroblasts (Ramey & Archer, 1993).

In conclusion, our data suggests that in contrast to their beneficial effects on trophoblastic outgrowth, activated macrophages and their secretory products are detrimental to sperm-egg interaction and result in arrested development of early stage embryos. Since macrophage activation may occur in inflammatory or infectious disorders in reproductive tract tissues, a variety of potentially embryotoxic products secreted by these cells may lead to events such as spontaneous abortion, or the endometriosis-associated infertility. Further investigations are required to determine the differences and the extent of activation of macrophages in physiological and pathological conditions which may be important in designing future treatment modalities directed to the infertile patient.

Materials and methods

All the culture supplies, fetal bovine serum, pregnant mare's serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), Limulus amebocyte lysate kit, lipopolysaccharide (LPS), phorbol myristate acetate (PMA), monoclonal antibodies to human cytokeratin 19, vimentin and smooth muscle α-actin, as well as all the other chemicals were purchased from Sigma Chemical Co. (St Louis, MO). Human specific interleukin-1α (IL-1α), tumor necrosis factor α (TNF-α) ELISA kits were purchased from Amersham Co. (Arlington Height, IL). Human granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-1β (IL-1β), prostaglandin E_2 (PGE_2, with 50% cross reactivity with PGE_1), thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) EIA kits were purchased from Advanced Magnetics Inc. (Cambridge, MS). ^{125}I-TGF-β1 (specific activity 93 μCi/μg) was purchased from Biomedical Technologies, Inc. (Stoughton, MA). Rhodamine (TRITC) conjugated affinipure IgGs were purchased from Jackson Laboratories Inc. (West Grove, PA). Macrophages (U-937) and CCL64 mink lung epithelial cells (mv1Lu) were obtained from American Type Culture Collection (Rockville, MD). Male and female $B_6D_2F_1$ mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and housed at 23–25°C under controlled illumination (14 h light and 10 h darkness. The experimental use of these animals was approved by the University of Florida Institutional Animal Care and Use Committee. All the culture wares were purchased from Costar (Cambridge, MS), and organ culture dishes (60 × 15 mm, Falcon) from Becton Dickinson Labware (Lincoln Park, NJ).

Uterine Tissue Collection and Stromal Cell Culture

Uterine specimens from nonpregnant premenopausal women undergoing hysterectomy for reasons other than cervical intraepithelial neoplasia (CIN) and endometrial cancer were collected at the University of Florida affiliated Shands hospital under approval of the University of Florida Institutional Review Board. The endometrial stromal cells were isolated and cultured in DMEM/Ham's F12 (v/v 1:1) containing 10% FBS and incubated at 37°C in a 5% CO_2 atmosphere as previously described (Tang *et al.*, 1994a). Purity of the cultures was determined by immunofluorescent staining using monoclonal antibodies to human vimentin, cytokeratin 19 and smooth muscle α-actin (Tang *et al.*, 1994a).

Macrophage culture

U937 cells, an established human monocyte-like cell line which retains many of the markers and activities of normal peripheral monocytes, were used in this study (Sundstrom & Nilsson, 1976; Lyons & Ashman, 1989). These cells were grown in 75 cm^2 flasks in RPMI 1640/MEM (v/v, 1:1) supplemented with 10% FBS.

Ova collection

Female mice were superovulated with intraperitoneal administration of 7 IU PMSG, followed 48 h later by 7 IU hCG. At 14 h post-hCG, mice were sacrificed by cervical dislocation and oviducts were excised, washed in culture medium and transferred to new dishes containing culture medium (Ham F10 nutrient mixture supplemented with sodium bicarbonate (2.1 g/l), calcium lactate (242 mg/l), penicillin G (37.5 IU/ml) and streptomycin sulfate (25 μg/ml). Medium was prepared using cell culture quality water (Irvine Scientific, Santa Ana, CA), osmolality adjusted to 281–285 mOsmol/kg H_2O, filtered through 0.20μ filters (Millipore Products Division, Bedford, MA) and equilibrated at 5% CO_2 in air in a humidified incubator for 24 h before use. Medium supplemented with 3 mg BSA (fraction V) per ml served as IVF medium as well as culture medium, whereas, medium supplemented with 30 mg BSA/ml served as capacitation medium. Cumulus oophorus complexes (COC) were recovered from each oviduct by puncturing the swollen ampulla using a 1-ml syringe fitted with 30G needle and a pair of fine forceps, washed and transferred to organ culture dish containing 1 ml culture medium. The quality of ova was determined for their *in vitro* fertilization ability (Juneja & Dodson, 1990).

2:Spermatozoa retrieval and capacitation

Mature fertile male mice (age, 10–14 weeks) were sacrificed at 12.5 h after hCG treatment of female mice. The caudae epididymides were excised and transferred to organ culture dishes containing 1 ml of capacitation medium (Juneja & Dodson, 1990). Spermatozoa was released gently by rupturing the caudae epididymides with fine forceps and a 1-ml syringe fitted with 30G needle and capacitated at a concentration of $5–10 \times 10^6$/ml capacitation medium for 90 min at 37°C in the incubator. Spermatozoa were washed using capacitation medium and collected by centrifugation at 800*g* for 15 min. The final concentration of BSA in the medium was maintained at 3 mg/ml.

Macrophage conditioned media

U937 cells were placed in 75 cm^2 flasks in serum free RPMI 1640/MEM (1:1), at densities of 10^6 cells/ml. After 24 h, cells were collected by centrifugation, washed in serum-free medium, and activated with 12.5 μg/ml LPS and 60 ng/ml of PMA for 1 h. The macrophages unexposed to LPS/PMA and activated macrophages were collected, washed three times and resuspended in RPMI/DMEM with 0.5% FBS. Culture conditioned medium from activated and unactivated macrophages were collected after 24 h of incubation, centrifuged, and either used for *in vitro* fertilization or stored at −80°C until assayed for cytokines and eicosanoids.

In vitro fertilization

Capacitated spermatozoa (5×10^5) were added to the ova and incubated in the presence of IVF medium (control), unactivated or activated macrophages culture conditioned media for 3 hrs. The ova were then washed and transferred to culture medium (Ham F10+3 mg BSA/ml). The rate of IVF and cleavage was determined by the expulsion of second polar body and pronuclei formation, and 2-cell formation, respectively.

Embryo collection

Female mice, superovulated as above were individually caged with fertile males. They were checked for a seminal plug in the vagina on the following morning (day of positive plug = day 1 of pregnancy) and were sacrificed by cervical dislocation for the recovery of 2-cell embryos from the oviducts on day 2, and blastocysts from the uteri on day 4 of pregnancy as previously described (Juneja & Dodson, 1990; Juneja & Williams 1993). Morphologically normal embryos were washed, pooled, then randomly distributed into culture dishes previously prepared with endometrial stromal cells, macrophages or both.

Embryo, macrophage and stromal cell coculture

Endometrial stromal cells were plated in organ culture wells and grown to confluence. The medium was removed and the cells were washed and incubated in DMEM/Ham's F12 under serum free conditions for an additional 48 h. Prior to coculturing, macrophages were also placed in serum free RPMI/MEM for 24 h, and were then activated with LPS and PMA as described above, washed three times and resuspended in DMEM/Hams's F12 with 2% FBS before being added to organ culture dishes at 1.5×10^5 cells/1.5 ml/dish. Ten to fifteen 2-cell embryos were added to each well containing stromal cells, activated or unactivated macrophages and their cocultures.

To determine the effect of macrophages on embryo development, 95 2-cell embryos were distributed into three coculture groups: (A) unactivated macrophages, (B) activated macrophages, and (C) control media alone. To determine if the addition of endometrial stromal cells altered the effects seen, 144 2-cell embryos were distributed into the following coculture conditions: (A) stromal cells, (B) stromal cells + activated macrophages, (C) stromal cells + unactivated macrophages, (D) activated macrophages, (E) unactivated macrophages, and (F) control media. In all the experiments, 25 2-cell embryos were placed in culture medium consisting of Ham's F10 containing 0.3% BSA, as a quality control (Juneja & Dodson, 1990). Embryo development was monitored every 24 h, with the final stage of development recorded at 80 h post initiation of coculture. Media was collected from all the cultures after the final reading, centrifuged and supernatant were stored at −80°C, and later analysed for endotoxin using a Limulus amebocyte lysate kit.

To determine trophoblast outgrowth, expanded to hatching blastocyst stage embryos were added one per well to 96-well plates with confluent stromal cells and/or activated or unactivated macrophages (2000/well). Attachment of embryos and outgrowth of trophoblast cells was determined at 108 h. Embryos were photographed individually at identical magnifications, and the surface area of trophoblast outgrowth determined by point counting (Wiebel, 1979).

Enzyme linked immunoassays

ELISA of macrophages conditioned media was performed using commercially available kits with the following reported detection limits: TNF-α, 4.8 pg/ml; IL-1α, 0.2 pg/ml; IL-1β, 4.3 pg/ml; GM-CSF, 2.5 pg/ml; PGE_2, 1.5 pg/ml; TXB_2, 4.3 pg/ml; LTB_4, 8.9 pg/ml. Conditioned media samples were analysed according to recommended procedures provided.

TGF-β radioreceptor assay

The level of TGF-β1 in activated and unactivated macrophages culture conditioned media was determined by competitive radioreceptor assay measuring binding of ^{125}I-TGF-β1 to CCL64 cells as previously described (Tang *et al.*, 1994b). Briefly, 2×10^5 CCL64 cells/well were seeded into 24 well plates in DMEM with 5% FBS for 24 h. The cells were washed with DMEM containing 1mg/ml of BSA, and the macrophages culture conditioned media as well as the media unexposed to cells containing 0.5% FBS and used in cell culture were individually added to CCL64 cells in the presence of 1nM ^{125}I-TGF-β and incubated for 2 h at room temperature. The culture conditioned media used in this assay were either untreated or treated with 1M acetic acid, which results in the activation of TGF-βs in the media (Tang *et al.*, 1994b). After incubation the cells were washed in ice-cold PBS containing 1mg/ml of BSA and extracted with 0.5 ml of 10 mM Hepes, 10% (v/v) glycerol and 1% triton X-100 (pH 7.0) for 30 min at 37°C (Tang *et al.*, 1994b). The intra and interassay variations were 7% and 11% respectively. The TGF-β level was determined in the conditioned media by comparison with known standards.

Statistical analysis

Statistical analysis of the data in Table I as well as Figures 3 and 4 was performed using the 2×2 Chi-Square test using contingency Tables and Yates correction, with *P values* <0.05 considered significant. Trophoblastic outgrowth areas are expressed as mean ± SEM and compared using the student's *t* test or ANOVA. The level of cytokines and eicosanoids in culture conditioned media were determined in triplicate and expressed as mean ± SEM and analysed using the student's *t* test.

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