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REVIEW ARTICLE

A ROLE FOR CYTOKINES IN EARLY PREGNANCY

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Abstract: Cytokines are expressed in a variety of cell types of the reproductive system, although in most instances their functions are not understood There are, however, a few instances where a role in early pregnancy has been established. First, preimplantation conceptuses of ruminant ungulate species, such as cattle, sheep and goat, secrete a unique Type I interferon (IFN-τ). By mechanisms that are still unclear, IFN-τ prevents the destruction of the corpus luteum and hence ensures the continued production of progesterone which is essential for continuation of pregnancy. Most like the IFN-τ prevent luteolysis by modulating the output of a luteolytic hormone, prostaglandin $F_{2\alpha}$, from the uterus. Depsite this involvement in pregnancy, the IFN-τ possess similar antiproliferative and antiviral activities to other Type I IFN, [1]λ *e.g.* IFN-α. There are 4-5 genes for IFN-τ in sheep and cattle whose promotor regions are highly conserved and distinct from those of other Type I IFN. These genens are not virally inducible and are expressed only in the trophectoderm (outer epithelium of the developing placenta) from the time of blastocyst hatching to implantation.

Leukemia inhibitory factor (LIF) is a multi-functional cytokine which is expressed by uterine endometrium of pregnant mice around day 4 of pregnancy. Female mice lacking a functional LIF gene are fertile but their blastocysts fail to implant, strongly implicating the cytokine in maternal control of implantation.

Colony stimulating factors (CSF) are a family of proteins (GM-CSF, CSF-1, G-CSF, and IL-3) that stimulate the cellular proliferation and induction of terminal differentiation of hemopoietic progenitor cells. CSF-1 is expressed in the uterine endometrium of the mouse and human during early pregnancy and its receptor, fms, is present on trophoblast. The osteopetrotic mouse, which represents a natural "knockout" of the CSF-1 gene, exhibits a low rate of fetal implantation and poor fetal viability. It seems likely that CSF-1 expression by the uterus influences growth and differentiation of the placenta.

Although different species may utilize different strategies for ensuring developmental and endocrinological coordination between the embryo and the mother, these three examples illustrate that cytokines are likely to be major participants as autocrine factors that direct the events of early pregnancy and not simply as modulators of the maternal immune system.

Key words: cytokines pregnancy interferon prostaglandins leukemia inhibitory factor LIF colony stimulating factors

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INTRODUCTION

Cytokines, which are normally produced by the cells of the immune system, also regulate the differentiation and proliferation of lymphocytes and macrophages (1, 2). Recent studies have shown that cytokines are also expressed in a variety of non-immune cell types, including the reproductive system (3-7). The first part of the review focuses on the role played by Type I interferons (IFN) in maternal recognition of pregnancy in cattle and sheep. The discovery of trophoblast interferons as antiluteolytic substances in cattle and sheep provided the first unequivocal demonstration of a role for any cytokine in a reproductive process. In the second part of the review, we discuss how the maternal system may control implantation in the mouse via endometrial expression of leukemia inhibitory factor (LIF) and colony stimulating factor-1 (CSF-1).

Rescue of the corpus luteum

In most mammals the lifespan of the corpus luteum (CL) must be extended during pregnancy so that progesterone production will be continued. This steroid hormone acts on the endometrium of the uterus to maintain an appropriate environment for the developing conceptus. The prevention of luteal regression is crucial and depends upon signals that originate from the conceptus. In primates, chorionic gonadotropin secreted by the trophoblast provides the luteotropic support to the CL during early pregnancy (8). In the absence of a luteotropic stimuli, the CL degenerates and a new follicle develops and eventually ovulates. Hysterectomy (removal of the uterus) in ruminants, but not in primates, extends the life span of the corpus luteum for almost as long as normal gestation (9). Thus, the uterus is considered to be the source of the substances that causes luteal regression when the animal is not pregnant. Experiments have shown that uterine release of prostaglandin $F_{2\alpha}$ during the late luteal phase of the estrous cycle is responsible for inducing CL regression in domestic farm animals (10). In ewes, for example, the uterus begins to produce prostaglandin $F_{2\alpha}$ on days 13 and 14 of the 17-day estrous cycle. If the animal is pregnant, the pulsatile release of prostaglandin $F_{2\alpha}$ is abolished and the CL continues to function (Fig. 1) (11).

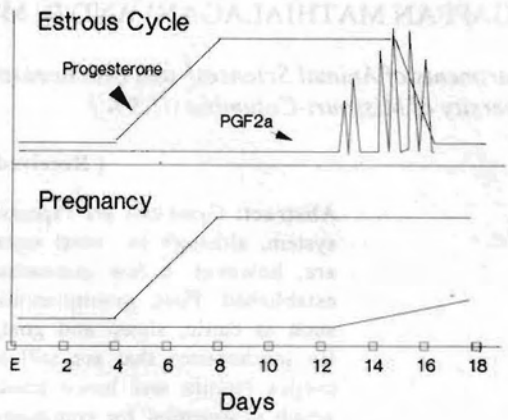

Fig. 1 : A diagram illustrating changes in plasma progesterone and concentration of prostaglandin $F_{2\alpha}$ in the utero-ovarian of ewes during the estrous cycle of pregnancy. Note that during pregancy, although prostaglandin $F_{2\alpha}$ concentrations may rise, pulsatile release from the uterus, which appears responsible for causing the regression of the corpus luterum of the ovary, is diminished. This figure is reproduced from ref. 36 with permission.

Trophoblast protein-1 secreted by the conceptus is the antiluteolytic signal in ewes and cattle

The conceptus product(s) involved in the rescue of CL in ewes must be produced around days 12-13, because embryo transfers to nonpregnant ewes up to that time lead to rescue of the CL. Removal of the embryos from pregnant ewes after day 14 delays a return to estrous (12). In addition, intrauterine infusion of day 14 to 15 conceptus homogenates can extend the luteal function in nonpregnant ewes by several days and occassionally by weeks (13). The active ingredient in homogenates is sensitive to heat and proteases and is not present in conceptuses much older than 20 days of age (14,15). In 1982, a protein was identified that is secreted into the medium when sheep conceptuses are cultured *in vitro* (16). The synthesis of this protein appears limited to the critical day 13-21 period when maternal recognition of pregnancy is mediated. This protein, when injected into uterine lumen of nonpregnant ewes between days 12 and 18, extends the CL function and prolongs progesterone secretion (17). The protein

was named ovine trophoblast protein-1 (oTP-1) and consists of at least four isoforms of Mr ~ 19,000 with pI 5·3 to 5·8. It is secreted maximally between days 13 and 21 and is the major translation product of conceptus during this period (18). oTP-1 is present in the uterine flushings of pregnant, but not nonpregnant ewes, and it has not been detected by immunoassay in maternal blood (19).

In cattle, maternal recognition of pregnancy is initiated at around day 15 (9), and at this time the bovine conceptus, like that of the sheep at day 13, begins to secrete large quantities of a protein which is immunologically related to oTP-1 and has become known as bovine trophoblast protein -1 (20). The bTP-1 consists of multiple isoforms of Mr 22,000 and 24,000. Cell-free translation studies and experiments in which either carbohydrate is removed from bTP-1 or its glycosylation is inhibited have shown that the bTP-1 polypeptide minus its carbohydrates is about the same size as oTP-1 (21). Intrauterine infusion of bTP-1 into cyclic cows between days 16 and 21 significantly extends the length of the interestrus interval, presumably by influencing the output of the uterine prostaglandin $F_{2\alpha}$ (22, 23).

The trophoblast proteins oTP-1 and bTP-1 are structurally related to Type I interferon

Molecular cloning and sequencing of oTP-1 and bTP-1 has shown that both proteins are represented by multiple mRNA whose sequences are very similar (>85% identity), with high a degree of conservation present throughout the molecules (24, 25). The mRNA for oTP-1 or bTP-1 is about 1 kb in length and possesses a 585-base open reading frame that codes for a polypeptide 195 amino acids in length. Each polypeptide was predicted to contain a 23-residue signal sequence, with residue 1 of the mature protein being cysteine. This prediction has been confirmed by amino acid sequencing (26, 27).

Surprisingly, both oTP-1 and bTP-1 cDNA have been shown to share sequence identity to Type I interferons (IFN-α and IFN-β). The trophoblast protein cDNA have about 30% nucleotide sequence identity with IFN-β and about 55-65% identity with a series of cloned cDNA and cloned genomic DNAs for IFN-α which code for mature secreted polypeptides of either 165 or 166 residues in length. However, the greatest degree of oTP-1 and bTP-1 nucleotide sequence identity (>80%) is with genes representing 172 amino acid residues, "long" bovine IFN. Such IFN have been categorized as either IFN-α_{II} (in contrast to the 165 to 166 residue IFN-α_I) or IFN-omegas (IFN-ω). The latter term is now the one recommended. The trophoblast proteins, due to their structural similarity to clear distinctiveness from Type I IFN, have been named IFN-τ (28, 29).

Biological properties of IFN-τ

Like other Type I IFN (IFN-α, IFN-β IFN-ω), IFN-τ also exhibit antiviral, antiproliferative and immunomodulatory properties (30). Daily intrauterine infusion of natural of recombinant preparations of IFN-τ into nonpregnant ewes has been shown to extend the estrous cycle length from days to weeks relative to control ewes (31-33). As previously pointed out, IFN-τ inhibit the pulsatile production of prostaglandin $F_{2\alpha}$, the uterine luteolysin, from the endometrium of cattle and sheep, thereby rescuing the corpus luteum of early pregnancy. The uterine endometrium of sheep and cattle possess receptors of IFN-τ appear to be identical to the ones that bind other Type I IFN and have roughly similar affinity for their ligands (34, 35). However, it remains to be confirmed whether some unusual properties of IFN-τ, such as their ability to extend the length of the estrous cycle, depend upon interaction with a secondary accessory protein on the receptor complex.

Expression of IFN-τ during early pregnancy

The large-scale production of ovIFN-τ occurs between days 13 to 21 when the conceptus is undergoing rapid morphological transformation from a spherical to an elongated form (Fig. 2). Transcripts for ovIFN-τ are detectable by in situ hybridization in days 10 and 11 spherical blastoysts, but increase markedly (5- to 10-fold) in concentration per cell by day 13. The concentration of mRNA then declines slightly by day 15 and precipitously thereafter, as judged by northern blot analysis (36-38). BoIFN-τ displays a pattern of expression similar to that of ovIFN-τ with synthesis peaking between days 17 and 19, thereby reflecting the comparatively later time frame of maternal recognition of pregnancy in cattle (38). This transient production of IFN-τ suggests that the initiation of

expression is triggered either by an autonomous developmental change within the trophoblast cells themselves or by an external stimulus provided at the appropriate time within the uterine environment.

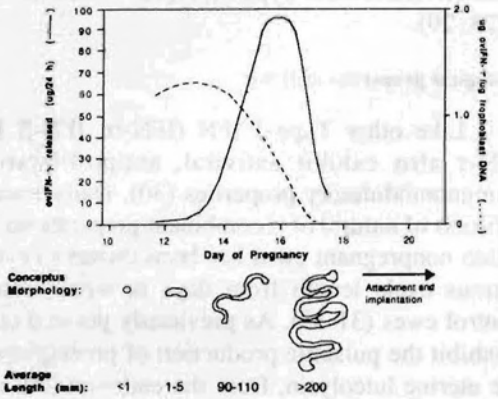

Fig. 2 : A diagram showing the relationship between day of ovine conceptus development and 1) amount of ovIFW-τ produced when individual conceptuses were placed in culture (S), 2). The amount of ovIFN-τ produced per μg of trophoblast DNA (broken line), and 3) the morphological changes of the conceptus during the preimplantation period (from ref. 56).

Bovine IFN-τ secretion (monitored by antiviral activity) and appearance of transcripts (detected by RT-PCR) can first be observed at the blastocyst stage when embryos (derived by *in vitro* fertilization procedures) begin to expand and hatch from the zona pellucida (39, 40). These observations suggest that the onset of boIFN-τ expression is a genetically programmed event. Although such *in vitro*-produced blastocysts continue to secrete boIFN-τ in small amounts for a few days, they fail to attach to the culture dish and form outgrowths and soon lose structural integrity. By contrast, when *in vitro*-dervied blastocysts are transferred to the uteri of synchronized cows, recovered 4 days later, and then cultured, they attach, form outgrowths and begin to produce large amounts of boIFN-τ (39). Therefore, while the onset of IFN-τ expression may be developmentally or regulated, some factor or factors in the luteal phase environment of the maternal uterus is conceivably required for full expression of the IFN-τ or to promote trophoblast growth and development, which in turn leads to increased IFN-τ production. In this regard, it has been noted that addition of GM-CSF (41), IGF-1 and IGF-2 (42) to the conceptus cultures promotes the increased synthesis and secretion of IFN-τ. Endometrial expression of GM-CSF mRNA during this period has also been shown in sheep uterus by *in situ* hybridization experiments (41).

Genes of IFN-α, -β, -ω, and -τ and their transcriptional control

Several Type I IFN genes of human (28), pigs, (43), sheep (44, 45) and cattle (28, 46, 54) have been cloned. They possess considerable sequence identity with the IFN-α genes upstream of the transcription start site (Fig. 3), and so it has been assumed that IFN-α and -ω genes share common transcriptional regulatory mechanisms. The transcriptional regulation of IFN-β has been intensively studied because it is represented in mouse and human by a single copy gene that is inducible in cultured fibroblasts (47). The transcriptional control of IFN-α and -β genes requires only the promoter region up to position 120 above the transcriptional start site for high levels of virus induction (48, 49). Within this region there are at least four overlapping but distinct positive regulatory consensus sequences (GAAANN) that have been implicated in virus inducibility (Fig. 3). Interferon regulatory factor-1 (IRF-I) IRF-2, NF-κB, Oct-1 and -2 are some of the transcription factors shown to interact with the purine-rich region of the IFN promotor (50 - 52). Type I IFN genes generally appear

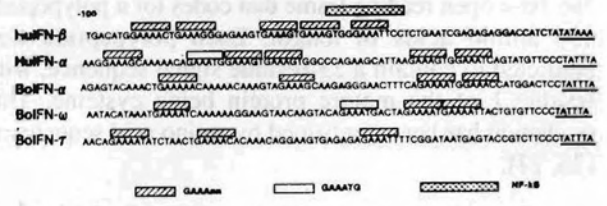

Fig. 3 : A comparison of the promoter regions for Type I IFN genes. The sequences upstream of the TATA box for human, IFN-β and IFN-α1 for the boIFN-ω1 and for the boIFN-τ genes are aligned. The position of the hexamers with the sequences AAATGA (which is thought to bind the transcription factors IRF-1 and IRF-2), GAAANN, GAAATG, and a motif that binds the NF-kB transcription factors are indicated by shaded rectangles (from ref. 56).

silent until induced by virus, and thus play a major role in host defense against viral infection (53). A range of other factors, including cytokines can also act as inducers in certain cell types (53). Even though primary regulation of IFN-α and -β is transcriptional, expression is variably transient, rarely lasting more than a few hours, even in continuous presence of inducing agent (49). Unlike IFN-α and -β, IFN-τ is weakly inducible by virus and constitutively expressed by the trophoblast over a period of 7 to 8 days.

Genomic sequences have now been reported for the IFN-τ of cattle (54), sheep (45, 55), goat (45) and musk ox (45), and of which are related species within the *Bovidae* family. There are minimum of four to five genes in each of the above species, except the musk ox which may only possess two (45). The proximal promoter regions of all the genes are highly conserved, both within and across species. Unlike other Type I genes, which usually start to diverge markedly from each other beyond about 120 bases upstream of the transcription start site, sequence similarities in the IFN-τ persist up to at least base position - 400, both within and across species (55, 56), suggesting that more distal regions may be important for promoter regulation. Though IFN-τ expression is trophoblast-specific, a comparison with other trophoblast-specific gene promoters (αCG and placental lactogen) reveals no resemblance to -450 bp of the IFN-τ promoter. Based on these observations, IFN-τ genes appear to have different transcriptional regulatory mechanisms than other Type I IFN and known trophoblast specific products (56).

Transfection experiments with IFN-α, -β and -ω genes have shown that 120 bp of the 5' promotor region are sufficient to drive viral responsiveness. While other Type I IFN genes are expressed in a variety of cell types, IFN-τ expression appears to be trophoblast specific. Transfection experiments with IFN-τ gene promotor-reporter gene constructs in several cell lines showed that only the trophoblast derived cell lines, JAR and BeWo (human choriocarcinoma cell lines), support the constitutive expression of the reporter genes. IFN-τ promotor base position - 126 from the transcription start site can increase the expression of human GH reporter gene 4- to 5-fold, relative to promoterless control. The upstream sequences between -126 and -450 possess additional enhancer activity (57). These results contrast with similar studies carried out with IFN-α and IFN-β genes, where sequence upsteam of about -120 appeared to be dispensable for conferring viral induction (48). Mapping of the spcific sequences and charactertization of the transcription factors that are involved in IFN-τ gene expression are currently under investigation.

Expression of IFN by other species during early pregnancy

Interferons are expressed by the conceptus and placental tissues of mice (58), pigs (59, 60, 61), horses (59) and rabbits (59). However, in species outside the *Bovidae* family, there is no evidence that Type I IFN have an antiluteolytic role. In addition, the amount of IFN expression in ruminant conceptuses is very much higher than the amount detected in other species. Perhaps the most interesting reports have been in the pig where antiviral activity, as in cattle and sheep, becomes detectable at the time of blastocyst expansion and elongation, *i.e.* around day 12. In this case, Type II IFN (IFN-γ) is responsible for a large part of the activity, but a novel Type I IFN is also produced simultaneously (60 - 62). However, infusion of total conceptus secretory proteins into the uteri of nonpregnant pigs during the same period that the IFN is produced does not lead to an extension of the estrous cycle (63).

In summary, the major proteins secreted by the preimplantation trophoblast of domestic ruminant species during the period of maternal recognition of pregnancy are Type I interferons. Their expression appears to be developmentally regulated. These trophoblast IFN, known as IFN-τ modulate the uterine release of luteolysin, prostaglandin $F2_{\alpha}$. They are structurally related to the IFN-α, -β and -ω with which they share many features, including an ability to confer protection against viral lysis, to inhibit cellular proliferation and to modulate the activities of immune cells. Although IFN production by the trophoblast is noted outside the non-ruminant mammals, there is no evidence that such IFN have any antiluteolytic function.

Cytokine production by the uterus

Uterine endometrium and its secretions are postulated to provide an environment that is conducive for the embryo implantation and development (64).

Glandular and luminal epithelium of the uterine endometrium produce several cytokines and growth factors in response to ovarian steroids (6, 65, 68). Estradiol and progesterone have also been reported to enhance the infiltration of leukocytes into the uterus (69-72) which in turn produce cytokines. In mice, the uterine luminal content of GM-CSF and IL-6 increases following mating (6, 73). Glandular epithelial cells of human endometrium express IL-6, TNF-α, CSF-1, EGF, TGF-α and PDGF, and also have receptors for PDGF and EGF (74-77, 84). Of the cytokines produced by the mouse uterus, leukemia inhibitory factor (LIF) and colony stimulating factor-1 (CSF-1) have been shown to influence the implantation of the mouse embryo.

Leukemia inhibitory factor

LIF or differentation inhibitory activity is a glycoprotein that occurs in various molecular weight forms (M_r 32,000 to 67,000) that have been purified from conditioned medium of wide variety of cell types (78). Mouse and human LIF cDNA have been cloned, and the availability of recombinant LIF has made it possible to study its biological role in various systems. LIF has multiple activities on cells. For example, it inhibits the differentation and proliferation of embryonic stem cells in vitro, regulates the differentation and proliferation of certain hematopoietic cells lines, and induces acute phase response in hepatocyte cultures (78).

The observation that LIF inhibits embryonic stem cell differentiation in virtro suggests that it might also play a role in regulating growth and development of early mouse embryos. LIF is expressed in low amounts in many different tissues, but the highest level of LIF mRNA epxression occurs in uterine endometrial glands. This expression of LIF is transient and occurs on day 4 of pregnancy, which coincides with the onset of implantation period in the mouse (79). During experimentally-induced, delayed implantation (bilateral ovariectomy at day 3 of gestation followed by progesterone treatment), embryos develop to the blastocyst stage and remain viable but do not implant. A single intraperitoneal injection of estradiol into the mother overcomes this delay, resulting in implantation. LIF mRNA has been detected only in the estradiol treated delayed mice (79). LIF expression is also noted in pseudopregant mice (produced by mating with vasectomized male mice) on day 4 of gestation. These observations suggest that LIF expression is under maternal control and possibly produced as a direct response to estrogen from the ovaries during day 3-4 of pregnancy (79).

Female mice lacking functional LIF gene are fertile, but blastocysts fail to implant (80). Homozygous mice lacking both LIF gene loci but provided with recombinant LIF delivered by intraperitoneally placed micro-osmotic pumps, exhibit normal implantation (80). Therefore, LIF produced by the maternal system seems to play a key role in the onset of implantation, despite the fact that blastocysts themselves also express small amounts of LIF (81). It is known that in mice and rats (and most probably in other animals as well) the maternal endocrine system prepares the uterine endometrium for implantation only during a short period following ovulation (the so called "window" of implantation), after which the endometrium becomes refractory. It seems likely that expression of LIF by the endometrium is a vital component of this phenomenon.

Colony stimulating factor-1 (CSF-1)

Colony stimulating factors (CSF) have been identified by their ability to stimulate the formation of colonies of differentiated cells in semisolid agar cultures of bone marrow progenitor cells (82). Granulocyte-macrophage CSF (GM-SCF), multi-CSF or interleukin-3 (IL-3), macrophage-CSF (M-CSF or CSF-1) and granulocyte-CSF (G-CSF) are the four major glycoproteins that have been characterized from mouse and human on the basis of their different biochemical properties and specificities for target cells (82-84). Activated macrophages, fibroblasts, bone marrow stromal cells and the endometrium of pregnant uterus (in mice and human) are the main source of CSF-1.

CSF-1 is a homodimer glycoprotein, (Mr 50,000 to 70,000) whose identical subunits are linked by disulphide bonds. Each subunit has a transmembrane domain at its carboxy terminal end, suggesting that some CSF-1 can be expressed on the cell surface (65, 84). Multiple transcripts are found for CSF-1 in fibroblasts and uterine decidual cells. Therefore, the mRNA for CSF-1 is probably subjected to alternative splicing (66, 84). Specific high affinity receptors for CSF-1 have been identified in several cell lines and on normal hemopoetic tissues (85, 86). The receptor is a

transmembrane tyrosine kinase and identical to c-fms, a proto-oncogene.

The expression of CSF-1 and its receptor (c-fms) at the maternal-fetal interface during pregnancy has been reported in mouse and human (85, 86). During pregnancy the uterine concentration of CSF-1 increases 1000-fold in mice (84). The glandular and luminal epithelial cells (maternal decidua) are the primary sources of this cytokine. An enhanced expression of CSF-1 mRNA is also observed following estradiol and progesterone treatment of ovariectomized mice (65). The receptor protein, c-fms, is primarily found on the surface of invading placental trophoblasts in mice (cells of ectoplacental cone), but also on preimplnatation embryos. Because the immediate precursor of soluble CSF-1 is a transmembrane glycoprotein found on the uterine epithelium, a complex between c-fms and CSF-1 may be implicated in cell - cell adhesion and implantation (85). A similar model has been proposed for TGF-α (a common ligand for EGF receptor), which is expressed on the uterine upithelium, and the EGF receptor, which is expressed on the surface of the mouse blastocyst (87, 88).

The osteopetrotic (op-/op-) mouse has a mutation in its CSF-1 gene and lacks CSF-1 (89). Homozygous mutant males have a near normal fertility when mated with op/+ heterozygous females, but when op-/op- males are mated with op-/op- females, no offspring are produced. Thus, CSF-1 expression by the mouse uterus, like LIF, may play a critical role in implantation (85). Interestingly, however, op-/op- female mice mated with heterozygous males exhibit a low rate of implantation and an increase in fetal resorption, suggesting that there may be some compensatory mechanisms that help to circumvent the lack of CSF-1 in such embryos during the period of implantation (85).

CSF-1 expression occur in human endometrium during the first trimester and increases 3-fold by the third trimester (84). As in the mouse, the receptor (c-fms) expression in primarily restricted to trophoblasts (86). Although CSF-1 is expressed in greater quantities in the decidua, human placenta also produces (CSF-1 (90). Addition of CSF-1 and other cytokines such as IL-1 and IL-6 stimulate the secretion of hCG from cultured human trophoblasts (66,91,92). IL-1 enhance the secretion of hCG by inducing the production of IL-6 from trophoblasts (91). These studies clearly establish CSF-1 as a component of a cytokine network in the placenta which influences its endorine functions.

In summary, cytokines play an important role as a local mediators in reproduction. In addition to a likely role in immunomodulation, they also function in maternal recognition of pregnancy, control of implantation and regulation of endocrine functions of the placenta.

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