Molecular Human Reproduction Vol.9, No.9 pp. 535–540, 2003 DOI: 10.1093/molehr/gag068

Macrophage inhibitory cytokine 1 in fetal membranes and amniotic fluid from pregnancies with and without preterm labour and premature rupture of membranes

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The placenta and fetal membranes are the site of expression of macrophage inhibitory cytokine (MIC-1), a member of the transforming growth factor (TGF)-β superfamily. We hypothesized that MIC-1 may act as an immune regulator in pregnancy complications associated with intrauterine inflammation. Decidual cells, chorionic trophoblasts and amnion epithelial cells were identified by immunohistochemistry as the predominant MIC-1-containing cell type in term membranes. Amnion and choriodecidual explants all produced MIC-1 in culture, the latter having the greatest production rate (206 ± 74.5 pg/mg tissue/24 h, *n* = 6; mean ± SEM). Production was not responsive to stimulation by pro-inflammatory cytokines. MIC-1 was detectable in 217 transabdominal amniotic fluid (AF) samples taken from 15 to 41 weeks gestation, concentrations ranging from 0.9–51.1 ng/ml. AF MIC-1 concentrations in pregnancies with premature rupture of membranes (PROM) or preterm labour, either with or without microbial invasion of the amniotic cavity, were not significantly different from those delivered at term either with or without labour. Treatment with MIC-1 (0.25–25 ng/ml) did not alter production of interleukin-6 or -8 by amnion or choriodecidual cells *in vitro*. We conclude that AF MIC-1 is derived from the fetal membranes and decidua, but that MIC-1 is unlikely to be involved in the pathophysiology of preterm birth or PROM.

Key words: amniotic fluid/MIC-1/pregnancy/premature rupture of membranes/preterm birth

Introduction

Macrophage inhibitory cytokine-1 (MIC-1) was identified and cloned on the basis of increased production with macrophage activation (Bootcov *et al*., 1997). The MIC-1 gene encodes a secreted 25 kDa protein with the structural characteristics of the transforming growth factor (TGF)-β superfamily, possibly representing the first member of a new sub-family (Bootcov *et al*., 1997). The most abundant source of MIC-1 mRNA transcripts is the human placenta, with levels of expression higher in the first trimester placenta compared with the placenta at term (Fairlie *et al*., 1999). Concentrations of MIC-1 in maternal serum increase with advancing gestation in normal pregnancies (Moore *et al*., 2000) but are unchanged with onset of normal spontaneous labour at term (Marjono *et al*., 2003). Its function in pregnancy remains unknown, although an immunomodulatory role has been proposed (Fairlie *et al*., 1999). MIC-1 is expressed in macrophages in response to a number of different activation agents, including interleukin (IL-1β) and tumour necrosis factor α (TNF-α). Purified recombinant MIC-1 was originally reported to inhibit lipopolysaccharide (LPS)-induced macrophage TNF-α production (Bootcov *et al*., 1997) although subsequent studies indicated that this effect was poorly reproducible and may not be real (Fairlie *et al*., 1999). Administration of TGF-β to pregnant rabbits has been shown to prevent LPS-induced preterm birth (Bry and Hallman, 1993), suggesting that this and related cytokines such as MIC-1 may have potentially important immunomodulatory roles in the uterus in the context of normal and abnormal parturition.

The present study was aimed at testing the premise that altered levels of MIC-1 release from the gestational membranes might occur with preterm labour and may play a role in the pathophysiology of the syndrome. To test this hypothesis, we performed immunohistochemical studies on gestational membranes, measured MIC-1 production by these tissues *in vitro*, and assessed MIC-1 concentrations in amniotic fluid (AF) samples from women with term and preterm labour, with or without premature rupture of membranes (PROM). The ability of MIC-1 to regulate decidual and amnion cytokine production *in vitro* was also examined.

Materials and methods

Materials

Culture medium (Ham's F-12/Dulbecco's modified Eagle's medium) was obtained from Irvine Scientific (USA). Fetal calf serum (FCS) was purchased

from Life Technologies Ltd (NZ). Bovine gamma-globulin (BGG) and bacterial lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (USA). Cytokines and anti-IL-6/-8 antisera were obtained from R&D Laboratories (USA). The donkey anti-goat IgG and anti-sheep IgG peroxidase conjugates were purchased from Jackson ImmunoResearch Laboratories Inc. (USA). Streptavidin biotin–alkaline phosphatase was purchased from Dako A/S, Denmark. Maxisorb plates for the IL-6/-8 assays, and disposable plastic tissue culture consumables were supplied by Nalge Nunc International, Roskilde, Denmark.

Recombinant human MIC-1 and MIC-1 antibodies

Recombinant MIC-1 was expressed in the yeast *Pichia pastoris* and purified to homogeneity using a multi-step procedure culminating in reverse phase HPLC as described (Moore *et al*., 2000). Sheep anti-MIC-1 polyclonal antibody (PAb) 233B3 was raised against recombinant human MIC-1 (rhMIC-1). Mouse anti-MIC-1 monoclonal antibody (MAb) 26G6H6 was generated from mice immunized with rhMIC-1 (Fairlie *et al*., 2001).

MIC-1 enzyme-linked immunosorbent assay (ELISA)

The MIC-1 sandwich ELISA was performed as previously described (Moore *et al*., 2000; Brown *et al*., 2002). MAb 26G6H6 was used for antigen capture and the sheep PAb 233B used for detection. The assay was calibrated against rhMIC-1 and had a limit of detection of ~8 pg/ml. Inter-assay and intra-assay precision were <5 and <11%, respectively.

Immunohistochemistry

Full thickness gestational membranes from term placenta delivered by Caesarean section prior to the onset of labour were fixed in 4% paraformaldehyde and embedded in paraffin. After cleaning and de-waxing in xylene/graded alcohols, sections (7 µm) were subjected to antigen retrieval (boiling for 20 min in 0.5 Tris–HCl pH 10) then blocked for 20 min with 1% H_2O_2 in 50% methanol. Antisera (1:2000 sheep anti-MIC-1 233B3) was applied to the sections in phosphate-buffered saline (PBS)/0.5% Tween-20/5% normal horse serum and incubated overnight at 4°C; negative controls were incubated with non-immune sheep serum. Following washing in PBS/0.5% Tween-20, immunodetection was accomplished using peroxidase-conjugated anti-sheep IgG (1:400) developed with diaminobenizidine (DAB). Sections were dehydrated and mounted, in some experiments after haematoxylin counterstaining to visualize cell nuclei. Digital photomicrographs were taken using a Nikon Eclipse E800 microscope fitted with a JBC TK-C1381 colour video camera. Negative images are displayed to enhance visual clarity (×200 and ×1000).

Amniotic fluid collection

AF samples were collected by transabdominal amniocentesis. All women provided informed consent prior to the collection of AF. The collection and use of AF was approved by the Human Investigation Committees of participating institutions (i.e. Wayne State University, Hutzel Hospital, MI, USA; Sotero del Rio Hospital, Puente Alto, Chile), and approved for research purposes by the IRB of the National Institutes of Child Health and Human Development. Several studies utilizing these fluids have been published recently which include full details of their collection and clinical categorization (Athayde *et al*., 1999, 2000). In brief, samples were divided into three main groups. Group 1 consisted of women with preterm labour and intact membranes subdivided into the following categories: (i) preterm labour without microbial invasion of the amniotic cavity (MIAC) who delivered at term (n = 29); (ii) preterm labour without MIAC who delivered preterm (<37 weeks) (n = 31); and (iii) preterm delivery with MIAC (n = 19). MIAC was defined as positive AF culture of aerobic/anaerobic bacteria or genital mycoplasmas (Athayde *et al*., 1999). Group 2 consisted of women with preterm PROM, either with (n = 29) or without (n = 43) MIAC. Group 3 consisted of women with intact membranes and term deliveries (i.e. >37 weeks of gestation) without MIAC, either before labour onset (n = 28) or in labour (n = 38).

Explant culture

Production of MIC-1 by gestational membranes *in vitro* was studied using an explant model to maintain tissue integrity and allow an accurate comparison of production rates between tissues. All placentas used in these studies were obtained after Caesarean section at term prior to the onset of labour (indications: previous section or malpresentation). Maternal consent was obtained according to the guidelines of the Auckland Human Ethics Committee. Explant cultures from amnion and choriodecidual tissues were prepared as previously described (Simpson *et al*., 1998, 1999), cultured in 24-well culture dishes in serum-free media (1 ml/well), and treated after a 24 h equilibration period with stimuli for a further 24 h. Media were stored at –20°C prior to assay, then thawed and diluted for assay as appropriate. Explant tissue wet weights (mg/well) were recorded at the end of the experiments for normalization purposes. Concentrations of stimuli (LPS 5 µg/ml, IL-1β 1 ng/ml, TNF-α 10 ng/ml) were based on previous studies using these tissues in which these doses have been shown to exert near maximal stimulatory effects on cytokine and prostaglandin production (Simpson *et al*., 1998, 1999).

Monolayer cell cultures

The concentration-dependent effects of MIC-1 on cytokine production by gestational tissues were investigated using monolayer cultures of amnion and decidual cells. This model exhibits better precision and increased sensitivity to stimuli/modulators compared to tissue explants, and also offers the opportunity to incorporate selective digestion and purification procedures to study a particular cell type. Amnion cells were isolated by collagenase/trypsin dispersion and cultured according to previously published methods (Keelan *et al*., 1997). Experiments were conducted on day 3–4 of culture, when medium was replaced with fresh medium without serum, supplemented with 0.1% (w/v) BGG and test substances added. Using this method, >90% of cells on day 3 of culture stained positive for cytokeratin, an epithelial cell-specific antigen. All cultures were incubated in a humid atmosphere of 5% CO_2/95% air. Four replicate wells per test treatment were used, and experiments were conducted on day 3 of culture over a 16 h treatment period on tissues from at least four different placentas. Media were removed after the treatment period and frozen prior to analysis. Decidual cells were isolated as described previously (Dudley *et al*., 1992; Keelan *et al*., 1998) with minor modifications. Decidual tissue was washed and gently scraped to remove adherent chorion, then cut into small pieces and digested for 2 h at 37°C in 0.012% collagenase in Hanks' balanced salt solution (HBSS). Liberated cells were isolated by centrifugation, then applied to a discontinuous Percoll gradient (10, 20, 30, 40 and 50%) and centrifuged for 20 min at 600 *g*. Cells recovered from the 10–50% interfaces were diluted in media, recovered by centrifugation, and plated out in 24-well culture dishes at 0.3×10^6 cells/ml in M199 media containing 10% FCS and antibiotics. Experiments were carried out on day 3 of culture in serum-free media as detailed above for amnion cultures. At the completion of experiments, cellular protein was measured using the bicinchoninic acid (BCA) method (Redinbaugh *et al*., 1986) with bovine serum albumin as standard.

Cytokine immunoassays

IL-6 and IL-8 were assayed by two-site ELISA using commercially available capture and detection antisera (R&D Systems, USA) with horseradish peroxidase detection and *O*-phenylenediamine (OPD) as a substrate as previously described (Keelan *et al*., 1997). Production rates were expressed as pg/µg cellular protein/24 h.

Statistical analysis and presentation of data

Production of MIC-1 in explant culture was derived as pg/mg wet weight tissue/24 h and expressed as % control to allow the results of multiple experiments to be pooled and analysed collectively. Similarly, production by amnion and decidual monolayer cultures was expressed as pg/µg cellular protein/16 h. Statistical significance was assessed by analysis of variance followed by Dunnett's test or Bonferroni *t*-test *post hoc*. Differences in AF concentrations of MIC-1 were assessed by Kruskal–Wallis test for non-parametric data. $P < 0.05$ was considered to be statistically significant.

Results

Production of MIC-1 by explants

Choriodecidual explants produced readily detectable amounts of MIC-1 over 24 h in culture, with concentrations in conditioned medium ranging from 4.2 to 42.0 ng/ml. Concentrations in amnion explant-

conditioned medium were approximately two orders of magnitude lower (<10–437 pg/ml). When normalized to weight of tissue, 24 h MIC-1 production rates were ~50-fold higher in choriodecidual explants (206 ± 74.5 pg/mg tissue/24 h, n = 6; mean ± SEM) compared to amnion (4.28 ± 2.2 pg/mg tissue/24 h, n = 5) (Figure 1). Production was not responsive to stimulation by LPS (5 μg/ml) or pro-inflammatory cytokines IL-1β (1 ng/ml) or TNF-α (10 ng/ml), although production of IL-6 was increased significantly by these agents in these experiments (data not shown).

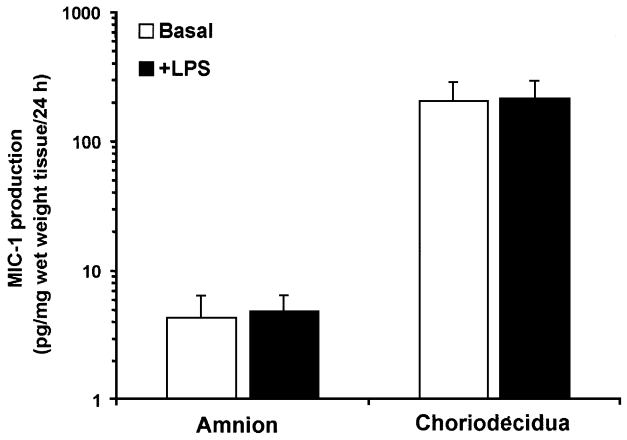

Figure 1. Production of macrophage inhibitory cytokine-1 (MIC-1) *in vitro* by amnion and choriodecidual explants. Explants were prepared from term gestational membranes as described in Materials and methods and cultured for 24 h in the presence or absence of lipopolysaccharide (LPS) (5 μg/ml). Media concentrations of MIC-1 were measured by enzyme-linked immunosorbent assay and production expressed as pg/mg wet weight tissue/24 h. The data shown are from n = 4 experiments performed in triplicate (mean ± SEM).

Immunolocalization of MIC-1 in gestational membranes

MIC-1 immunoreactivity was present in amnion, chorion and decidual membranes (Figure 2A). Staining was cytoplasmic, although some sporadic nuclear staining was observed in the amnion epithelium. Strong immunoperoxidase staining was observed in chorionic trophoblasts (C) and decidual cells (D); staining of amnion epithelial cells, while distinct, was generally less intense (E). Amnion mesenchymal cells and chorionic reticular cells were also sporadically stained. Staining was absent in negative controls incubated with normal sheep serum (B).

Amniotic fluid MIC-1 levels

MIC-1 was readily detectable in all AF samples (n = 217), with concentrations ranging from 0.9 to 51.1 ng/ml. Median AF MIC-1 concentrations in women presenting with preterm labour were independent of gestational age at delivery or MIAC (Table I). AF MIC-1 concentrations were also similar in pregnancies complicated by PROM, either at term/preterm or with/without MIAC (Table I). MIC-1 concentrations in pregnancies sampled in preterm labour were

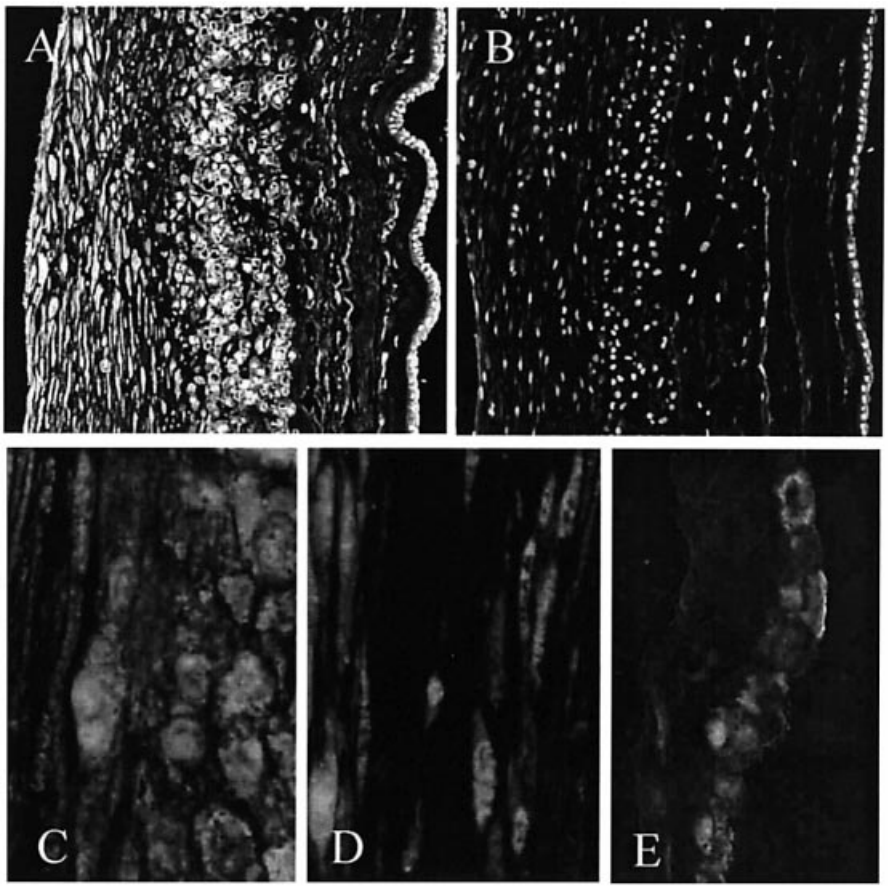

Figure 2. Immunohistochemical localization of macrophage inhibitory cytokine-1 (MIC-1) in term gestational membranes. Immunoperoxidase staining of full thickness term gestational membranes (prior to labour) was performed after antigen retrieval using a sheep polyclonal anti-MIC-1 antibody (**A**, **C**, **D**, **E**) or non-immune sheep serum (**B**) as a negative control. Tissues in **A** and **B** (full thickness membranes ×200 magnification) were counterstained to allow visualization of nuclei. **C**, **D** and **E**: chorion, decidua and amnion epithelium at ×1000 magnification without counterstaining. Images are shown as negatives to enhance visual clarity.

Table I. Amniotic fluid macrophage inhibitory cytokine-1 (MIC-1) concentrations [median (range)] in term and preterm pregnancies with or without preterm labour, microbial infection of the amniotic cavity (MIAC) or premature rupture of membranes (PROM)

Gestational group	*n*	Gestational age at amniocentesis	*P**	MIC-1 concentrations (ng/ml)	*P**
Preterm labour			NS[a]		NS[b]
Term delivery	29	29.3 (21.2–34.6)[a]		9.5 (0.9–38.3)[b]	
Preterm delivery	31	29 (20–34)[a]		15.2 (1.1–28.6)[b]	
+ MIAC	19	27 (20–36)[a]		15.5 (1.4–42.9)[b]	
Preterm PROM			NS[c]		NS[d]
No MIAC	43	30.6 (22–35.5)[c]		15.1 (3.0–31.2)[d]	
+ MIAC	29	29 (21.4–35.4)[c]		10.3 (3.5–51.1)[d]	
Term delivery			NS[e]		NS[f]
No labour	28	38.5 (37–41.5)[e]		8.8 (1.4–35.9)[f]	
In labour	38	38.3 (37–41.5)[e]		7.5 (1.7–25.3)[f]	

All values are shown as median and range.
*Non-parametric statistical analysis was performed on groups designated by common suffix.
NS = not significant.

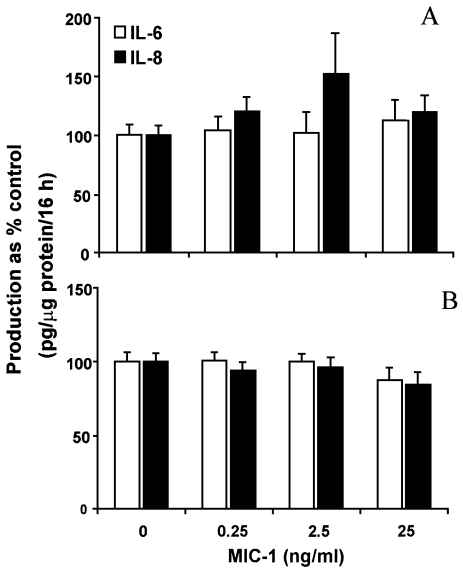

Figure 3. Effects of macrophage inhibitory cytokine-1 (MIC-1) on production of interleukin (IL)-6 and IL-8 by decidual cells *in vitro*. Decidual cells were isolated and cultured as described in Materials and methods, and treated with MIC-1 over 16 h at the concentrations indicated in the presence (**B**) or absence (**A**) of lipopolysaccharide (LPS) (5 μg/ml). Production of IL-6 (open bars) and IL-8 (solid bars) was determined by enzyme-linked immunosorbent assay, expressed as pg/mg protein/16 h, and normalized to control. The data shown are derived from $n = 3$ experiments performed in quadruplicate (mean ± SEM). Production rates of IL-6 and IL-8 (mean ± SEM) in the presence of LPS were 27.0 ± 5.2 and 566.8 ± 137.2 pg/μg protein/16 h, respectively (2- to 3-fold greater than baseline controls).

not significantly different from those sampled during term labour, and there was no evidence of a correlation between AF MIC-1 concentrations and gestational age at amniocentesis.

MIC-1 effects on cytokine production

Decidual cells grown in monolayer culture were stimulated for 24 h with 5 μg/ml LPS (decidua) in the presence of MIC-1 (0.25, 2.5, 25 ng/ml) or vehicle control. While production of both IL-6 and IL-8 was stimulated by LPS (3- and 2-fold, respectively), MIC-1 had no significant effect on their production under either basal or stimulated conditions (Figure 3). Similarly, production of IL-6 and -8 by amnion cells under both basal and IL-1β-stimulated conditions was not responsive to MIC-1 (not shown). The cells responded to IL-1β with a 20- and 90-fold increase in IL-6 and IL-8 production, respectively. The presence of MIC-1 in the stock reagent used to treat the cultures was confirmed by ELISA.

Discussion

The present study was founded upon the hypothesis that MIC-1/placental TGF-β may play a role in the pathogenesis of preterm labour. It was prompted by initial findings from a cDNA expression array study (Marvin *et al*., 2002) showing abundant expression of MIC-1 in gestational membranes both at term and preterm. Ample evidence suggests that preterm labour is, in many cases, associated with intrauterine infection and/or elaboration of an inflammatory response in the gestational membranes (Gomez *et al*., 1997). We, and many others, have studied various immunological components of this process, including the presence and role of anti-inflammatory cytokines such as IL-10 and a variety of TGF-β superfamily members (Goodwin *et al*., 1998; Keelan *et al*., 1998, 2000; Simpson *et al*., 1998). We have postulated that some cases of preterm labour might be associated with an over-vigorous inflammatory response arising, perhaps, as a result of inadequate control by local immunosuppressive factors. MIC-1 appeared to be an ideal candidate for such a factor, being expressed in large amounts in the human placenta (which is susceptible to the effects of products of an inflammatory reaction) and reported to be capable of suppressing TNF-α production by LPS-stimulated macrophages (Bootcov *et al*., 1997). To explore this hypothesis we studied the production of MIC-1 by the gestational membranes and the effects of inflammatory mediators, and measured MIC-1 levels in AF to determine the relationship between term and preterm labour and intrauterine MIC-1 concentrations. We also assessed the ability of MIC-1 to suppress amnion and decidual cytokine production.

Amnion and choriodecidua were identified as being sites of production of MIC-1, in support of mRNA expression data derived from cDNA array studies. Choriodecidual tissue was a much greater source of MIC-1 protein than amnion, as might be expected based on the assumption that the trophoblast would be the major site of MIC-1 synthesis (as it is in the placenta). To some extent our immunohistochemical data support this, with strong immunoperoxidase staining being identified in the chorionic trophoblast layer. However, decidual cells were also strongly immunostained and would be expected to contribute a considerable amount to the total MIC-1 production capacity of the membranes. A recent study by Marjono *et al*. (2003), published while this manuscript was under review, reported similar

immunohistochemical findings. In our explant model chorion and decidua are left intact to preserve tissue integrity, since the two layers are interdigitated and difficult to separate efficiently and without damage. Because of this limitation we have not been able to assess the relative MIC-1 production rates of these two tissues. Amnion production of MIC-1 was much lower than the choriodecidua; the levels of production were considerably lower than those of IL-6 or activin A, for example, by amnion explants under similar conditions (Keelan *et al*., 1998; Simpson *et al*., 1999). Surprisingly, production of MIC-1 by the membranes was not regulated by inflammatory stimuli. Production of other TGF-β members by these tissues has been shown to be responsive to the effects of pro-inflammatory cytokines such as IL-1β and TNF-α (Keelan *et al*., 1998), so these negative data indicate that production of MIC-1 in the gestational membranes may be constitutive and, in this regard, atypical.

MIC-1 was measured by ELISA in a large number of AF samples. Levels of MIC-1 in AF were similar regardless of gestational age, in agreement with preliminary data from a previous study (Moore *et al*., 2000); similar concentrations were found in fluids taken preterm (20–36 weeks) or at term. This negative observation contrasts with measurements of maternal serum MIC-1 levels which showed an increase with gestational age (Moore *et al*., 2000). The difference is likely to reflect the different source of MIC-1 in the two compartments, with the placenta contributing the majority of circulating MIC-1, production of which rises as placental size and hormonal production capacity increases during pregnancy. We also found that AF MIC-1 levels were not elevated in pregnancies complicated by MIAC, PROM or preterm labour. Neither did we find any evidence of an increase in AF levels with spontaneous term labour, in accordance with measurements of MIC-1 in maternal serum (Marjono *et al*., 2003). Taken together, these negative data argue against the postulate that MIC-1 plays a role in parturition or PROM, either at term or preterm, although it should be borne in mind that AF levels do not necessarily reflect biological activity in tissues at the site of production. However, these negative findings could still be of significance if MIC-1 exerted a maintenance/protective role in the membranes, as a lack of response by MIC-1 could then result in the elaboration of rampant inflammatory activation. In this light, we assessed the immunomodulatory effects of MIC-1 in amnion and decidua using monolayer models that have been widely used to study concentration-dependent effects of cytokines and other immunomodulators on gestational membranes. The dose range of MIC-1 employed (0.25–25 ng/ml) was chosen to cover the effective concentration range of TGF-β and MIC-1 reported in previous studies (Bry *et al*., 1993; Bootcov *et al*., 1997). Cytokine (IL-6 and IL-8) production in either tissue was not inhibited by MIC-1, under either basal conditions or in the presence of stimulation by IL-1β (amnion) or LPS (decidua). These findings do not lend support, therefore, to the notion that MIC-1 might be involved in maintaining some form of immunological homeostasis in these tissues, although the possibility cannot be excluded that alternative culture/treatment conditions (MIC-1 pre-treatment prior to stimulation, extended incubation times, increased MIC-1 concentrations, tissue explants) might have yielded different findings. It remains to be determined whether MIC-1 plays any role in modulating placental growth or survival, as might be suggested by its reported involvement as a mediator of apoptosis (Kannan *et al*., 2000; Li *et al*., 2000).

In conclusion, we report that MIC-1 is an abundant component of AF, is produced by cells of the amnion, chorion and decidua, yet there is no evidence of an association between MIC-1 and the pathophysiology of preterm labour or premature rupture of membranes. The functional significance of MIC-1 in gestational membranes in late pregnancy remains unresolved.

Acknowledgements

The authors gratefully acknowledge the financial support of the Health Research Council of New Zealand, the National Health and Medical Research Council of Australia, New South Wales Health Research and Development infrastructure grant, and St Vincent's Hospital Sydney, Meriton Apartments Pty Ltd through an R&D syndicate arranged by Macquarie Bank Limited. The generous assistance of the nursing and theatre staff of the National Women's Hospital, Auckland, and the assistance of Miss S Khan with the immunohistochemical data, is also gratefully acknowledged.

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Submitted on February 25, 2003; accepted on May 13, 2003