Amnion-derived cells express intercellular adhesion molecule-1: regulation by cytokines

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ABSTRACT

We have examined the expression of the intercellular adhesion molecule-1 (ICAM-1) mRNA in primary and established amnion-derived cell cultures and regulation of this expression by tumour necrosis factor- α (TNF- α) and interleukin (IL)-1 β . TNF- α (50 ng/ml) and IL-1 β (1.0 ng/ml) induced 18- and 11-fold increases respectively in expression of the ICAM-1 mRNA in WISH cells (an amnion epithelium-derived cell line). The increase was detectable within one hour of treatment and peaked by two hours. The protein synthesis inhibitor, cycloheximide (10 μ g/ml) did not inhibit this induction. Increased levels of ICAM-1 protein were detected in the cells within 4 h after initiation of treatment with either cytokine. By 16 h of treatment with IL-1 β or TNF- α ICAM-1 reached 40 and 73 pg/ μ g cellular protein, representing 6- and

11-fold stimulations respectively. In primary amnion cells, basal expression of ICAM-1 mRNA was undetectable. However, TNF- α (50 ng/ml) induced ICAM-1 mRNA within two hours, peak expression being reached between four and eight hours after initiation of treatment. The present report demonstrates for the first time that amnion derived cells can express ICAM-1 and, further, that this expression is regulated by pro-inflammatory cytokines. This has implications for the amnion as a possible source for soluble ICAM-1, for this gene product as a marker for preterm labour, and for participation of the amnion, additional to its reported secretory role, in inflammatory processes of the fetal membranes.

Journal of Molecular Endocrinology (1999) **22**, 193–205

INTRODUCTION

The principal cause of perinatal morbidity and mortality is preterm delivery (McCormick 1985, Committee to Study the Prevention of Low Birthweight, Division of Health Promotion and Disease Prevention, Institute of Medicine 1985). While preterm delivery occurs at a rate of 10% of live births (Creasy 1993), preterm infants account for approximately 80% of perinatal mortality (Copper *et al.* 1993). Furthermore, those surviving have a 22-fold higher morbidity rate than term infants (Scott & Spiker 1989, Morrison 1990). Thus, studies into the mechanisms of preterm labour address a very significant health problem.

Although drug intervention into the process of preterm labour may permit a significant maturation of the fetal lungs and other organs (Gardner & Goldenberg 1995), success in reducing the incidence of preterm labour has been minimal (Higby

et al. 1993). In addition, clinical diagnosis of preterm labour based on contractions and other symptoms suffers from low positive predictive values and sensitivities (Creasy 1993, Iams *et al.* 1994, Lockwood 1995, Mercer *et al.* 1996). Furthermore, a high percentage (30–50%) of women presenting with apparent preterm labour ultimately deliver at term. Thus, both better understanding of the processes underlying preterm labour and improvement of diagnostic tools are required to assist in the management and treatment of women presenting with preterm labour.

Studies of abnormal localisation or expression of cell adhesion molecules or extracellular matrix proteins show considerable promise in these areas. Fetal fibronectin (fFN) measurement has received the most attention as a diagnostic tool. The presence of this extracellular matrix protein in cervical vaginal fluid predicts preterm delivery among women at high risk but with intact membranes

(Lockwood *et al.* 1993, Inglis *et al.* 1994). Cell adhesion molecules in the intrauterine environment, on the other hand, have principally been studied in relation to implantation and early placentation (Damsky *et al.* 1993, 1994, Kwee *et al.* 1995, Yang *et al.* 1995), or pre-eclampsia and intrauterine growth restriction (IUGR) (Labarrere & Faulk 1995, Lyall *et al.* 1995). Notably, in one such study the concentration of the soluble form of intercellular adhesion molecule-1 (ICAM-1) in the amniotic fluid correlated strongly with shortened gestation and IUGR (Salafia *et al.* 1993).

ICAM-1, an immunoglobulin superfamily cell adhesion molecule, is well known as an inflammatory mediator promoting the adhesion of neutrophils or other leukocytes to the endothelium prior to emigration into inflamed tissues (Springer 1990, 1994, Carlos & Harlan 1994). Like the genes for prostaglandin H synthase 2 (PGHS-2) (Tazawa *et al.* 1994) and the cytokine interleukin (IL)-1 β (Hiscott *et al.* 1993), the ICAM-1 gene has a cytokine inducible promoter (Ledebur & Parks 1995, Roebuck *et al.* 1995). All three promoters contain nuclear factor- κ B responsive elements, which partially mediate this effect (Hiscott *et al.* 1993, Tazawa *et al.* 1994, Ledebur & Parks 1995).

Since secretion of inflammatory cytokines in the intrauterine environment is often associated with preterm labour (Inglis *et al.* 1994), especially in association with intrauterine infection (Romero *et al.* 1989a,b), ICAM-1 expression would be expected in a much broader range of preterm deliveries than so far reported (Salafia *et al.* 1993). Further, since there have been several reports of ICAM-1 expression in epithelia or epithelial cells, particularly during inflammation (Sippy *et al.* 1995, Ciprandi *et al.* 1996, Khair *et al.* 1996, Iwata *et al.* 1997), it is reasonable to suggest that the amnion might also be able to express this gene. In order to test this hypothesis, we have studied the ability of the inflammatory cytokines IL-1 β and tumour necrosis factor- α (TNF- α) to regulate the expression of ICAM-1 in cultures of WISH cells (an amnion epithelium-derived cell line) and primary cultures of amnion cells.

METHODS AND MATERIALS

Cell culture

Primary amnion cell cultures were established as previously described (Keelan *et al.* 1997a). Briefly, placentae were obtained, with informed consent, from women undergoing repeat caesarean section at term prior to labour at National Women's Hospital, New Zealand. The amnions were peeled from the

chorion laeve and dispersed by successive treatment with 0.024% collagenase (Boehringer Mannheim NZ Ltd, Auckland, New Zealand) and 1.2% trypsin (Life Technologies Inc., Gaithersburg, MD, USA). The dispersed cells were plated in 1:1 Ham's F12:Dulbecco's modified Eagle's medium (F12/DMEM, Irvine Scientific, Irvine, CA, USA) supplemented with 10% heat treated fetal bovine serum (FBS, Life Technologies Inc.) at 1.2×10^5 /cm² in 6-cm dishes (Falcon, Becton-Dickenson, Lincoln Park, NJ, USA). Media were changed 24 h after plating and at 48-h intervals thereafter. Ethical approval for collection and use of human placental tissue was obtained from the Ethics Committee of the Northern Regional Health Authority of New Zealand and the North Health Division of the Transitional Health Authority of New Zealand.

WISH cells (Hayflick 1961), a kind gift from Dr Les Myatt, University of Cincinnati, USA, were grown in F12/DMEM supplemented as for amnion cell culture. Media changes were performed every 48 h (Hansen *et al.* 1998). The plating densities were 3×10^5 cells/well of a 24-well culture plate or 3×10^6 of a 6-cm dish. HL60 cells, a kind gift from Dr Kathryn Crosier, University of Auckland, New Zealand, were grown in RPMI 1640 supplemented with 20% heat-treated fetal calf serum at a density of 1×10^6 cells per ml.

Experimental conditions

TNF- α , a kind gift from Dr John Fraser, University of Auckland, New Zealand, and IL-1 β (Immunex, Seattle, WA, USA) were prepared as 100-fold concentrates in 12 mM Na₂HPO₄, 3.9 mM KH₂PO₄, 145 mM NaCl, 1% bovine serum albumin fraction V (BSA) (SERVA, Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany). Cycloheximide (CHX) and retinoic acid (RA), both purchased from Sigma Chemical Co. (St Louis, MO, USA) were dissolved in ethanol at 10 mg/ml and 10^{-5} M respectively. Confluent cultures of amnion (day 5 of culture) or WISH cells were treated with TNF- α or IL-1 β as noted in the figure legends. When used CHX was applied at 10 μ g/ml, 30 min prior to experimental treatment. HL60 cells were treated with 5×10^{-8} M RA for four days to induce differentiation.

Preparation of RNA

Total RNA was isolated by the guanidinium thiocyanate (GTC)-acid phenol method (Chomczynski & Sacchi 1987). Adherent cell cultures were directly scraped in the GTC lysis solution and suspension cells were collected by centrifugation before direct

resuspension in that solution. The RNA was dissolved in formamide (Chomczynski 1992) and stored at -70°C .

Preparation of ICAM-1 cDNA

Reverse transcription PCR (RT-PCR) was used to prepare ICAM-1 cDNA from total RNA isolated from RA-induced HL60 cells. The reported sequence for ICAM-1 cDNA (GenBank Accession g184532, Tomassini *et al.* 1989) was retrieved with the aid of the Wisconsin Package version 9.1, Genetics Computer Group (GCG) (Madison, WI, USA) sequence analysis software. Primer design was performed using Oligo version 4.01 (National Bioscience, Plymouth, MN, USA). The primer sequences are listed in Fig. 1. One microlitre ($0.5\text{ }\mu\text{g}$) RNA and 10 pmol primer P3 were combined in $12\text{ }\mu\text{l}$ diethylpyrocarbonate (Sigma Chemical Co.)-treated milliQ (Millipore, Bedford, MA, USA) purified water. The RNA was denatured for 1 min at 95°C , annealed at 58.5°C for 5 min and transferred to ice. Reverse transcription was performed with 200 U Superscript II RT (Life Technologies Inc.) in a $20\text{ }\mu\text{l}$ reaction at 42°C for 1 h. Primary amplification to produce the 2.9 kb cDNA was performed with the Expand Long Template System (Boehringer Mannheim NZ Ltd) using $1\text{ }\mu\text{l}$ of the reverse transcription reaction and 10 pmol each of primers P1 and P3. After a 2-min denaturation step at 94°C , PCR was performed for 30 cycles of 1 min at 94°C , 1 min at 63°C , and 2 min at 68°C . The initial 2.9 kb product was gel purified and used as the template for re-amplification with P1 and P2 to prepare the 1.5 kb probe or P1 and P3 to prepare the 2.9 kb probe.

Restriction analysis

Restriction endonuclease digests were prepared using 40–60 ng PCR product and one unit of enzyme per reaction. Digestions were performed by incubating for 1 h at the temperature recommended by the enzyme supplier. The restriction endonucleases used, EcoRI, ApaI, KpnI and SmaI, were obtained from Boehringer-Mannheim NZ Ltd. Digestion products were separated on a 1% agarose, Tris-acetate-EDTA buffered gel. Predicted fragment sizes were determined using the Wisconsin Package version 9.1, (GCG) sequence analysis software.

Northern blot analysis

Fifteen micrograms RNA were loaded per lane on 1% agarose, MOPS-formaldehyde gels (Sambrook

et al. 1989). The RNA was transferred from the gels to Gene Screen Plus (NEN Life Science Products, Boston, MA, USA) by the capillary method (Sambrook *et al.* 1989), and crosslinked to the wet membrane by ultraviolet light, 1200 J/m^2 , using a Stratalinker (Stratagene, La Jolla, CA, USA). cDNA probes were labelled with $[\alpha^{32}\text{P}]\text{d-CTP}$ (Amersham International plc, Amersham, Bucks, UK) by random primed synthesis using a Mega Prime Kit (Amersham International plc). Hybridisation was carried out as described (Vollberg *et al.* 1991, Marvin *et al.* 1992) or, where noted, at 68°C in QuikHyb solution (Stratagene). Progressive stringency washing was performed using $2\times$, $0.5\times$ and $0.1\times$ SSC with 0.1% sodium dodecylsulphate (Serva, Boehringer Ingelheim Bioproducts Partnership) at 22, 55 and 60°C respectively (Vollberg *et al.* 1991, Brown & Mackey 1997). Detection was performed by electronic autoradiography at $200\text{ }\mu\text{m}$ resolution using a Storm 860 phosphor imager (Molecular Dynamics, Sunnyvale, CA, USA) or, where noted, $500\text{ }\mu\text{m}$ resolution using an InstantImager (Packard, Canberra, Australia). The manufacturer's image analysis software was utilised for quantitation. The probes used were the 1.5 kb ICAM-1 cDNA (unless otherwise noted) and a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Tso *et al.* 1985). The size of the detected mRNA was estimated from its mobility and those of the components of a 0.24–9.5 kb RNA ladder (Life Technologies Inc.) marker using the GROWTH function of the EXCEL spreadsheet program (Microsoft Corp., Redmond, WA, USA).

Cell lysis and extraction for protein analysis

Cellular extracts were prepared with Reporter Gene Assay Lysis buffer (Boehringer-Mannheim) supplemented with 0.2 mM phenylmethylsulphonyl fluoride (Sigma Chemical Co.), $2\times 200\text{ }\mu\text{l}$ /well of a 24-well culture dish, with the aid of a rubber policeman. This is a hypotonic lysis buffer containing 0.5% Triton-X 100. The lysates were rapidly frozen and stored at -70°C until assayed. Prior to assay the samples were rapidly thawed and centrifuged for 5 min at $15\,000\text{ g}$ at 4°C .

Extracted cellular protein was measured by the bicinchoninic acid (BCA) method (Redinbaugh & Turley 1986). The assay was calibrated with BSA. Optical densities were measured on a Spectramax UV ELISA plate reader (Molecular Devices, Palo Alto, CA, USA) at 562 nm.

ICAM-1 immunoassay

Enzyme-linked immunosorbent assay (ELISA) for ICAM-1 was performed using a matched pair of anti-human ICAM-1 monoclonal antibodies, clone 15·2 and clone 8·4A6 (biotinylated) (Ancell Corp., Bayport, MN, USA). These recognize domains D1 and D2 respectively of ICAM-1. FluoroNunc ELISA plates (Nunc A/S, Roskilde, Denmark) were coated with clone 15·2 antibody (50 µl/well of 2 µg/ml) in 35 mM Na₂HPO₄, 18 mM Na₂CO₃, 14 mM NaCl, 0·02% NaN₃, pH 8·8 overnight at 4 °C. Residual binding sites were blocked with the assay diluent (10% heat-treated FBS in 10 mM NaH₂PO₄-Na₂HPO₄, pH 7·4, 130 mM NaCl, 0·5% NP-40, 0·02% NaN₃). Recombinant human soluble (s)ICAM-1 (R&D Systems, Minneapolis, MN, USA) was used as a standard. Samples, diluted as required, and serial dilutions of the standard were prepared such that the final buffer was composed of equal proportions of the assay diluent and lysis buffer. The standards and samples (50 µl/well) were incubated in the coated ELISA plates overnight at 4 °C. The plates were washed in 50 mM Tris-HCl, pH 7·2, 300 mM NaCl, 0·5% Tween 20 and the biotinylated antibody was added (50 µl/well of 500 ng/ml antibody in 80 mM NaH₂PO₄-Na₂HPO₄, pH 7·0, 6·4 mM EDTA, 27 mM NaCl, 0·5% Tween-20, 0·3% BSA, 0·02% thiomersalol). After incubation for 2 h at room temperature, StreptABComplex/AP (Dako Corp., Carpinteria, CA, USA) was applied (50 µl/well at a 1 in 25 dilution) and the plates incubated for a further 1 h at room temperature. After a final series of washes, 100 µl/well methylumbelliferyl phosphate (Sigma Chemical Co.) (0·01 mg/ml in 0·1 NaHCO₃-Na₂CO₃ buffer, pH 9·5) were added. Fluorescence was measured after 4 h with a Victor multilabel counter (Wallac OY, Turku Finland). Analysis of the data was performed using MultiCalc software (Wallac OY). The sensitivity of the assay was 0·3 ng/ml sICAM-1 with an upper limit of at least 20 ng/ml. Intraplate and interplate variability were 10% and 13% respectively. Human plasma and a non-recombinant ICAM-1 standard extracted from cultured human cells (Ancell) have been used to further verify the assay.

Expression of data and statistical analysis

ICAM-1 mRNA data are expressed as percentage of a common reference sample (RNA extracted from WISH cells treated for two hours with 1·0 ng/ml IL-1β) for all experiments. ICAM-1 protein is expressed as ng sICAM-1/µg cellular protein (i.e. not corrected for the difference in the molecular

FIGURE 1. Map of the locations of the primers used to prepare the RT-PCR derived ICAM-1 cDNA probes on the reported cDNA sequence for ICAM-1. (■) coding region; (—) untranslated regions; (→) primers. Forward primers are noted above the cDNA and reverse primers below the map. Several restriction sites useful for characterization of ICAM-1 cDNA are indicated.

weights of the soluble and membrane spanning forms). Statistical significance was assessed by use of one-way analysis of variance followed by application of the Student-Newman-Keuls test.

RESULTS

Characterization of the ICAM-1 PCR products

A map of the locations of the primers on the reported sequence for ICAM-1 (GenBank Accession g184532, Tomassini *et al.* 1989) is shown in Fig. 1. The recognition sites for the restriction endonucleases used to confirm the identity of the products are also indicated. No EcoRI sites are present in the reported cDNA. Both ApaI and KpnI would be expected to cleave the full length ICAM-1 cDNA into two major fragments, but the latter would cut outside the smaller, 1·5 kb, PCR product. Finally, five recognition sites for SmaI are predicted in ICAM-1 cDNA, four within the range of the 1·5 kb product.

As shown in Fig. 2, restriction endonucleases EcoRI, ApaI, KpnI and SmaI give the anticipated digestion patterns of the PCR-derived ICAM-1 probes. EcoRI cuts neither PCR product. Furthermore, KpnI cuts only the larger of the two PCR products, resulting in the expected two fragments. Again as expected, ApaI cleaves both products yielding the appropriate sized fragments. Finally, SmaI cleaved the smaller PCR product entirely to fragments of 0·5 kb or less. The expected 1·3 kb fragment was observed when the larger PCR product was digested with this enzyme.

Both PCR products detected a major transcript on northern blots of WISH and HL60 total RNA estimated from four independent northern blots

at 3.2 ± 0.2 kb (mean $\pm$ standard deviation), and a minor, smaller transcript of 2.3 ± 0.1 kb (data not shown). The larger probe also detected a 0.2 kb RNA but the smaller probe did not (data not shown).

TNF- α (50 ng/ml) and IL-1 β (1.0 ng/ml) both positively regulated the expression of ICAM-1 mRNA in WISH cells, as shown in the depicted time course studies (Figs 3 and 4 respectively). In both cases, significantly elevated levels of ICAM-1 mRNA were detectable by 1 h and persisted through 16 h. The maximum expression after treatment with either cytokine was attained by 2 h. For the TNF- α - and IL-1 β -treated cultures these maxima were 18 ± 3 - and 11 ± 3 -fold (mean $\pm$ S.E.M.) respectively over the expression in the corresponding vehicle-treated controls. In the latter case expression at later time points had declined significantly from that at 2 h. TNF- α , however, consistently stimulated persistent maximal

Concentration dependence of change in ICAM-1 mRNA in WISH cells treated with inflammatory cytokines

Significant stimulation of ICAM-1 mRNA expression was observed with as little as 0.3 ng/ml TNF- α or 0.1 ng/ml IL-1 β (Fig. 5A and B respectively). A concentration-dependent stimulation was observed through 50 ng/ml of the former and through 1 ng/ml of the latter. While higher concentrations of TNF- α might stimulate yet further, stimulation by IL-1 β was maximal. The trend towards less

FIGURE 3. Time course of the stimulation of ICAM-1 mRNA expression in WISH cells following treatment with TNF- α . WISH cells were treated with either 50 ng/ml TNF- α or vehicle and total RNA was extracted after 0, 0.5, 1, 2, 4, 8, or 16 h. (A) Representative northern blot probed for ICAM-1 and GAPDH: the treatments and times were as indicated above the blot. (B) Quantitative representation of ICAM-1 mRNA expression over the above time course. The data have been normalised to GAPDH and expressed relative to a common reference sample. They are plotted as means $\pm$ S.E.M. ($n=4$ independent experiments at the 2-h time point, $n=3$ at all other points); * $P<0.05$, significantly greater than the zero hour and corresponding vehicle-treated controls (Student-Newman-Keuls test). The 2-, 8-, and 16-h treated samples were also significantly greater than the 1-h, and the 8-h significantly greater than the 4-h samples ($P<0.05$, Student-Newman-Keuls test).

expression of ICAM-1, observed in Fig. 5, for IL-1 β concentrations above 1 ng/ml was not statistically significant ($P>0.05$ for the 3 and 10 ng/ml treated samples vs the 1 ng/ml sample).

Response of the stimulation of expression of ICAM-1 mRNA to inhibition of protein synthesis

In order to determine whether protein synthesis was required for up-regulation of ICAM-1 mRNA by TNF- α , WISH cells were pretreated with CHX 30 min before the addition of 50 ng/ml TNF- α . As shown in Fig. 6, the stimulation of ICAM-1 mRNA expression in WISH cells by TNF- α was not inhibited by this treatment. Control treatments with CHX alone resulted in no enhancement of ICAM-1 expression.

Production of ICAM-1 protein in WISH cells treated with TNF- α and IL-1 β

To evaluate the extent to which the increase in ICAM-1 mRNA stimulated by the pro-inflammatory cytokines is reflected in an increase in ICAM-1 protein, cellular lysates were assayed for ICAM-1 by ELISA. A hypotonic lysis buffer containing a non-ionic detergent compatible with the assay was used to suspend the membrane and cytoplasmic components of the cell. As shown in Fig. 7, ICAM-1 protein becomes significantly greater by 4 h of treatment in WISH cells exposed to either 1.0 ng/ml IL-1 β or 50 ng/ml TNF- α than in cells receiving vehicle alone. Although the response to the two cytokines is the same through 8 h of treatment, by 16 h the response to the latter exceeds that to the former by nearly twofold. At

FIGURE 4. Time course of the stimulation of ICAM-1 mRNA expression in WISH cells following treatment with IL-1 β . WISH cells were treated with either 1.0 ng/ml IL-1 β or vehicle and total RNA was extracted after 0, 0.5, 1, 2, 4, 8, or 16 h. (A) Representative northern blot probed for ICAM-1 and GAPDH: the treatments and times were as indicated above the blot. (B) Quantitative representation of ICAM-1 mRNA expression over the above time course. The data have been normalised to GAPDH and expressed relative to the same common reference sample as in Fig. 3. They are plotted as means $\pm$ S.E.M. ($n=3$ independent experiments); * $P<0.05$, significantly greater than the zero hour and corresponding vehicle-treated controls (Student-Newman-Keuls test); ** $P<0.05$, significantly greater than at all other time points (Student-Newman-Keuls test).

this time point ICAM-1 protein levels were 6 ± 0.8 - and 11 ± 1 -fold (mean $\pm$ S.E.M.) respectively for the two cytokines, over the corresponding vehicle-treated controls. No statistically significant change could be detected in the basal level of ICAM-1 over the entire 24-h time course. Shedding of sICAM-1 into the media in response to the above cytokines over this time was not detected (data not shown).

Treatment of amnion primary cultures with TNF- α

TNF- α also stimulates expression of ICAM-1 in primary amnion cells in culture (Fig. 8). This increased expression became detectable at 2 h, with the 8-h time point exhibiting the highest expression.

Primary cultures of amnion cells did not exhibit detectable basal expression of ICAM-1. As with the WISH cells, TNF- α induced expression of ICAM-1 by the amnion cells remained elevated through 16 h.

A constitutively expressed mRNA migrating slightly slower than the 3.2 kb ICAM-1 message was faintly detectable on amnion cell RNA northern blots (Fig. 8). Use of the 1.5 kb probe did not eliminate the band. As observed with WISH cell RNA, the 1.5 kb probe did eliminate detection of a very small (0.2 kb) RNA (not shown).

DISCUSSION

Expression of ICAM-1 mRNA and the regulation thereof have been evaluated for the first time in

FIGURE 5. Concentration dependence of the stimulation of ICAM-1 expression in WISH cells by TNF- α or IL-1 β . WISH cells were treated for 2 h with (A) vehicle or 0.1, 0.3, 1, 3, 10 or 50 ng/ml TNF- α , or (B) vehicle or 0.1, 0.3, 1, 3 or 10 ng/ml IL-1 β , and northern analyses for ICAM-1 mRNA were performed on total RNA extracted from the cells. The data have been normalised to GAPDH and expressed relative to the common reference sample (means $\pm$ s.e.m., $n=3$ experiments). Representative blots are shown as insets.

* $P<0.05$, significantly greater than expression in cells treated with vehicle or all lower concentrations of cytokine (Student-Newman-Keuls test).

FIGURE 6. Northern blot analysis of RNA extracted from confluent cultures of the WISH cells which were treated for 1 h with 50 ng/ml TNF- α or carrier in the presence or absence of 10 μ g/ml CHX 30 min prior to addition of TNF- α . The times of collection noted above the blot are relative to the time of addition of TNF- α . The blot was probed in Quikhyb solution with 2.9 kb ICAM-1 and the GAPDH probes. Exposed at 500 μ m resolution. (Representative of 2 independent experiments.)

amnion cell cultures. We have also recently demonstrated the expression of this mRNA in amnion tissues taken during the latter half of gestation (Marvin *et al.* 1998). The cDNA probes utilised in this study were prepared by RT-PCR from RNA from appropriately treated HL60 cells. These cells are known to express ICAM-1 following induction to differentiate (Staunton *et al.* 1988). Restriction analysis indicated that the desired PCR products had been obtained. Furthermore, the major and minor transcripts detected on northern blots (3.2 and 2.3 kb respectively) correspond within experimental error to those reported previously (3.3 and 2.4 kb respectively) (Staunton *et al.* 1988).

Regulation by pro-inflammatory cytokines of ICAM-1 mRNA in both amnion cell culture models was consistent with previous studies on endothelial cells (Poher *et al.* 1986, 1987, Dustin & Springer 1988, Springer 1994) and on other epithelial culture systems (Sippy *et al.* 1995, Ciprandi *et al.* 1996, Khair *et al.* 1996, Iwata *et al.* 1997). Specifically, TNF- α and IL-1 β , used at concentrations previously demonstrated in this laboratory and by others to stimulate prostaglandin production and expression of PGHS-2 mRNA (Albert *et al.* 1994, Perkins & Kniss 1997, Gilmour *et al.* 1998, Hansen *et al.* 1998), dramatically increased levels of ICAM-1 mRNA in the WISH cell model. As with PGHS-2 this enhanced expression was

FIGURE 7. ELISA analysis of the time course of the production of ICAM-1 protein in WISH cells following treatment with IL-1 β or TNF- α . Cellular proteins collected from WISH cells after incubation with vehicle (open bars), 1.0 ng/ml IL-1 β (diagonal striped bars), or 50 ng/ml TNF- α (solid bars) for the times indicated were assayed for ICAM-1 by ELISA and normalised for total protein estimated by the BCA method. Results of three independent experiments with three replicate samples per condition were combined and expressed as means $\pm$ S.E.M.; * P <0.05, significantly greater than the zero hour and corresponding vehicle-treated controls (Student-Newman-Keuls test). TNF- α also stimulated significantly more at the 16- and 24-h time points than IL-1 β .

independent of protein synthesis. TNF- α similarly stimulated ICAM-1 expression in primary amnion cell cultures, albeit with several hours delay. Although the cause of this delay, which has also been observed with PGHS-2 (W R Hansen & M D

FIGURE 8. Northern blot analysis of RNA extracted from primary cultures of human amnion cells treated at confluence (day 5 of culture) with TNF- α for the times (h) indicated above. Probed in QuikHyb solution and exposed at 500 μ m resolution. (Representative of 3 independent experiments.)

Mitchell, unpublished data), has yet to be determined, it is clear that the capacity to express ICAM-1 in response to TNF- α is not peculiar to the transformed cell line.

These results are pertinent to the study of preterm labour. First, the concentration in amniotic fluid of one of the ICAM-1 gene products, sICAM-1, has been reported to correlate to the degree of prematurity in cases of IUGR-associated preterm labour (Salafia *et al.* 1993). Secondly, a massive secretion of pro-inflammatory cytokines, including those used in the present study, has been shown to accompany intrauterine infection-associated preterm labour (Romero *et al.* 1989*a,b*). Finally, a number of reports have suggested that elevated levels of these cytokines also accompany cases of preterm labour in which there is no apparent association with clinical or pathological signs of infection (Romero *et al.* 1990, Dudley *et al.* 1996*a,b*). The present study shows that amnion-derived cells are capable of expressing ICAM-1 and suggests that inflammatory cytokines reaching the amnion would likely result in enhanced expression of this gene. Consequently, a broader range of aetiologies of preterm labour than previously reported (Salafia *et al.* 1993) would be expected to result in its expression.

The classic role of ICAM-1 is in recruitment of leukocytes to sites of inflammation (Carlos & Harlan 1994, Springer 1994). The engagement of ICAM-1 on the surface of activated endothelium by α_L - or α_M -/ β_2 -integrin expressing leukocytes promotes their transendothelial migration (Smith *et al.* 1988, 1989, Furie *et al.* 1991, 1992, Meerschaert & Furie 1995). Chemoattractants increase this interaction by regulating the expression or adhesiveness of these integrins on the circulating cells (Smith *et al.* 1989, Tonnesen *et al.* 1989, Huber *et al.* 1991, Brady *et al.* 1995). Interestingly, the ICAM-1-dependent adhesion and penetration phases are distinct. The latter phase can be blocked by interfering with cytoplasmic Ca^{2+} transients which occur in the endothelial cells (Huang *et al.* 1993). In addition, the small inflammatory mediators, leukotriene (LT) C_4 and histamine, initiate similar transients. Either the binding of the small mediators or of leukocytes increases the permeability of endothelial cell monolayers to both ions and albumin (Huang *et al.* 1993).

In allergic or infectious inflammation of lung, nasal or conjunctival tissues, epithelial as well as endothelial expression of ICAM-1 is observed (Khair *et al.* 1996, Canonica *et al.* 1997). This situation may be more analogous to that in fetal membranes that are expressing this adhesion molecule. Inhibition of the accumulation of leuko-

cytes in these tissues or in the fluids on their apical surface occurs not only with topical histamine H1 receptor antagonists, which inhibit ICAM-1 expression (Ciprandi *et al.* 1996) or with antagonists to the receptors for the chemoattractant, LTB_4 (Allen *et al.* 1996), but also with anti-ICAM-1 antibodies applied to the epithelium (Wegner *et al.* 1990). This suggests that engagement of ICAM-1 on these epithelia by leukocytes plays a significant role in the inflammatory response.

The fetal membranes and cells derived from them also possess considerable biosynthetic capacity for leukocyte chemoattractants. Under conditions of intrauterine infection the concentration of the chemokine, IL-8, in amniotic fluid rises dramatically (Saito *et al.* 1993). WISH and amnion cells have been demonstrated in culture to secrete IL-8 in response to pro-inflammatory cytokines (Trautman *et al.* 1992, Keelan *et al.* 1997*a,b*). In addition, fetal membrane tissue explants have been demonstrated to secrete LTB_4 (Ticconi *et al.* 1995).

The availability of leukocytes to bind ICAM-1 expressing amnion epithelium would appear to be limited, because of its remoteness from the circulation. However, the stroma of the amnion contains resident fetal macrophages (Bulmer & Johnson 1984). The neighbouring chorion also contains resident macrophages and, in addition, granulocytes and T-lymphocytes. Furthermore, infiltration of the chorion by neutrophils is diagnostic of chorioamnionitis (Maudsley *et al.* 1966, Zaaizman *et al.* 1982, Lopez Bernal *et al.* 1989). Recruitment by the amnion of resident or infiltrating leukocytes from the chorion or decidua would require passage across the intervening chorionic cytotrophoblast layer. ICAM-1-dependent migration of leukocytes across this layer is conceivable, since intravascular cytotrophoblasts have been reported to express ICAM-1 (Labarrere & Faulk 1995). In addition, infiltrating neutrophils extensively damage the chorionic cytotrophoblast layer (Van Meir *et al.* 1996). A final potential pool of leukocytes which might interact with the amnion would be those in amniotic fluid (Romero *et al.* 1991). The latter, however, correlate to intra-amniotic infection. They, therefore, would most likely have been recruited from the fetal lung and possibly the fetal membranes as part of the inflammatory response.

A direct role for ICAM-1 expression by the amnion in preterm labour is not immediately obvious. However, altered permeability of the amnion to cytokines and chemokines in the amniotic fluid or augmented infiltration of leukocytes through the amnion could influence inflammatory reactions and structural integrity of the fetal

membranes. Furthermore, increased permeability of the membranes to protein might contribute to the presence of fFN and other diagnostic indicators in the cervico-vaginal fluid of women who are likely to go into preterm labour. From the present results it is clear that ICAM-1 expression by the amnion would be expected during infection-associated preterm labour. To the extent that this is reflected in sICAM-1 production under *in vivo* conditions, this gene product itself may serve as a valuable indicator of preterm labour. Evaluation of this potential will require further investigation.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr Les Myatt, University of Cincinnati, and Dr Kathryn Crosier, University of Auckland, for providing the cell lines used in this study and Dr Jeffrey Keelan, University of Auckland, for reviewing this manuscript. This work was supported by grants from the Health Research Council of New Zealand (grant number 97/017), the Royal Society of New Zealand Marsden Fund (grant number UOA-605), and the New Zealand Lottery Grants Board (grant number AP 57964).

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REVISED MANUSCRIPT RECEIVED 15 October 1998