Expression of adrenomedullin mRNA is altered with gestation and labour in human placenta and fetal membranes

Alfia AL-GHAFRA∗†, Shaun P. BRENNECKE∗†, Roger G. KING‡ and Neil M. GUDE∗†

∗Department of Perinatal Medicine, Royal Women’s Hospital, Carlton VIC 3053, Australia, †Department of Obstetrics and Gynaecology, University of Melbourne, Parkville, Australia, and ‡Department of Pharmacology, Monash University, Clayton, VIC 3800, Australia

ABSTRACT

The aim of the present study was to investigate whether placental and fetal membrane AdM (adrenomedullin) mRNA expression changes with gestation and human labour, as we have previously found labour-associated changes in AdM content in fetal membranes [Al-Ghafra, Gude, Brennecke and King (2003) Clin. Sci. 105, 419–423]. Placentas and fetal membranes were collected either at term or pre-term from women either in-labour or not-in-labour, and AdM mRNA abundance was measured in tissue extracts by Northern blot analysis. Increases were found in the relative abundance of amniotic tissue AdM mRNA in both in-labour and not-in-labour groups at term compared with those at pre-term, and there were positive correlations with gestational age. Relative abundance of choriodecidual tissue AdM mRNA was also significantly elevated in the not-in-labour groups between pre-term and term tissues, although there was no significant correlation with gestational age. However, placental AdM mRNA expression was neither significantly increased at term (compared with pre-term) nor correlated with gestational age. In addition, there were significant increases in AdM mRNA in amnion and choriodecidua in the in-labour group compared with the not-in-labour group for both pre-term and term gestations. There was no difference in AdM mRNA in placental tissues between labour groups. In conclusion, the present study provides evidence that AdM production by fetal membranes is increased in amniotic and choriodecidual tissues at term, compared with pre-term, and in response to labour.

INTRODUCTION

We have shown previously [1] that AdM (adrenomedullin) content of fetal membranes is increased in both term and pre-term labour. We therefore wished to determine whether AdM mRNA expression is altered in placenta or fetal membranes during gestation and/or labour. AdM mRNA is strongly expressed in the reproductive system of rats and humans and its concentrations are altered in normal pregnancy [2]. The abundance of mRNA in pregnant rat uterus is double that in the non-pregnant animal. Many reports have shown that the plasma levels of AdM increase in normal human pregnancy at as early as 8 weeks of gestation and are elevated as pregnancy progresses [3–5]. Furthermore, a previous study [6] has demonstrated that, in maternal plasma, total AdM concentration increased progressively throughout pregnancy and decreased after delivery, whereas mature AdM levels increased until the second trimester of pregnancy and then remained stable until term. AdM is produced from an inactive intermediate precursor, which is then converted into an active mature AdM by enzymatic amidation. Total AdM is the sum of intermediate and mature AdM. These data suggest that AdM plays a role in maternal

Key words: adrenomedullin, gestational tissue, parturition, placenta, pregnancy.

Abbreviations: AdM, adrenomedullin; IL, interleukin.

Correspondence: Dr Neil M. Gude, at the Department of Perinatal Medicine, Royal Women’s Hospital, 132 Grattan Street, Carlton, VIC 3053, Australia (email neil.gude@rwh.org.au).
and perinatal cardiovascular adaptation, especially in the second and third trimester of pregnancy.

In humans, mRNA for both AdM and its specific receptors are present in the amnion, chorion and endothelial cells of the placental villi [7]. This distribution has also been confirmed by immunohistochemical analysis [8,9]. Moreover, evidence suggests that AdM staining appears to be greater in fetal membranes (in epithelial cells and fibroblasts of the amnion and in trophoblast cells of the chorion) than in placenta [8]. In addition, another study revealed that the intensity of AdM expression in the amniotic membranes in the first trimester of pregnancy is similar to that in term pregnancy, suggesting that amniotic membranes may be a site of production or binding of AdM during pregnancy [6]. Also, the importance of AdM in placental and fetal growth and development has been demonstrated in rat pregnancy [10]. Continuous infusion of AdM antagonists caused fetal growth restriction, placental size reduction, gross necrosis of placental margins and amniotic membranes, histologically deficient fetal vessel development in the labyrinth and fetal oedema. This study [10] has suggested that AdM plays multifunctional roles during pregnancy, such as regulation of angiogenesis and vascular tone, as well as functioning as a growth factor.

The role of AdM in human parturition has been examined by Di Iorio et al. [11], who demonstrated that human labour is associated with changes in AdM concentrations in maternal or fetal plasma and in fetal-placental tissues. The concentration of amniotic fluid AdM was found to increase in patients with pre-term labour. Maternal plasma AdM concentration peaked at the beginning of the active stage of labour and decreased with advancing cervical dilatation. AdM output by cultured amniotic epithelial cells and trophoblast cells of the chorion was up-regulated in term labour. Our group has reported previously [1] the measurement of AdM protein in human amnion, chorioidecidua and placenta, which was at a similar concentration (90–140 pg/mg of DNA) for the three tissues collected at term before labour. The same study also found significantly increased concentrations of AdM protein in amnion and chorioidecidua in association with labour at either term or pre-term gestations. Thus, to determine the possible source of the labour-associated increases of AdM in fetal membranes as well as any gestational changes, we measured AdM mRNA by Northern blot analysis in tissue extracts of labour and not-in-labour placental and fetal membrane samples obtained at pre-term and term gestations.

MATERIALS AND METHODS

All experimental procedures were approved by the Royal Women’s Hospital Research and Ethics Committees and in accordance with the National Health and Medical Research Council of Australia Guidelines. Written informed consent was obtained from all participants recruited into this study.

Tissue collection
Placentas were collected either at elective Caesarean section without spontaneous onset of labour (not-in-labour) or after normal vaginal deliveries or at Caesarean section with spontaneous onset of labour (in-labour) from women with singleton pregnancies. Placentas were obtained at both pre-term (between 24–36 weeks) and term (>36 weeks) gestations. The indications for pre-term (elective) not-in-labour Caesarean sections were placenta praevia and fetal distress; for pre-term (emergency) in-labour Caesarean sections, indications were fetal distress, breech presentation and spontaneous rupture of membranes and labour onset in patients with a history of previous Caesarean section. The indications for elective Caesarean section at term were breech presentation, placenta praevia and previous Caesarean section. Labour was defined as the onset of regular rhythmic painful uterine contractions accompanied by dilatation of the cervix and descent of the presenting part of the fetus. Labour onset was spontaneous in all cases. Patients were excluded from the study if there was clinical evidence of infection at the time of sampling, evidence of major pathology and PROM (premature rupture of membranes) without labour onset within 3 days.

Tissue extraction and Northern blot analysis
Placentas were dissected and tissue samples [placenta, reflected amnion (i.e. amnion not covering the placenta) and chorioidecidua] were obtained for total RNA extraction. Each fresh sample was sliced less than 0.5 cm thick and approx. 1 g was stored in at least 10 vol. of RNA later RNA stabilization reagent at 4°C. RNA was extracted within 4 weeks by using a total RNA extraction kit (RNeasy Maxi Kit; Qiagen). RNase-free water was used to dissolve the final RNA pellets, and the RNA was stored at −80°C until processing further.

Total RNA (20 μg from each chorioidecidual and placental sample or 30 μg from each amniotic sample) was denatured and size-fractionated by agarose-gel electrophoresis at 100 V for 3.5 h [2.7% (w/v) formaldehyde/1.3% (w/v) agarose] using a 500 ml gel casting tray with two 26-well combs. Two lanes were loaded with 0.16–1.77 kb RNA molecular-size markers on each gel to estimate the transcript size. Separated nucleic acid fragments were transferred by capillary blotting from the agarose gel on to a sheet of nylon membrane (Hybond-N; Amersham Biosciences). RNA was fixed to the membranes by using a UV cross-linking procedure. The blots were then wrapped and stored at −20°C prior to prehybridization.
Blots were initially pre-hybridized for 3 h at 42°C, and hybridization was carried out overnight at 42°C in hybridization buffer (50% (v/v) deionized formamide, 5× SSC (where 1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 0.5% (w/v) SDS, 5× Denhardt’s solution [0.1% BSA/0.1% Ficoll/0.1% poly(vinylpyrrolidone)] and 0.1 mg/ml denatured herring sperm DNA) with 25 ng of 32P-labelled human cDNA probes, first to AdM and then, after stripping the blot, to 18S oligonucleotide. A hybridization probe for AdM mRNA was generated by RT (reverse transcription)-PCR using the forward primer 5′-TCTAAGCCACAAGCACAC-3′ and the reverse primer 5′-AAACACACTCACATCCAC-3′. This resulted in a 692 bp fragment specific for exon IV of the human AdM gene. Following hybridization, Northern blots were washed with 2× SSC/0.1% SDS buffer at room temperature for 5 min and, then, twice with 0.1× SSC/0.1% SDS for 15 min each at 42°C. The filters were autoradiographed by exposure to Kodak X-OMAT® AR film at −80°C for 1 day. Filters hybridized with 18S rRNA oligonucleotide probes were placed into cassettes at room temperature for 15–30 min. The relative density of hybridization bands on the autoradiographs was determined by laser densitometry (Molecular Dynamics) and image analysis software (ImageQuant; Molecular Dynamics). Results are expressed as the ratio of the relative absorbance of the AdM mRNA hybridization signal and the 18S rRNA hybridization signal.

**Statistical analysis**

Statistical analyses were performed using the statistics software package Statgraphics (STSC). Data were tested for homogeneity of variance using Bartlett’s test and were found to be homogeneous. Data were analysed by ANOVA and multiple range tests (Newman–Keuls). Data are presented as means ± S.E.M. *P < 0.05 was considered significant.

**RESULTS**

Clinical characteristics of patients that participated in the present study are shown in Table 1. There were no differences found between the not-in-labour or in-labour groups in gestational length, birth weight, placental weight either for the term groups or for the pre-term groups. However, as expected, there were significant differences in each of the clinical characteristics between the labour-matched pre-term and term groups (significance shown in Table 1).

**Effect of labour status on AdM mRNA expression in gestational tissues**

cDNA for AdM hybridized with a single band of mRNA of 1.6 kb as reported previously [12,13]. The absorbance ratio of AdM mRNA to 18S rRNA was significantly greater in the amniotic samples of the labour groups compared with the not-in-labour groups at both pre-term and term gestations (Figure 1). Also, there were significant differences in amniotic AdM mRNA between pre-term and term gestations in both in-labour and not-in-labour groups. In addition, there were significant increases in choriodecidual AdM mRNA expression in Table I General characteristics of the study subjects

<table>
<thead>
<tr>
<th></th>
<th>Pre-term</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not-in-labour</td>
<td>In-labour</td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>32.5 ± 0.5</td>
<td>31.4 ± 0.6</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>1970 ± 125</td>
<td>1772 ± 157</td>
</tr>
<tr>
<td>Placenta weight (g)</td>
<td>445 ± 30</td>
<td>424 ± 28</td>
</tr>
</tbody>
</table>

![Figure 1 mRNA expression of AdM in human fetal membranes and placental tissue extracts in term and pre-term groups in association with labour](image)

Upper panel, representative Northern blot analyses for mRNA expression in pre-term not-in-labour (PNIL), pre-term in-labour (PIL), term not-in-labour (TNIL) and term in-labour (TIL) groups. Lower panel, quantification data are presented as a ratio of AdM mRNA abundance relative to 18S rRNA, and are expressed as means ± S.E.M. *P < 0.05 and **P < 0.001. Significant differences between term and pre-term gestations are shown by the dotted (P < 0.05), solid (P < 0.01) and dashed (P < 0.001) lines respectively.
Figure 2  Effect of gestational age on AdM mRNA expression in fetal membranes and placenta

association with human labour in both pre-term and term samples. A significant difference in AdM mRNA was found in choriodecidual between pre-term and term gestations in the not-in-labour groups, but not in the in-labour groups. However, there were no differences in AdM mRNA found in placental AdM mRNA expression in the different labour or gestation groups.

AdM mRNA expression in pre-term and term gestational tissues
To investigate whether there was any association between AdM expression in intrauterine tissues and gestational age, AdM mRNA expression was plotted against gestational age (Figure 2). Labour groups were kept independent in order to compare intrauterine AdM expression at pre-term and term gestations without the interference of labour. In amnion, linear regression analysis showed that relative abundance of AdM mRNA in both not-in-labour ($r^2 = 0.229, P < 0.01$) and in-labour ($r^2 = 0.622, P < 0.05$) groups were significantly correlated with gestational age. On the other hand, in both choriodecidual and placenta, there were no significant correlations between AdM mRNA and gestational age (either in-labour or not-in-labour; Figure 2).

DISCUSSION

The present study shows for the first time that the relative abundance of AdM mRNA increases between pre-term and term gestation in amnion in both not-in-labour and in-labour groups and in choriodecidual in not-in-labour groups, but not in placenta. Moreover, in amnion, relative abundance of AdM mRNA was correlated with gestational age in both not-in-labour and in-labour groups. Up-regulation of AdM expression occurs during the last weeks of pregnancy, suggesting that AdM expression by human fetal membranes is important during late gestation. The specific roles of amnion- and choriodecidual-derived AdM have yet to be elucidated. The identification of AdM mRNA, immunoreactive protein and its receptors in fetal membranes suggest autocrine roles, which may include modulation of cell growth and/or proliferation. Alternatively, AdM produced in amnion may have paracrine functions in adjacent tissues. Amniotic epithelial cells may be the source of the high concentrations of AdM detected in amniotic fluid at term. In this regard, Di Iorio et al. [3] reported that amniotic fluid AdM concentrations increase greatly after 20 weeks of gestation. These data could also suggest that amnion-derived AdM may be of physiological importance to the fetus. Furthermore, fetal membranes are adjacent to the myometrium. Thus AdM produced in fetal membranes may also affect the uterus; indeed, the density of AdM-binding sites and its mRNA are greater in pregnant compared with non-pregnant uterus [14].

Although, other studies have measured AdM protein, to our knowledge this is the first study that also demonstrates labour-associated increases in production of AdM mRNA by human fetal membranes. In the present study we have shown that amniotic and choriodecidual concentrations of AdM mRNA are augmented in association with either pre-term or term labour.

Few studies have been done to elucidate the involvement of AdM in human parturition. A previous study [15] examined the mature/total AdM ratio in plasma from the maternal cubital vein and umbilical vein after term deliveries either vaginally or by Caesarean section. In the infants delivered vaginally, fetal ratio of mature/total AdM was significantly higher than its maternal ratio. In contrast, in newborns delivered by Caesarean section,
there were no significant differences between fetal and maternal mature/total AdM ratios. This study [15], however, failed to detect any significant differences in maternal or fetal plasma total or mature AdM levels between the different labour groups. That was in contrast with the study by Boldt et al. [16], who reported that the concentrations of total AdM in cord artery and vein in newborns delivered vaginally were significantly higher than in newborns delivered by elective Caesarean section at term, suggesting that during or immediately prior to labour there are significant increases in the production and/or secretion of AdM. The authors postulated that increased AdM was involved in vascular adaptation of the newborn.

Our present findings are supported by the previous work of Di Iorio et al. [11], who measured immuno-reactive AdM levels in amniotic fluid and maternal plasma. The amniotic fluid concentration was much higher in samples collected from women in pre-term labour than those in pre-term not-in-labour. The study also identified that protein concentrations are significantly higher in plasma collected after the onset of labour, with peak values occurring at 4 cm of cervical dilatation, compared with the pre-labour values. Furthermore, as cervical dilatation progressed, circulating AdM levels decreased gradually and, at delivery, reached the same values recorded before the onset of labour. In addition, basal output of AdM by amniotic and choriodecidual cultured cells was greater from samples obtained after term spontaneous delivery compared with those from term Caesarean section.

AdM peptide and mRNA are predominantly expressed in fetal membranes and placenta and less in the umbilical artery and uterine muscle [17]. In contrast, it has been demonstrated that mRNA for AdM receptor components [RAMP2 (receptor activity modifying protein 2) and CRLR (calcitonin receptor-like receptor)] are expressed predominantly in the placenta, uterine muscle and umbilical artery, rather than in the fetal membranes [17]. Thus it is possible that increased AdM from fetal membranes in association with labour may be released into amniotic fluid and/or towards the uterus and may be involved in fetal and/or maternal adaptations to labour.

AdM is a potent smooth muscle relaxant and increased production of AdM throughout pregnancy may help to maintain a quiescent uterus. Several studies have assessed the ability of AdM to inhibit uterine contractility in vitro. In isolated non-pregnant rat uteri, AdM has been shown to attenuate the contractile response of uterine muscle to galanin [14], suggesting a relaxant role for AdM in the uterus. AdM was also shown to reversibly inhibit spontaneous and bradykinin-induced periodic rat uterine contractions in a concentration-dependent manner [18].

Fetal membrane AdM released towards the uterus could exert a vasodilatory effect on blood vessels. AdM has been shown to be 16 times more potent than prostaglandin I₂ as a vasodilator in the sheep uterine circulation[19]. AdM has marked and prolonged vasodilator activity and, thus, may have a role in regulation of uterine blood supply during pregnancy and labour. It has been postulated previously [1] that AdM, acting in an autocrine or paracrine manner, dilates vessels and facilitates blood flow during parturition, compensating for the local-acting vasoconstrictor substances, the synthesis and release of which are increased during labour (for example prostaglandins, endothelin and thromboxane).

At birth, pulmonary vascular resistance decreases dramatically, allowing pulmonary blood flow to increase approx. 8–10-fold and oxygen exchange to occur in the lungs. It is possible that AdM could play a role in the transitional changes in fetal pulmonary blood flow that commence during labour. Exogenous AdM administered into the left pulmonary artery causes vasodilatation in fetal sheep and increases pulmonary blood flow significantly, with less effect on the systemic circulation [20]. The same authors demonstrated that this effect of AdM was largely dependent on nitric oxide release and ATP-dependent potassium channel activation [21,22]. Thus these findings support the hypothesis that, in the fetal lung late in gestation, endogenous AdM activity could influence basal vascular tone and contribute to changes in the pulmonary circulation at birth.

AdM may have anti-apoptotic effects on fetal membranes. It has been shown in vivo that inhibition of endogenous AdM action leads to induction of apoptotic changes in trophoblast cells in placenta and decidual cells of the uterus, resulting in fetal growth restriction in rats [23]. Apoptosis is a component of normal development and differentiation in most tissues. Apoptosis also occurs in term amnion and chorion [24], and Hsu et al. [25] have demonstrated that apoptosis in human amnion is significantly increased in association with labour at term.

It is not known what regulates AdM production during labour. The onset of parturition is associated with an increase in pro-inflammatory cytokines [e.g. TNF-α (tumour necrosis factor-α), IL (interleukin)-1β and IL-6] and chemokines (e.g. IL-8) in fetal membranes and decidua (for example, see [26]). At the same time, AdM gene expression and release has been shown to be regulated by inflammatory-related substances including cytokines (for example, see [27]). It would therefore be of interest to determine whether pro-inflammatory cytokines regulate AdM expression in human fetal membranes.

In summary, our present findings provide evidence that amniotic AdM mRNA expression in both not-in-labour, and in-labour groups and choriodecidual AdM mRNA expression in not-in-labour groups, significantly increase at term compared with pre-term. In addition, the present study has identified increased expression of AdM mRNA by amnion and choriodecidual, but not by placenta, in association with human labour either at
pre-term or term gestations. These changes in mRNA parallel those in protein expression reported previously. Although a number of hypotheses have been suggested for the function of increased production of AdM during pregnancy and parturition, further work needs to be done to substantiate these hypotheses.

ACKNOWLEDGMENTS

This work was supported by a Royal Women’s Hospital Research Scholarship. We gratefully acknowledge the help of the Clinical Research Midwife Sue Nisbet, the midwifery and obstetric staff of the Royal Women’s Hospital, and the technical support of Vicki Doherty.

REFERENCES

14 Upton, P. D., Austin, C., Taylor, G. M. et al. (1997) Expression of adrenomedullin (ADM) and its binding sites in the rat uterus: increased number of binding sites and ADM messenger ribonucleic acid in 20-day pregnant rats compared with nonpregnant rats. Endocrinology 138, 2508–2514

Received 6 October 2005/21 November 2005; accepted 29 November 2005
Published as Immediate Publication 29 November 2005, doi:10.1042/CS20050303