Increased adrenomedullin protein content and mRNA expression in human fetal membranes but not placental tissue in pre-eclampsia

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Introduction

Since the discovery of AdM in human phaeochromocytoma in 1993 (Kitamura et al., 1993) many studies have been done to elucidate the role of this 52-amino acid peptide in the human body. AdM was shown to be widely distributed and to have multiple biological effects (Hinson et al., 2000). It is one of the secretory products of various cells including vascular endothelium, smooth muscle, cardiomyocytes and cardiac fibroblasts (Horio et al., 1998; Isumi et al., 1998). Consequently, AdM mRNA and gene expression are found in most tissues, according to degrees of vascularity. AdM is also a circulating hormone, and its concentration has been investigated in a range of disease states. Plasma AdM concentrations are increased in association with cardiovascular diseases such as hypertension, congestive heart failure, renal failure and septic shock. Thus, AdM has been postulated to be another factor involved in the physiology and pathophysiology of cardiovascular and body fluid homeostasis (Schell et al., 1996; Kitamura and Eto, 1997).

Many studies have been conducted to ascertain the roles of AdM in the human reproductive system and pregnancy. Cameron and Fleming (1998) demonstrated that AdM is highly expressed in uterus and ovary, and that concentrations exceed those expressed in the adrenal medulla. Thus, they hypothesized the involvement of AdM in reproductive function. Plasma AdM concentration is elevated during the follicular phase and decreased during the luteal phase of the menstrual cycle (Marinoni et al., 2000). In rat uterus, AdM expression is found to be closely correlated with circulating estradiol levels (Cameron et al., 2002). Circulating AdM concentrations increase in women during pregnancy; concentrations progressively rise during normal pregnancy and fall post-partum (Di Iorio et al., 1997; Di Iorio et al., 1999; Kobayashi et al., 2000; Kanenishi et al., 2001; Hoshimoto et al., 2002). High AdM concentrations have been detected in cord blood and amniotic fluid (Macri et al., 1996; Kanenishi et al., 2001). Also, AdM causes a dose-dependent relaxation of placental arteries in normal pregnancies (Hoeldtke et al., 2000). In rats, AdM was shown to regulate normal placental function and fetal development (Witlin et al., 2002). AdM gene knockout models have shown that lack of AdM is associated with vascular abnormalities during embryonic development (Shindo et al., 2001). Caron and Smithies (2001) also demonstrated extreme hydrops fetalis in the AdM−/− rat embryos. Furthermore, recent data has suggested that AdM plays a vascular protective role through a NO-dependent pathway of eNOS activation in rats (Ando and Fujita, 2003).

AdM was also shown to be involved in some pregnancy-associated diseases such as PE, pregnancy-induced hypertension (PIH), intrauterine growth retardation (IUGR) and oligohydramnios. Occurring in
5–7% of pregnancies, PE remains a major cause of perinatal and maternal morbidity and mortality worldwide. PE is characterized by increased peripheral vascular resistance, increased blood pressure, placental ischaemia, platelet aggregation and endothelial dysfunction with an imbalance of vasodilating (such as prostacyclin, NO) and vasoconstricting substances (e.g. thromboxane A2, endothelin-1). PE leads to reduced uteroplacental blood flow. A role of AdM in PE was investigated in 1997 by Hata et al., who demonstrated lower plasma AdM concentration in pre-eclamptic women compared to normotensive third-trimester pregnant women. Since then other studies have reported somewhat conflicting findings, which have been summarized by Di Iorio et al. (2003). Some reports showed that maternal plasma AdM concentrations did not differ significantly between women with PE and normal pregnant women in the third trimester (Di Iorio et al., 1998; Minegishi et al., 1999; Jerat et al., 2001), whereas one study demonstrated increased maternal plasma level of AdM in pre-eclamptic patients at term (Lauria et al., 1999). Differing findings were also reported using immunohistochemistry, where one study reported that the intensity of AdM expression in placentas obtained from pregnancies with PE was significantly decreased compared with expression in placentas from normal pregnancies (Kanenishi et al., 2000). In contrast, another study showed no difference in the staining for AdM between pre-eclamptic and normotensive placentas (Di Iorio et al., 1998).

A substantial decrease of AdM mRNA in placental villi was detected from pregnancies with PE, whereas its AdM receptor mRNA was not affected (Knerr et al., 2002). A recent study also demonstrated significantly decreased production of AdM by cytotrophoblast cultures in PE patients (Minegishi et al., 1999). However, AdM concentrations in amniotic fluid and umbilical vein plasma were found to be much higher in PE compared to normotensive pregnant women (Di Iorio et al., 1998). Furthermore, a recent study demonstrated that cerebroplacental fluid AdM levels are significantly higher in patients with PE than in normotensive pregnant women (Celik et al., 2003).

In order to further clarify the expression of AdM during PE, we measured fetal membrane and placental AdM protein concentration and mRNA expression in samples obtained from patients with PE and compared them with normal pregnancies. Because there are labour-associated changes in tissue AdM concentrations (as we previously reported, Al-Ghafra et al., 2003), in contrast to other studies, samples were divided into labour and not-in-labour groups.

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 patients participating in the study.

Tissue collection

Term placentas were collected either at elective Caesarean section (not-in-labour) or after normal vaginal deliveries or at Caesarean section during labour (in-labour) from women with singleton pregnancies. The women either had otherwise uneventful antenatal courses or they were diagnosed with PE. PE was defined as the development of hypertension (blood pressure: systolic = 140 mmHg and /or diastolic = 90 mmHg) and proteinuria (protein in the urine: ‘2+’ with dipstick testing or >300mg/24hr) occurring after the twentieth week of pregnancy. Placentae were obtained at both term (>36 weeks gestation) and preterm (between 24 and 36 weeks gestation) gestations. Preterm placentae were collected at emergency Caesarean section with no signs of labour (not-in-labour). The indications for preterm Caesarean section were PE (for PE group), placenta praevia or spontaneous rupture of membranes with previous Caesarean section and fetal distress (for control group). Patients with premature rupture of membranes without labour onset within 3 days (PROM) were excluded from the study. Labour onset was spontaneous in all cases. The tissues collected at elective Caesarean section prior to the onset of spontaneous labour were defined as not-in-labour. The indications for elective Caesarean section at term were breech presentation, placenta praevia and previous Caesarean section. The indications for emergency Caesarean section at term were obstructed labour, intrapartum bleeding, fetal distress (for control group) and PE (for PE group). None of the patients had clinical evidence of infection at the time of sampling. Patients were excluded from the study if there was evidence of major pathology other than PE or if there was spontaneous rupture of membranes of greater than 3 days without delivery.

Tissue extraction and radioimmunoassay

Placentas were dissected and tissue samples, placenta, reflected amnion (i.e. amnion not covering the placenta) and chorionic decidua were obtained for total RNA and protein extraction. For subsequent protein extraction, tissues were snap frozen in liquid nitrogen and stored at –80°C. Frozen tissue of 1 g was processed for AdM measurement and for DNA assay, as previously reported (Al-Ghafra et al., 2003). AdM was measured using a commercial radioimmunoassay kit (Phoenix Pharmaceuticals, Mountain View, CA, USA) based on a polyclonal rabbit antiserum against synthetic 1–52 human AdM. The cross-reactivity of the antibody was as follows: AdM1-52, 100%; AdM13-52, 15%; C-GRP 0.7% and amylin, endothelin-1, alpha-atrial natriuretic peptide, B-type natriuretic peptide-32 and C-type natriuretic peptide-22, 0%. The intra- and inter-assay coefficients of variation were 5.9% and 12.7%, respectively, and the sensitivity of the assay was 5 pg/tube. Total tissue DNA content was measured in aliquots of the initial tissue homogenates by a modified diphenylamine method (Al-Ghafra et al., 2003). AdM concentrations were expressed as pg per mg of DNA.

Total RNA extraction

Each fresh sample was sliced less than 0.5 cm thick and approximately 1g was stored in at least 10 volumes of RNAlater RNA stabilization reagent (Qiagen Pty Ltd) at 4°C. Within 4 weeks they were extracted by using a total RNA extraction kit (RNeasy Maxi Kit, Qiagen Pty Ltd). RNAse free water was used to dissolve the final RNA pellets and total RNA was quantified by measuring the absorbance at 260nm. All samples were stored at -80°C prior to Northern blot analysis.

For both amnion and chorioidecidua, samples were routinely taken from multiple sites of each tissue and pooled for protein and RNA extraction. For placenta, irAdM and AdM mRNA expression was found not to be significantly different when sampled from multiple sites of the basal plate of normal tissue (unpublished data, n = 4). Thus, for all subsequent experiments tissue was taken from a single macroscopically normal site for each placenta.

Northern blot analysis

Total RNA samples were electrophoretically size fractionated on 1.3% (w/v) agarose gel containing 2.7% (v/v) formaldehyde. Of the total RNA, 20 μg from each sample was mixed with 18.5 μl of sample running buffer (1 μl 10× MOPS, 3.5 μl of filtered formaldehyde, 10 μl of deionised formamide, 2 μl of ethidium bromide and 2 μl of RNA running dye: 50% (v/v) glyceral, 1 mM EDTA, 0.4% (w/v) bromophenol blue and 0.4% (w/v) xylene cyanol) and denatured at 65°C for 10 min, then chilled on ice briefly. Samples and 0.16–1.77 kb RNA ladder were loaded onto wells and electrophoresed using MOPS buffer at 100 V for 3.5 h. At the completion of the electrophoresis the gels were examined on a UV light box and photographed using Polaroid film.

Capillary transfer of RNA (Northern blotting)

The agarose gel was blotted with a sheet of nylon membrane (Hybond – N, Amersham Pharmacia Biotech UK Limited). The RNA was fixed to the membrane by using an optimized UV cross-linking procedure, and the position of the bands of the RNA ladder were marked on the filter. The blot was then wrapped and stored at -20°C prior to prehybridization.

Prehybridizing and hybridizing

The blots were rolled and placed in 30 ml of hybridization buffer (deionized formamide 50% (v/v), 5× SSC, 0.5% (v/v) SDS, 0.1 mg/ml denatured herring sperm DNA, 5× Denhardt’s solution) for 3 h at 42°C in a rotating hybridization oven (Ratek Instruments, Boronia, Victoria, Australia, Model 3060) for prehybridizing. Then blots were hybridized with 20 ml of fresh hybridization buffer and 25 ng of radiolabelled human cDNA probes first to AdM and then, after
stripping the blot, to 18S-oligonucleotide, overnight at 42°C. Following hybridization, blots were briefly rinsed twice with 50 ml of 2× SSC/0.1% SDS buffer at room temperature, and then placed in 2× SSC/0.1% SDS buffer at room temperature for 5 min. Two washes were performed with 0.1× SSC/0.1% SDS for 15 min each at 42°C. The filters were autoradiographed.

Stripping of probes from blots for rehybridization
Blots were initially hybridized with the human AdM cDNA probe and then with the human 18S ribosomal RNA probe. To remove hybridized cDNA probes from the blots, 0.1% SDS was boiled, added to the container with filters and left until it cooled down. Removal of probes was checked by following overnight autoradiography.

Autoradiography and densitometry
Autoradiography was performed by exposure of Kodak® X-OMAT® AR film (24 × 30 sm) to the blots. Blots hybridized with AdM cDNA were placed into cassettes with intensifying screens (Lightening Plus, Dupont, Wilmington, DE, USA) at -80°C for 1 day. Filters hybridized with 18S ribosomal RNA oligonucleotide probes were placed into cassettes at room temperature for 15–30 min. The films were developed, rinsed in water and fixed in Hypam Rapid paper and film fixer for 2–3 min. The relative density of hybridization bands on the autoradiographs was determined by using laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA) and image analysis software (ImageQuant Version 3.3, Molecular Dynamics). The AdM mRNA densities were expressed relative to the 18S rRNA densities.

Statistical analysis
Statistical analyses were performed using the statistics software package Statgraphics (SPSS, Lead Technologies, USA). Data were tested for homogeneity of variance using Levene’s test. Where data were found to be homogeneous, they were analysed by one-way ANOVA and multiple range tests (Student Newman-Keuls). When data were found to be non-homogenous, comparisons were made using non-parametric statistics (Cochran chi-square and Hotelling’s T-square tests). Within tissue comparisons between pre-eclamptic and control groups were made using Student’s t-tests. The group data are presented as mean ± SEM; P<0.05 was considered significant.

Results
Gestational age, infant weight and placental weight for each group are presented in Table I. Comparison of the two preterm groups showed that infant and placental weights were significantly lower in the PE group compared to control. Comparison of term gestational groups revealed that there were no significant differences between control and PE groups in gestational length and in placental weight. However, there were significant differences in infant weight between control and PE for both the in-labour and not-in-labour groups.

AdM expression in preterm pre-eclampsia
Previously, we have demonstrated that fetal membrane AdM concentrations increase with labour during normal pregnancy at both preterm and term gestation (Al-Ghafra et al., 2003). Therefore, in this study we assessed the results for both normal pregnancy and PE according to labour status. We found that there were increases in AdM protein concentration with PE at preterm gestation in non-labouring tissues (Figure 1). A two-fold increase (P<0.01) in irAdM content was present in preterm choriodecidual pre-eclamptic samples and a smaller, but statistically significant (P<0.05), increase was seen in amniotic pre-eclamptic tissues compared with controls. Placental concentrations of AdM were not altered in PE at preterm gestations.

AdM mRNA expression was also measured in amniotic, choriodecidual and placental tissues of the preterm not-in-labour PE group and compared with the relevant uncomplicated pregnancies. The cDNA for AdM hybridized with single band of messenger RNA of 1.6 kb as reported previously (Makino et al., 1999; Minegishi et al., 1999). Figure 2 shows that there was a significant increase in AdM mRNA expression in choriodecidual tissues during PE. Amniotic AdM mRNA relative abundance normalized to its expression of 18S rRNA was not significantly different in preterm pre-eclamptic women compared to preterm controls, even though there appeared to be an almost two-fold increase. In addition, Figure 2 shows that placental AdM mRNA expression was not significantly changed with PE. The transcript was most abundant in placental tissues and least in amniotic samples.

AdM expression in term pre-eclampsia
In term samples, there were also significant increases in irAdM protein concentration in choriodecidual and amnion of women with PE (both not-in-labour and in-labour groups), compared with those that had uncomplicated pregnancies (Figure 3). As was the case at preterm, there were no significant alterations in protein content of AdM in placentas from women with PE who delivered at term compared with controls. Furthermore, similar to controls, significant labour-associated differences were seen in amniotic and choriodecidual immunoreactive protein content between the two pre-eclamptic groups, i.e. not-in-labour and in-labour. irAdM content in placenta was not altered with labour onset in controls or pre-eclamptics.

Discussion
In the present study we investigated whether the placenta and fetal membrane production of AdM is altered in PE. This study demonstrated that amniotic and choriodecidual protein concentration of

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### Table I. General characteristics of the study subjects

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>N</th>
<th>Gestation (weeks)</th>
<th>Birth weight (g)</th>
<th>Placenta weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrT Control NIL</td>
<td>10</td>
<td>31.9 ± 0.6</td>
<td>2061 ± 108</td>
<td>457 ± 32</td>
</tr>
<tr>
<td>PrT PE NIL</td>
<td>10</td>
<td>30.6 ± 0.6</td>
<td>1314 ± 128a</td>
<td>334 ± 30a</td>
</tr>
<tr>
<td>T Control NIL</td>
<td>14</td>
<td>38.1 ± 0.3</td>
<td>3278 ± 67</td>
<td>560 ± 24</td>
</tr>
<tr>
<td>T PE NIL</td>
<td>8</td>
<td>37.5 ± 0.3</td>
<td>2810 ± 188a</td>
<td>521 ± 63</td>
</tr>
<tr>
<td>T Control IL</td>
<td>14</td>
<td>39.1 ± 0.4</td>
<td>3623 ± 163</td>
<td>676 ± 41</td>
</tr>
<tr>
<td>T PE IL</td>
<td>8</td>
<td>38.4 ± 0.6</td>
<td>3018 ± 156a</td>
<td>632 ± 60</td>
</tr>
</tbody>
</table>

IL, in-labour; NIL, not-in-labour; PE, pre-eclampsia; PrT, preterm; T, term.

aSignificantly different for corresponding control group, P < 0.05.

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**Figure 1.** AdM protein content in human fetal membranes and placental tissue extracts of preterm not-in-labour pre-eclamptic and control groups. The data are presented in pg per mg of DNA as mean ± SEM. Significant increases in AdM content for the pre-eclamptic groups compared with control groups in amnion and choriodecidual are indicated by an asterisk (control – open bar; pre-eclamptic – closed bar). N values and other patient data are shown in Table I.
AdM was increased in patients with PE compared to women with uncomplicated pregnancies at both preterm and term gestations. Furthermore, we showed that choriodecidual AdM mRNA expression is elevated in preterm PE. The study did not, however, find any differences in either AdM protein concentration in preterm and term placental tissues or mRNA expression in preterm placental tissues obtained from women with PE compared to normal pregnancy.

Conflicting results have been reported for tissue and plasma AdM concentrations between PE patients and normotensive pregnant women. The variability of the different studies measuring AdM concentrations with PE is summarized by Di Iorio et al. (2003). Possible explanations for the different results obtained in those studies include differences in the characteristics of women recruited, diagnostic criteria for PE, mode of delivery, as well as differences in methodologies used (such as placental sampling, handling of samples and storage). In this study we tried to reduce possible confounding factors by using strict diagnostic criteria for patients (only severe PE patients were admitted to the study), by classifying samples according to gestation, i.e. term (>36 weeks) and preterm (26–36 weeks), and, also, by classifying samples in accordance with patients’ labour status. The grouping of patients according to labour status has not been well described by other studies that compared AdM concentrations in PE and normotensive patients. We have previously shown marked increases in AdM concentrations in amnion and choriodecida with onset of labour (Al-Ghafra et al., 2003). Thus, measurement of AdM in these tissues, and possibly maternal blood and amniotic fluid (Di Iorio et al., 2001), may be influenced by labour status, which, if not taken into account, may greatly increase the variability of the measurements. Finally, some technical considerations were taken into account, such as minimizing the time of sample storage and avoiding use of samples that had been previously thawed.

Makino et al. (1999) showed increases in irAdM in the fetal membranes and umbilical artery, and AdM mRNA in the umbilical artery (but not in placenta or uterine muscle) in patients with pregnancy-induced hypertension. They also showed that expression of RAMP2 (a component of the specific AdM receptor) mRNA was increased in the fetal membranes, despite its decrease in the umbilical artery and uterus (Makino et al., 2001). In addition, one study demonstrated that AdM induces relaxation in the placental circulation (stem villous artery) in both normotensive and PE pregnancies, suggesting that PE arteries retain their ability to respond to AdM (Jerat et al., 2001). Thus, on the basis of our findings, together with results of studies discussed above, we postulate that increased fetal membranes, production of the vasoconstritory peptide AdM may counteract arteriolar vasoconstriction found in PE as part of a compensatory response. For instance, increased production of AdM by fetal membranes may compensate for the vasoconstrictor actions of endothelin-1, the immunoreactive concentrations of which are significantly higher in the amnion, chorion leave and placental plate chorion during PE (Singh et al., 2001).

Our present findings are consistent with the results obtained by Gratton et al. (2003), where AdM mRNA expression was significantly increased in choriodcida in pregnancies complicated by severe PE. Furthermore, our data agree with that of Di Iorio et al. (1998), who reported higher concentrations of AdM in amniotic fluid in non-labour pre-eclamptic patients compared with normotensive not-in-labour pregnant women (25–38 weeks of gestation). These authors also reported absence of any significant differences in either the percentage or intensity of AdM staining between pre-eclamptic and normotensive placentas. This is in line with our findings of no significant differences in placental irAdM concentrations. Similarly, we also found that AdM mRNA expression in placenta was not changed with severe preterm PE, which is in agreement with the report of Minegishi et al. (1999). In contrast, Gratton et al. (2003) found localized increases in AdM mRNA at syncytial knots and cytotrophoblasts in the regions surrounding placental infarcts in
placentas from PE. Nevertheless, our study and that of Minegishi et al. (1999) measured total placental AdM mRNA from macroscopically normal tissues, rather than localizing it to necrotic/affected regions within the placenta, and this may account for the different observations.

The biological function of the augmented production of AdM by fetal membranes in PE still needs to be elucidated. Increased expression of RAMP2 mRNA and no change in CRLR in PIH patients in fetal membranes have been reported (Makino et al., 2001), and AdM-specific receptors may also be increased with PE. If that is the case, it provides further evidence that the localized effect of this peptide on fetal membranes is up-regulated during PE. Recent reports have suggested that AdM is a regulator of cell growth and differentiation and plays a potent protective role, for example, by inhibiting cell death by the up-regulation of Bcl-2, the anti-apoptotic protein (Oehler et al., 2001). Some authors postulate that one role of AdM is to prevent apoptosis and maintain normal implantation, placental development and fetal growth during early gestation (Penchalaneni et al., 2004). They demonstrated in vivo that the inhibition of endogenous AdM action leads to induction of apoptotic changes in trophoblast cells in the labyrinth zone of the placenta, and in decidual cells of the uterus resulting in fetal growth restriction in rats. Thus, it is possible that AdM may have similar effects in cells of fetal membranes, and may possibly inhibit the increased placental apoptosis that has been reported in PE (Di Federico et al., 1999).

In addition to its effects on fetal membranes, AdM produced by choriodecidua may be released towards the uterus and play a role in the regulation of local vascular reactivity in a compensatory manner. For example it may play a protective role against the vascular response to injury (Kawai et al., 2004). Makino et al. (2001), however, found that AdM-specific receptor mRNA was down-regulated in umbilical and uterine arteries with PIH. If AdM receptors are also down-regulated in these vessels with PE, it suggests that umbilical and uterine arteries may be excluded from any compensatory role of increased AdM production. Indeed, Makino et al. found that there was a significant negative correlation between RAMP2 mRNA in both umbilical and uterine arteries and systemic blood pressure. Finally, AdM produced by the amnion may be released into amniotic fluid and play a protective role in fetal physiology during PE, as it has been shown that AdM concentration in amniotic fluid is increased with PE (Di Iorio et al., 1998).

The mechanisms generating the increase in the production of AdM by fetal membranes in women with pregnancy complications by PE are not known. Local production of cytokines may be involved. Leucocyte and monocyte-derived cytokine secretion in both peripheral blood and the placenta, especially in fetal membranes, are changed during and even before clinical manifestation of PE (Bowen et al., 2002). It is known that increased concentration of AdM in plasma positively correlates with the plasma levels of mediators such as tumour necrosis factor (TNF)-alpha, interleukin (IL)-6 and IL-8 (Ueda et al., 1999; Hoßbauer et al., 2002), and that in various cells AdM production has been shown to be increased by a wide range of inflammatory cytokines and growth factors (Minamino et al., 1995; Horio et al., 1998; Isumi et al., 1998). Thus, it is possible that inflammatory cytokines that are increased in PE may be involved in regulation of local AdM production by fetal membranes.

In conclusion, in the present study we have shown that AdM protein content is elevated in fetal membranes during PE at both term and preterm gestation, and that AdM mRNA is elevated in preterm choriodecidua with PE. It is possible that AdM acts as an autocrine and/or paracrine factor affecting fetoplacental function.

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