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The expression and localization of the human placental prorenin/renin-angiotensin system throughout pregnancy: roles in trophoblast invasion and angiogenesis?

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ABSTRACT

The renin-angiotensin system (RAS) is thought to regulate placentation, however, the expression and localization of RAS pathways in early gestation human placenta is not known. Here we describe the expression of prorenin (*REN*), (pro)renin receptor (*ATP6AP2*), angiotensinogen (*AGT*), angiotensin converting enzyme 1 and 2 (*ACE; ACE2*), angiotensin II type 1 and 2 receptors (*AGTR1; AGTR2*) and angiotensin 1–7 receptor (*MAS1*), as well as the angiogenic factor, vascular endothelial growth factor (*VEGF*), and transforming growth factor-β1 (*TGF-β1),* in early gestation (6-16 weeks) and term (>37 weeks) human placentae. We also describe the location of all of the key RAS proteins in the early gestation placentae. The highest levels of *REN, ATP6AP2, AGT, AGTR1* and *ACE2* mRNAs were found in early gestation, whereas *ACE1* mRNA was highest at term. *AGTR2* and *MAS1* mRNA expression were low to undetectable in all samples. *REN, ATP6AP2* and *AGTR1* mRNA levels were correlated with *VEGF* expression, but not with *TGF-β1* mRNA. In early gestation placentae, prorenin, (pro)renin receptor and the angiotensin II type 1 receptor (AT_1R) were localized to extravillous trophoblast cells, suggesting they play a key role in trophoblast migration. ACE2 in syncytiotrophoblasts could regulate release of Ang 1-7 into the maternal circulation contributing to the vasodilation of the maternal vasculature. ACE was only found in fetal vascular endothelium and may specifically target the growing fetal placental vessels. Because *REN*, *ATP6AP2* and *AGTR1* show strong correlations with expression of *VEGF* this pathway is likely to be important in placental angiogenesis.

INTRODUCTION

Intrauterine growth restriction, with or without preeclampsia, affects approximately 10% of all pregnancies and is known to be a major risk factor for cardiovascular disease, hypertension and diabetes mellitus in adult life [1]. Placental dysfunction is a major causal factor of intrauterine growth restriction. Normally, placentation requires the formation of a branching network of fetal vessels within the chorionic villi as well as trophoblast growth, invasion, and remodelling of the uterine spiral arterioles [2]. These processes are essential for the provision of an adequate blood flow to the placenta to support normal fetal growth. Because placental insufficiency is probably the major cause of failure to thrive 'in utero', it is surprising that, although a role for the renin-angiotensin system (RAS) in placental development has been suggested [3], there has been no systematic investigation into the expression and localization of the RAS system in early gestation placenta, or its function throughout pregnancy.

Although the RAS is mainly considered an endocrine system that regulates blood pressure, water and electrolyte homeostasis, it is clear that tissue-based renin angiotensin systems (RASs) exist, including those within the human conceptus. Active renin catalyses the conversion of angiotensinogen (AGT) to Angiotensin (Ang) I. This peptide is then cleaved to Ang II by angiotensin-converting enzyme (ACE). Ang II acts via type 1 or type 2 angiotensin receptors (AT_1R or AT_2R). Most of the actions of Ang II, including vasoconstriction, angiogenesis and cell growth are mediated by the Ang II/AT_1R interaction. Ang II acting on AT_2R has, in general, opposing effects [4]. An alternate ACE, ACE2, terminates the action of Ang II by converting it to Ang 1-7, which, acting via a *Mas* G-coupled protein receptor, opposes the action of Ang II on AT_1R [5].

In other tissues and cell lines, activation of the AT_1R by Ang II leads to potent induction of vascular endothelial growth factor (VEGF) [6-8], which is thought to act locally to establish the fetoplacental circulation [9]. The Ang II/AT_1R interaction also stimulates cell proliferation [10], and decreases trophoblast invasion by increasing transforming growth factor (TGF)-β1 and plasminogen activator inhibitor-1 (PAI-1) [11, 12]. It is very likely therefore, that the placental RAS may be a key mediator of placental angiogenesis, trophoblast proliferation and invasion and in view of the often opposing actions of Ang II by AT_1R and AT_2R and the actions of Ang 1–7 at Mas it is likely that dysregulation of expression of components of the placental RAS could affect placental development and lead to placental insufficiency.

Until recently, prorenin, the precursor of active renin was considered to have little or no biological activity despite normal circulating levels being 10 times higher than active renin [13]. A (pro)renin receptor [(P)RR], however, has been identified that binds both renin and prorenin [14]. Prorenin can directly stimulate intracellular signalling via the (P)RR or can be activated conformationally within the receptor complex and so generate Ang I from AGT [14]. Only the kidney secretes active renin, whereas prorenin is constitutively secreted by other tissues. To have biological activity prorenin secreted by non-renal tissues must bind to the (P)RR or be activated by proteases. Therefore, if (P)RR is present, prorenin could act in a placental RAS, as well as in many other tissue RASs.

The highest biological levels of prorenin are found in gestational fluids during early pregnancy [15]; they are 1000 times normal plasma levels and are highest at ~6 weeks of gestation in both plasma [16] and gestational fluids [15]. We and others have found high levels of (P)RR in syncytiotrophoblast (STB) of term placenta, decidua, amnion and chorion [14, 17] and all of the RAS components have been shown to be

present in term human placenta [17, 18]. To date, however, no studies have systematically examined the expression and localization of the placental RAS in early gestation human placenta at the time when maternal circulating prorenin levels are highest. As a necessary first step in studying the functions of a placental RAS in early gestation we have determined the expression and localization of all of the components of the RAS, including the more recently identified (P)RR and ACE2. To begin to understand the role of the RAS in regulating placental angiogenesis and trophoblast invasion we also determined if there is any association between the expression of RAS genes and the expression of VEGF or TGF-β1. This descriptive analysis of early gestation placental RAS and its relationships with VEGF and TGF-β1 pathways, which are likely to be involved in trophoblast invasion and angiogenesis, is the first of its kind.

METHODS

Tissue Collection

This study was approved by the University of Newcastle's Human Ethics Committee and Hunter New England Health's Research Ethics Committee. Early gestation placentae (6-16 weeks) were collected from women undergoing elective termination of pregnancy. Samples were collected in glycerol substituted artificial cerebrospinal fluid (g-ASCF) containing 250 mM glycerol, 26 mM $NaHCO_3$, 11 mM glucose, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, and 2.5 mM $CaCl_2$ and bubbled with Carbinox (95% O_2, 5% CO_2) to achieve a pH of 7.3. All samples were transported on ice and arrived in the laboratory within 30 minutes of the termination procedure. Placental tissue was then collected in either 4% paraformaldehyde or RNA*later*® solution (Invitrogen) for subsequent protein and RNA analysis, respectively. Term placentae

(37-41 weeks) were collected from healthy pregnant women undergoing elective caesarean sections at the John Hunter Hospital within 30 minutes of delivery. Women being treated with non-steroidal anti-inflammatory drugs, or with a history of infection, chorioamnionitis, or asthma, or undergoing induction of labour were excluded from the study.

Quantitative real-time RT-PCR (qPCR)

Total RNA was extracted from intrauterine tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and DNase treated (Qiagen). RNA was reverse transcribed using a SuperScript III RT-kit with random hexamers (Invitrogen). qPCR was performed in a Applied Biosystems 7500 Real Time PCR Machine (Applied Biosystems) using SYBR Green for detection. Each reaction contained 5 µl of SYBR Green master mix (Invitrogen), primers, cDNA (10 ng reverse-transcribed total RNA) and water to 10 µl. The primers for RAS mRNAs and *TGF-β1* have been described previously [18]. The *VEGF* primer sequences were: 5' CTA CCT CCA CCA TGC CAA GT 3' (forward) and 5' GCA GTA GCT GCG CTG ATA GA 3' (reverse). Cycling conditions were as follows: incubation at 50ºC for 2 minutes, followed by 95ºC for 10 minutes, 40 cycles of 15 seconds at 95ºC and 1 minute at 60°C. mRNA abundance was calculated relative to β-actin (*ACTB*) mRNA using the ΔCT method. Comparisons of mRNA abundance were made by incorporating a calibrator sample in each run and determining relative abundance as 2-ΔΔCT [19]. A cDNA sample from a term placenta was used as the calibrator for all mRNAs. Dissociation curves, to detect non-specific amplification, were generated for all reactions, and no-template control samples were

included. The predicted sizes of the PCR products were verified by agarose gel electrophoresis (data not shown).

Immunohistochemistry (IHC)

Paraformaldehyde-fixed paraffin embedded sections (4 μm thick) were deparaffinized, and antigen retrieval for prorenin, (P)RR, ACE, AGT, AGTR1 was performed using a microwave oven with Epitope Retrieval Solution™ pH 6.0 (Novacastra) for 10 min. No antigen retrieval was required for ACE2. Antibodies used were: ACE (Novacastra, NCL-CD143), ACE2 (Abcam, ab15348), AGT (R&D Systems, AF3156), AT_1R (Abcam, ab9391), renin propeptide (R&D Systems, MAB4447) and (P)RR (Abcam, ab64957). Positive control tissue included small intestine, basal forebrain, kidney and heart from normal adult humans. Matched samples lacking the primary antibody were used as negative controls. Sections were blocked with 2% skim milk powder in Tris-buffered saline (TBS) and then incubated for 1 h with primary antibody. Immunostaining was performed on a Bond-XTM automated immunostainer (Vision BioSystems) with the Bond Polymer Refine Detection System (Leica MicroSystems) consisting of polymer conjugated anti-mouse/rabbit secondary antibody. The site of antibody binding was visualized with diaminobenzidine (DAB) and counterstained with haematoxylin. Images were captured and analyzed using the Aperio Scanscope XT slide scanner (Aperio Technologies).

Data Analyses

Data were tested for normality using the skewness and kurtosis test. Data that failed these tests were logarithmically transformed to satisfy assumptions for analysis of

variance [20]. Comparisons between gestational ages were made by one-way analysis of variance followed by the Bonferroni post-hoc test to determine where differences in gestational ages existed. Pearson correlations to determine relationships between RAS genes, *VEGF* and *TGF-β1* were calculated on logarithmically transformed data. The SPSS statistical package (SPSS Release 17.0.0. Chicago) was used for the analyses. Significance was set at $P<0.05$.

Results

Placental RAS pathways in early gestation human placenta

Early gestation human placentae expressed most components of the RAS: specifically, *REN*, *ATP6AP2*, *AGT*, *ACE*, *ACE2* and *AGTR1* mRNAs. *AGTR2* mRNA abundance was very low in 11/25 early gestation placenta samples (data not shown). *MAS1* mRNA abundance was not detected in any sample (data not shown).

REN mRNA abundance was highest in 6-9 week placentae (compared to 10-13 week and 14-16 week placentae; $P=0.002$ and $P<0.001$, respectively, Figure 1A). *ATP6AP2* mRNA expression was also highest in 6-9 week placentae compared to placentae collected at 14-16 weeks (P=0.037, Figure 1B).

AGT mRNA abundance was low in all samples and was not different between the early gestation groups (Figure 1C). *AGTR1* mRNA abundance was highest in 10-13 week placentae compared with those collected at 14-16 weeks ($P=0.019$; Figure 1D), but not different from levels observed in 6-9 week placentae. *ACE* and *ACE2* mRNA was found in all early gestation samples but levels did not differ between the different early gestational age groups (Figure 1E and 1F).

RAS protein localization in early gestation placentae

In early pregnancy prorenin protein was localized to the syncytiotrophoblasts (STBs), cytotrophoblasts (CTBs) and extravillous cytotrophoblasts (EVTs) of the placenta (Figure 2A). (P)RR displayed a similar pattern localizing to STBs and EVTs, however it was not found in CTBs (Figure 2B). The intensity of immunostaining for prorenin and the (pro)renin receptor in placentae did not appear to change throughout early pregnancy.

The antibody against AGT protein displayed weak immunoreactivity in STBs, CTBs and villous stroma (Figure 2E). ACE protein was specifically localized to the fetal vascular endothelium of the placental villi and did not change during early gestation (Figure 2I). In contrast ACE2 was not present in the fetal vascular endothelium but was abundant in the STB layer and villous stroma (Figure 2J). Less intense ACE2 immunoreactivity was also observed in CTB. Immunostaining for AT_1R was found in STB, CTB and villous stroma as well as EVTs (Figure 2F).

Placental RAS mRNA abundance throughout gestation

REN mRNA abundance was highest in early gestation placentae (collected between 6 and 16 weeks gestation) compared to term placentae ($P<0.001$; Figure 3A). *ATP6AP2* mRNA expression was also higher in early gestation placentae compared to term placentae ($P<0.001$; Figure 3B) and was highly correlated with *REN* mRNA abundance ($r=0.75$, $P<0.001$ Figure 3C). *AGT* mRNA abundance was low in all samples but higher in early gestation compared to placentae at term ($P=0.022$; Figure 4A). *AGTR1* mRNA abundance was highest in 10-13 week placentae compared with those collected at term ($P=0.020$), and appeared to be higher overall in early gestation placentae (6-16 weeks)

compared to term placenta, but this did not reach statistical significance (P=0.059; Figure 4B). *ACE2* mRNA expression was also highest in early gestation placentae compared to those at term (*P*<0.001; Figure 4D) whereas *ACE* mRNA levels were highest in term placentae (P<0.001; Figure 4C). Interestingly, there was a negative correlation between *ACE* and *ACE2* mRNA abundance (r=-0.61, P=0.001; Figure 4E). *AGTR2* mRNA abundance was very low (data not shown) and there was no significant difference between *AGTR2* mRNA levels in early and late gestation placentae.

Placental Expression of *VEGF* and *TGF-β1* mRNA throughout gestation and their associations with RAS gene expression

Although placental *VEGF* mRNA levels appeared to decrease with advancing gestational age, there was no significant difference between the different age groups (Figure 5A). *VEGF* mRNA abundance was, however, correlated with *REN* (r=0.44, *P*=0.010), *ATP6AP2* (r=0.60, *P*<0.001) and *AGTR1* (r=0.51, *P*=0.003) mRNA levels (Figure 5C-E). The abundance of *TGF-β1* mRNA in the placenta remained stable throughout gestation (Figure 5B) and was not significantly correlated with RAS mRNA levels.

DISCUSSION

We have described for the first time the expression and localisation of RAS pathways in the early gestation and term human placenta by measuring mRNA expression of RAS genes and antibody labelling of key RAS proteins. Our data show several RAS pathways are present in the early gestation human placenta.

The localisation of prorenin, the (pro)renin receptor and AT_1R to EVTs coupled with their high mRNA expression in very early gestation when placental invasion is maximal, suggest that prorenin plays a key role in regulating trophoblast migration and vascular remodelling. This could occur directly through binding and intracellular signalling via the (P)RR, or indirectly through (P)RR activation and subsequent Ang II generation acting through the AT_1R [14].

The ACE protein was specifically localised to the fetal endothelium and *ACE* expression increased with advancing gestational age. This is likely the result of increased placental vascularisation as pregnancy progresses and we propose that ACE may specifically target the placental RAS to growing vessels. Indeed, placental *VEGF* mRNA abundance also appeared to be highest in early pregnancy and the strong correlations between *VEGF* expression and that of *REN*, *ATP6AP2* and *AGTR1*, as well as significant evidence that the Ang II/AT_1R pathway regulates the VEGF system [6-8], indicates a role for the RAS pathway in placental angiogenesis in early pregnancy. The localisation of ACE2 to the STB of the placental villi means that ACE2 in STBs could regulate the release of Ang 1-7 into the maternal circulation and contribute to the vasodilation of the maternal vasculature.

AGT mRNA levels are low in the placenta and the protein was weakly expressed. Abundant AGT protein, however, is found in term placenta [18]. Since the STB is bathed by maternal blood entering the intervillous space, it is possible that these cells take up AGT from maternal blood.

The absence of ACE protein in trophoblasts does not mean that production of Ang II is limited to fetal placental vascular endothelium. ACE is abundant in the maternal blood that bathes the placental villi. Alternatively, Ang I may be converted to Ang II

through the action of chymase, by an ACE independent pathway [21]. Chymase is expressed in the human placenta, its activity is increased in placentae from women with preeclampsia [22], and it may increase Ang II production [23].

In contrast to ACE, *ACE2* mRNA was most abundant in early gestation placenta, and the protein was present in STBs and villous stroma. Since ACE2 is also exposed to circulating maternal Ang II, its localization in STBs could indicate that it plays a role in converting maternal Ang II to Ang 1-7. Placental production of Ang 1-7, which is a powerful antagonist of Ang II/AT_1R [5], may be important for controlling maternal blood pressure and salt and water balance.

We found high levels of *AGTR1* mRNA and AT_1R protein at all ages examined, suggesting that the Ang II/AT_1R pathway is the predominant pathway in both early gestation and term placenta, as reported previously [24]. Furthermore, *AGTR1* mRNA abundance was highest in placentae collected during early pregnancy compared to those at term. Neither *AGTR2* nor *MAS1*, however, were detected at significant levels in the placenta in this study. This is not surprising as, in contrast to Ang II/AT_1R interactions, Ang II/AT_2R inhibits VEGF [25] and Ang 1-7/Mas inhibits tumour angiogenesis because it down regulates VEGF mRNA and protein levels [26]. It is well documented that AT_2R is downregulated in maternal tissues in pregnancy (e.g. myometrium and endometrium, where it is the dominant Ang II receptor in the non-pregnant state, [27]). We have found AT_2R mRNA and protein in term placenta [18], as previously described [3, 24, 28]. Although it is recognized that levels in placenta are low [3].

The co-localisation of (P)RR and prorenin in trophoblasts of the early gestation placenta means that prorenin could act via the RAS or independently of the classical RAS pathway. Non – Ang II dependent targets for the (P)RR include cell signaling via

ERK1/2 [29] or HSP27/p38 [30] and interaction with the Wnt pathway through the action of (P)RR as an adaptor (see [31]). Given that levels of *REN* and *ATP6AP2* mRNA are highest during early pregnancy (Figure 1), these alternate pathways could also be essential for early placental growth and trophoblast invasion as the (P)RR knockout is embryo lethal (see [32]).

Ang II/AT_1R interactions have been shown to inhibit trophoblast invasion by stimulating TGF-β1 [11, 12]. We were not able to show any gestational changes in the expression of *TGF-β1* mRNA, which is known to inhibit trophoblast migration [33, 34] nor, surprisingly, was its expression correlated with RAS mRNA levels. Rats overexpressing the human (P)RR transgene have increased renal TGF-β1 [35] but again we did not find any significant correlation between *TGF-β1* and either *REN* or *ATP6AP2* mRNA levels. A role for the RAS in regulating TGF-β1 and inhibiting trophoblast invasion may be more evident in the maternal decidua because it is thought to produce factors, including TGF-β1, that counteract the pro-invasive trophoblast cells [36]. Indeed, we have found previously that term human decidua has the highest levels of expression of *REN* [17] and secretes large amounts of prorenin when grown *in vitro* [37].

In conclusion, we have provided the first systematic description of the expression and localization of all the major components of the RAS in early gestation human placenta at a critical period in placentation. Its association with the expression of VEGF, its high expression during early placentation, and the expression of key proteins of the renin/(P)RR and Ang II/AT_1R pathway in invading cells suggests the placental RAS plays a key role in placental angiogenesis, proliferation and trophoblast invasion.

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FIGURE LEGENDS

Figure 1. mRNA expression of RAS components in early gestation placenta. (A) *REN* mRNA levels were highest at 6-9 weeks gestation. (B) *ATP6AP2* mRNA levels were higher at 6-9 weeks compared to 14-16 weeks gestation. (C) *AGT* mRNA levels did not vary in early gestation. (D) *AGTR1* mRNA levels were higher at 10-13 weeks gestation than at 14-16 weeks. (E) *ACE* and (F) *ACE2* mRNA levels were not different between the different gestational age groups. Different superscripts denote differences between groups, $P<0.05$. N=5-11 per gestational age group.

Figure 2. Immunolocalization of RAS proteins in early gestation placentae. (A) Prorenin was localized in STBs, CTB and EVTs. (B) (P)RR was also localized to STBs and EVTs but not to CTBs in placental villi. (E) AGT was localized to STB, CTB and villous stroma. (F) Low levels of immunostaining for AT_1R was found in villous stroma, CTB, STB and EVTs. (I) ACE protein was only expressed in the fetal endothelium of the placental villi, whereas (J) ACE2 was abundant in the STB and villous stroma. Negative controls for prorenin, (P)RR, AGT, AT_1R, ACE and ACE2 are shown in (C), (D), (G), (H), (K), and (L), respectively. N=14 early gestation placenta (6-14 weeks gestation).

Figure 3. mRNA expression of *REN* and *ATP6AP2* in early and late gestation placenta. (A) *REN* and (B) *ATP6AP2* mRNA levels were higher in early gestation placentae (6-16 weeks gestation) compared to term (>37 weeks). * denotes significant difference to early gestation placentae, $P<0.05$. N=27-29 early gestation placentae and N=9 term placentae. (C) Plot showing relationship between *REN* and *ATP6AP2* mRNA levels.

There was a significant correlation between placental *REN* and *ATP6AP2* mRNA levels (r=0.75, P<0.001).

Figure 4. mRNA expression of RAS components in early and late gestation placenta. (A) *AGT* mRNA was higher in early gestation placentae compared to term. (B) Placental *AGTR1* mRNA expression appeared to be higher in early gestation placentae compared to term (P=0.059) (C) *ACE* mRNA levels were highest in term placenta, whereas *ACE2* expression (D), like the other RAS genes, were highest in early gestation placenta. * denotes significant difference to early gestation placentae, $P<0.05$. N=22-26 early gestation placentae and N=7-9 term placentae. (E) Plot showing relationship between *ACE* and *ACE2* mRNA levels. There was a significant negative correlation between *ACE* and *ACE2* mRNA levels (Pearson's correlation; r=-0.61, $P=0.001$).

Figure 5. mRNA expression of *VEGF* and *TGF-β1* mRNA levels in early and late gestation human placenta. There was no significant difference between the different gestational age groups in (A) *VEGF* or (B) *TGF-β1* mRNA levels. N=8-12 per gestational age group. VEGF mRNA abundance was however correlated with (C) *REN* (r=0.44, $P=0.010$), (D) *ATP6AP2* (r=0.60, $P<0.001$) and (E) *AGTR1* (r=0.51, $P=0.003$) mRNA levels.