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Potential role of Intermedin/Adrenomedullin 2 in early embryonic development in rats

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Abstract

Adrenomedullin2 (ADM2), also referred to as Intermedin (IMD) is expressed in trophoblast cells in human placenta and enhances the invasion and migration of first trimester HTR-8/SV-neo cells. Recently we demonstrated that infusion of IMD antagonist in pregnant rats causes feto-placental growth restriction suggesting a role for IMD in maintaining a successful pregnancy. Therefore, this study was undertaken to assess if IMD has a functional role in embryo implantation in a rat model. We show that IMD mRNA is expressed in rat implantation sites and its expression is significantly higher on day 15 in placenta compared to days 18 – 22. Infusion of IMD antagonist IMD_{17–47} from day 3 of pregnancy causes a significant decrease in the weights of day 9 implantation sites as well as serum levels of 17 β -estradiol, progesterone, nitric oxide and serum MMP2 and MMP9 gelatinase activity. Further, expression of MMP2, MMP9, VEGF and PLGF protein levels are significantly downregulated in the implantation sites of IMD antagonist treated rats. This study suggests a potential involvement of IMD in regulating the factors that are critical for implantation and growth of the embryo and thus in establishment of normal rat pregnancy.

Keywords

Intermedin; implantation sites; nitric oxide; matrix metalloproteinases; growth factors

1. Introduction

During implantation, the blastocyst undergoes initial inner cell mass differentiation, while the trophoblast initiates interactions with the uterus [1]. The trophoblast–uterine interaction involves several progressive stages as the blastocyst moves into the endometrium. During this time, luteal hormones transform the surrounding uterine stroma into metabolically active, enlarged decidual cells [2–6]. There are reports that some of the vasodilatory substances produced and released under the influence of oestrogen, induce or modulate endogenous NO [7–9]. Oestrogen also stimulates uterine generation of NO by inducing available arginine [10]. The vasoregulatory role of NO is well documented [11]. It has been hypothesized that NO is involved as an effector molecule in mediation of the effect of oestrogen on the uterus [12]. Progesterone also appears to influence uterine NO production

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because antiprogestins have been reported to reduce the NOS II isozyme, the major isoform of NOS that is responsible for upregulation of NO production during pregnancy [13].

MMPs are also thought to play a role in implantation, since increased expression of several prominent types of MMPs that aid in the invasive process have been observed during the periimplantation period in mice [14, 15]. Furthermore, several studies indicate that nitric oxide (NO) directly regulates MMPs through feedback mechanisms [15–17]. Evidence suggests that components of extracellular matrix, and hormones all play a role, and either alone or in combination may alter NO concentrations [16, 18].

Adrenomedullin2 (ADM2) also referred to as Intermedin (IMD) is a 47 amino acid peptide recently discovered as a member of Calcitonin gene related peptide alpha/Calcitonin gene related peptide beta (CALCA/CALCB) family peptides. IMD has ~28% structural homology to adrenomedullin (ADM) and <20% with CALCB. IMD is abundantly expressed in rat ovary, uterus, and placenta, along with its expression in other tissues such as brain, heart, and pituitary gland [19, 20]. Recently we reported that plasma levels of immunoreactive IMD are elevated during pregnancy and that IMD induced vasodilatation is greater during pregnancy in rats [21]. In addition, we have previously shown that blocking of endogenous effects of IMD during mid gestation in rats through infusion of IMD antagonist (IMD_{17–47}), causes fetoplacental growth restriction as well as a significant decline in the expression of NOS 1/2/3 in rat placenta on day 15 of gestation [22].

Therefore, this study was undertaken to assess if infusion of IMD antagonist affects implantation in rats. First we demonstrate the expression of IMD mRNA in the implantation sites on day 9 and in placenta throughout gestation and that expression of IMD is higher in day 15 placenta compared to days 18 – 22. Infusion of IMD antagonist from day 3 of pregnancy caused a significant decline in the serum levels of 17 β -estradiol, progesterone, NO and serum gelatinase activity. Further IMD antagonist infusion caused a significant decline in the weights of implantation sites on day 9 along with a decline in mRNA expression of MMP2, MMP9, vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) proteins in day 9 implantation sites. Therefore, this study demonstrates a potential involvement of this novel peptide in regulation of molecules that are known to play a role in embryo implantation and early placental development.

2. Materials and Methods

2.1 Animal

Adult female rats (*Rattus norvegicus*), obtained from Harlan Sprague Dawley (Houston, TX) were fed with standard chow pellets and water *ad libitum*. All animals were housed in a climate-controlled room with a 12L:12D schedule. Two females were paired with a male overnight and the next morning, males were removed and females were assessed for the presence of sperm in the vaginal flush. Animals with positive sperm in the flushes are designated as day 1 of gestation. All procedures were approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX) in accordance with National Institute of Health “Guide for the Care and Use of Laboratory Animals.” Five pregnant rats were used in each experimental group.

2.2 Treatments

[In our previous work we have shown that IMD antagonist infusion during mid gestation in pregnant rat causes fetoplacental growth restriction. Function of IMD during periimplantation and early placental development are not known. Therefore we were interested to know if IMD had any effects on the growth and rate of embryo implantation as well as, on the factors involved in early placental growth (day 9 implantation sites which

precede the formation of placenta). Therefore to block the endogenous function of IMD during periimplantation period, osmotic minipumps (model 2ML1, 10 μ l/h; Alza, Palo Alto, CA) containing saline alone or with saline containing IMD₁₇₋₄₇ were inserted s.c. into the dorsum of pregnant rats from Day 3 to day 9 of gestation while animals were under anesthesia to deliver IMD₁₇₋₄₇ (200 μ g per rat per day). Anesthesia consisted of a combination of ketamine (45 mg/kg of body weight; Fort Dodge Laboratories, Fort Dodge, IN) and xylazine (5 mg/kg of body weight; Burns Veterinary Supply, New York, NY). Based on the pumping rate and the duration of infusion, we prepared the drug concentrations in the pumps to provide the specified daily dose of the drug. All rats were killed on Gestational Day 9 using a CO₂ inhalation chamber. The dose selected in this study was based on previous investigations performed with day 15 pregnant rats [22]. Implantation sites were carefully dissected out from the uterine horn, counted and their weights were recorded. Placentas were also collected from control rats on day 15, 18 and 22 of pregnancy (n=4 in each group) and flash frozen to isolate RNA. Implantation sites were either frozen in liquid nitrogen and stored at -80°C for further analysis of various proteins or fixed in buffered formalin for immunohistochemistry. Blood was collected followed by serum extraction for measurement of 17 β -estradiol and progesterone and stored until used at -80°C.

2.3 Radioimmunoassay

RIA of 17 β -estradiol and progesterone was performed in accordance with manufacturer's instructions (DSL, Webster, Texas).

2.4 Nitrite determination by Greiss assay

The blood samples were centrifuged to isolate plasma. Aliquots of plasma were stored at -80°C until used. Total nitrates, as indicator of NO production in the serum were determined using the Greiss reaction. The serum nitrates are reduced with nitrate reductase and the total nitrites were measured using the Greiss Colorimetric Assay (Cayman Inc., Ann Arbor, Michigan, USA). Absorbance was read at 540 nm.

2.5 RNA extraction and RT-PCR

Trizol reagent was used to isolate total RNA from IMD₁₇₋₄₇-treated and control rat implantation sites as per the manufacturer's protocol (Invitrogen, CA). The RNA was dissolved in 20 μ l of ribonuclease (RNase)-free water containing deoxyribonuclease (DNase) I buffer and 2 units of amplification grade DNase I. The DNase I was removed by phenol chloroform extraction. The RNA was dissolved in RNase-free water and was stored at -70°C until use. Using total RNA, first-strand cDNA was synthesized by RT in a 20- μ l reaction volume containing PCR buffer, reverse transcriptase, RNase inhibitor, 2 μ g of RNA, 5 mM MgCl₂, 1 mM deoxyribonucleotide triphosphate (dNTP) mixture, and random primers as described by the supplier (Ambion Inc., Austin, TX). For RT, samples were placed into a thermal cycler for 1 cycle at 28°C for 15 min, 42°C for 45 min, 99°C for 5 min, and 4°C for 5 min. The cDNA was stored at -20°C.

The PCR reactions were initiated for IMD, by the specific primer set designed based on the published DNA sequence (NM_201426-1). Briefly, 1 μ l of cDNA was mixed with a PCR mixture containing 2.5 mM MgCl₂, 1 $\times$ of 10 $\times$ PCR buffer, 5 U/100 μ l of 1 mM dNTP mixture, and 0.2 μ M of the gene-specific forward primer -5'-AGTTGCTGATGGTCACGGTAA-3' and reverse primer -5'-ATAACTGTGGGGGTGCTG-3'. The PCR cycle involved an initial denaturing step at 95°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 50°C for 30 sec, 73°C for 2 min, and 72°C for 7 min. Amplification of the housekeeping gene 18S was also performed for the same samples using a standard 18S primer pair (Ambion). The PCR reactions were carried out on a GeneAmp PCR system 9700

(Perkin Elmer, Branchburg, NJ). The PCR products were visualized on a 1.4% agarose gel containing 0.5 µg/ml of ethidium bromide, run in 0.5× Tris-borate-EDTA buffer at 100 V for 1.5 h. Gels were placed on a UV light box and imaged, and expression of IMD transcripts were analyzed relative to the 18S with the FluorChem 8000.

2.6 Gelatin Zymography

The protocol used for gelatin zymography was similar to that used in our previous study [23]. Briefly, two microgram of total serum protein was mixed with sample buffer and loaded onto a 12% SDS-PAGE gel containing gelatin from Invitrogen (Invitrogen, CA) without boiling. The electrophoretic gels were washed with 2.5% Triton X-100 and 50 mM Tris-HCl (pH 7.5) to remove SDS and then incubated at 37°C in reaction buffer (150 mM NaCl, 5 mM CaCl₂, and 50 mM Tris-HCl at pH 7.5) for 2–3 days and processed as per the manufactures instructions. Thereafter, gels were stained with 0.1% Coomassie Brilliant blue R-250 for 30–60 min and destained in 10% methanol with 5% glacial acetic acid. The gelatinolytic activity could be seen as a clear band on a uniform blue background.

2.7 Tissue Homogenization and Western blot

Proteins were extracted from the implantation sites on day 9 of pregnancy from rats infused from day 3 as well as controls by homogenization in buffer containing: 50 mM Tris with IMD_{17–47} (pH 7.4), 1 mM EDTA, 150 mM NaCl and proteinase inhibitors (1 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 1 µg/ml leupeptin). The homogenates were centrifuged at 2000 × *g* for 15 min at 4° C. The supernatants were separated and their protein concentrations were measured. Equal amounts of protein (20 µg) were separated on 12% SDS-PAGE and electro transferred to nitrocellulose membranes. Membranes were blocked with TTBS buffer (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk for 1 hr and probed with MMP2/MMP9/VEGF and PLGF antibodies. After exposure to secondary antibodies (diluted 2000-fold to 5000-fold) for 1 h, horseradish peroxidase-conjugated anti-rabbit IgG (PLGF), or anti-mouse IgG (MMP2, MMP9 and VEGF), blots were washed and developed by enhanced chemiluminescence (ECL kits; Amersham Life Science, Piscataway, NJ). Each blot was stripped with 100 mM glycine, pH 2.3, and was reprobed with β-actin to normalize for any variations incorporated in protein loading. Densities of each protein of interest were expressed as a ratio to that of β-actin on the same blot.

2.8 Statistical Analysis

The weights of implantation sites in each rat are averaged and the implantation site numbers and weights are expressed as mean ± SEM for each group. Statistical analysis between the two groups for all parameters was performed using the Student *t*-test. One way ANOVA was used for assessing the differences in IMD mRNA expression during different days of gestation. Values were considered significant at *P* < 0.05.

3. Results

3.1 Expression of IMD in rat placenta

Figure 1 demonstrates that IMD mRNA is expressed in implantation sites in rats on day 9 of gestation and in the placenta throughout gestation. As shown in this figure, placental expression of IMD is significantly higher (*p* < 0.05) on day 15 compared to days 18 – 22.

3.2 Effect of IMD_{17–47} on the implantation sites

Implantation sites were carefully dissected out and counted for the total number in both the uterine horns and weighed. As shown in figure 2, infusion of IMD_{17–47} to pregnant rats from

day 3 caused a decrease in the weights of implantation sites obtained on day 9 ($p < 0.05$). However, the differences in the number of implantation sites between control and antagonist treated are not significant.

3.3 Effect of IMD₁₇₋₄₇ on the serum levels of sex steroid hormones

To assess if IMD antagonist alters the synthesis of estrogens and progesterone during pregnancy, we measured 17β estradiol and progesterone in the serum from the control and IMD₁₇₋₄₇ treated rat on day 9 of gestation. As shown in figure 3, infusion of IMD₁₇₋₄₇ to pregnant rats from day 3 caused a significant decline ($p < 0.05$) in the levels of both 17β -estradiol and progesterone on day 9 suggesting a role for IMD in the regulation of serum levels of sex steroid hormones during early pregnancy in rats.

3.4 Effect of IMD on the serum Nitrite levels

In our previous report we showed that IMD antagonist causes a significant decrease in NOS enzymes in placenta on day 15 of gestation in rats [22]. Nitric oxide regulates activity of several molecules critical for embryo implantation and trophoblast invasion which include matrix metalloproteinases and growth factors [17, 24, 25]. Therefore, in this study, using Greiss reagent we assessed the effect of IMD antagonists on serum total nitrites in control and IMD antagonist treated rats on day 9 of gestation. Figure 4 shows that infusion of IMD₁₇₋₄₇ caused a significant decline ($p < 0.05$) in the serum nitrite levels suggesting that IMD antagonist reduces NO synthesis in early gestation in the rat.

3.5 Regulation of matrix metalloproteinases and growth factors by IMD at the maternal fetal interface

We have previously shown that IMD increases the invasive capacity of 1st trimester human trophoblast cells [26]. Trophoblast invasion is believed to be mediated by autocrine trophoblastic factors as well as paracrine uterine factors. Several types of regulatory factors have been investigated and extra-cellular matrix proteinases such as MMP2 and MMP9 and growth factors such as VEGF and PLGF have been shown to be important for remodeling of the uterine components and angiogenesis during implantation and early placental development. Therefore, we investigated the effect of IMD₁₇₋₄₇ infusion on the expression of MMP2/MMP9/VEGF and PLGF proteins in the implantation sites on day 9 of gestation. As shown in figure 5, IMD significantly downregulated the expression of MMP2, MMP9, VEGF and PLGF in the implantation sites ($p < 0.05$) sites compared to the controls.

3.6 IMD induces serum gelatinolytic activity in pregnant rats

As the placental expression of MMP2 and MMP9 protein was downregulated by IMD antagonist we investigated the effects of IMD antagonist on the serum gelatinolytic activity by measuring serum MMP2 and MMP9 activity using gelatin zymography. Figure 6 shows a substantial decrease in both MMP2 as well as MMP9 activity (72 kDa and 92 kDa) in the IMD₁₇₋₄₇ infused pregnant rats on day 9.

4. Discussion

IMD is a recently discovered peptide with emerging roles in reproduction. We recently reported that IMD regulates vascular tone and feto-placental growth in rats and stimulates the invasive potential of 1st trimester trophoblast cells in humans [21, 22]. However it is not known if the effects of IMD in pregnancy start as early as embryo implantation in the female. Therefore using a rat model in this study, we investigated the effects of IMD antagonist on implantation and decidual growth. We first demonstrate the expression of IMD mRNA in day 9 implantation sites and show that the expression of IMD mRNA in

placenta is significantly higher on day 15 compared to days 18 – 22. In addition, this study shows that infusion of IMD antagonist from day 3 of pregnancy caused a significant decrease in the weights of implantation sites without significant effect on the number of implantation sites. Further this IMD antagonist treatment caused a decrease in serum levels of 17 β -estradiol and progesterone. Serum nitrite levels and MMP2 gelatinolytic activity are also significantly downregulated along with a significant decrease in expression of MMP2, VEGF and PLGF proteins in implantation sites on gestational day 9. Therefore this study suggests a potential role for IMD during embryo implantation and growth via regulation of matrix metalloproteinases and perhaps NO system in early gestation in rats.

Successful implantation, placentation and subsequent gestation require coordinated vascular development and adaptation on both sides of the maternal-fetal interface. Specifically several temporally distinct vascular processes occur enabling successful pregnancy to ensue. Implantation of embryo into the uterine wall is regulated by various factors which include matrix metalloproteinases, hormones and angiogenic growth factors [15, 17, 25, 27–29]. The fertilized ova arrives in the rat uterus as blastocyst about 4.5 days after mating and embryo implantation is initiated on day 5.5 and completed on day 7.75 [6, 30]. Although many molecules are involved in the process of implantation and placental development at different time points, the specific mechanisms associated still remains to be determined. In this study, the weights of day 9 implantation sites were significantly reduced in rats infused with IMD_{17–47} from day 3, suggesting that the IMD may play a role in normal implantation and growth of decidua. This study also demonstrates that IMD is expressed in implantation sites and its expression is significantly ($p < 0.05$) greater on day 15 compared to days 18 – 22 in rat placenta coinciding with early embryonic development and placental growth. We have shown earlier that infusion of IMD antagonist on day 15 causes fetoplacental growth restriction [22]. Because the IMD inhibition does not significantly reduce the number of implantation sites and causes reduced weights in implantation sites and placenta, we suggest that IMD may be important for the growth of fetoplacental tissues.

Nitric oxide (NO) production is increased at the time when implantation begins. NO regulates production and activity of matrix metalloproteinases (MMPs) responsible for the remodeling of the uterine extracellular matrix during embryo implantation [17, 24, 31, 32]. Uterine-derived MMP2 is an essential component of implantation and initiation of placentation [14, 17, 33]. Abnormal tissue levels of MMPs are present in several pregnancy complications including spontaneous abortions and preeclampsia. The embryos also express iNOS at the peri-implantation stage [17]. It is suggested that iNOS expressed at peri-implantation would lead to enhanced NO production, which could act as a vasodilator and an angiogenic mediator. Our previous study demonstrated that IMD regulates NOS in rat placenta [22]. In this study we show that inhibition of IMD activity in early gestation in rats significantly reduces the level of NO production, measured as total nitrites in serum, (figure 4). Since IMD is shown to stimulate trophoblast invasion and migration [26] and NO levels are downregulated in IMD_{17–47} treated rat serum, we then assessed if regulators of extracellular matrix integrity, MMP2 and MMP9 and angiogenic growth factors VEGF and PLGF are altered. Figure 5 demonstrate that inhibition of IMD activity in early pregnancy significantly downregulates the protein levels of these factors in implantation sites on day 9 of gestation. Decreases in MMP2 and MMP9 protein are correlated with the decreased serum gelatinolytic activity of MMP2 and MMP9. Thus IMD through the stimulation of NO production may play a role in activating MMPs and thus implantation and growth of decidua.

Serum levels of 17 β -estradiol and progesterone are also significantly ($P < 0.05$) down regulated in the IMD antagonist treated rats (figure 3) suggesting regulation of sex steroid hormone levels by IMD in pregnant rats. Coordinated action of ovarian estrogen and

progesterone has been shown to play a major role in mediating implantation, decidualization and placental growth [27, 28]. Progesterone alone or in combination with estrogen leads to uterine stromal cell proliferation which is further potentiated by periimplantation surge in estrogen levels on day 4 of pregnancy [3, 18]. The periimplantation ovarian estrogen is also necessary for increased endometrial capillary permeability at the location of the blastocyst, a prerequisite event in the initiation of implantation. It is thought that estrogen and/or progesterone mediated events are accomplished by the expression of a unique set of genes in the uterus. While steroid hormones can directly regulate several genes including prolactin and progesterone receptors they can also modulate expression of several growth factors and their receptors in the uterus in spatiotemporal manner [34]. The decreased weights of implantation sites in IMD antagonist treated rats could be related to the reduced growth factors including VEGF and PLGF, and reduced steroid hormones may also mediate these effects. Thus, further studies involving treatments with estrogen and progesterone to the IMD₁₇₋₄₇ infused animals will identify whether specific targets of IMD actions are mediated through estrogen and progesterone. IMD is expressed in nonpregnant rat uterus [20]. In the rat after fertilization, embryo enters the uterine lumen on day 4 of pregnancy and may potentially be influenced by maternal IMD for at least 24 h before implantation on day 5. IMD₁₇₋₄₇ infusion could also affect the growth and differentiation of the embryos, and thus affect embryo's ability to implant at the specified time. Since there are no decreases in the number but only decreases in the weights of implantation sites we suggest the effects are limited to the decidualization.

5. Conclusion

Current findings suggest a regulatory role of IMD at the maternal fetal interface which may have a broader spectrum of activity at early stages of embryonic development.

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Research Highlights

- Intermedin antagonist reduces weights of rat implantation sites
- Intermedin regulates serum levels of 17- β estradiol and progesterone in rat early pregnancy
- Intermedin antagonist down regulates serum levels of nitric oxide and MMP2 gelatinase activity
- Intermedin regulates MMP2, VEGF and PLGF at the maternal fetal interface in early rat pregnancy

Figure 1.

Expression of IMD in rat placenta: RT-PCR demonstrating expression of IMD in (A) day 9 implantation site (IS) and (B) placenta on different days (D15, D18, D20 and D22) of gestation in rat. Bottom panel shows the densitometric analysis of the placental IMD mRNA expression on different days of gestation normalized to respective 18S mRNA. Bar represents means $\pm$ SEM values from five animals in each group. Asterisk (*) indicate $p < 0.05$ compared to day 15.

Figure 2.

Effect of infusion of IMD₁₇₋₄₇ on weight and number of implantation sites: Rats received a continuous infusion of IMD₁₇₋₄₇(200µg/day) or vehicle on day 3 and were sacrificed on day 9. Weights (A) and total number (B) of implantation sites were recorded. Bars are mean $\pm$ SEM values for 5 replicate animals in each group. Asterisk (*) Indicates significantly different compared with the controls.

Figure 3.

Regulation of serum levels of sex steroid hormone by IMD: 17 β -estradiol (A) and Progesterone (B) concentration were assessed by radioimmunoassay in serum collected on gestational day 9 in rats infused with IMD 17-47(200 μ g/day) or vehicle alone from day 3 (n=5 in each group). Asterisks (*) indicates significantly different compared with the controls.

Figure 4.

Effect of IMD₁₇₋₄₇ infusion on plasma nitric oxide concentration: IMD₁₇₋₄₇ was infused in pregnant rat on day 3 of gestation and serum was collected on day 9 for the assay. Inhibition of IMD activity in IMD₁₇₋₄₇ infused rats caused a significant decline in the NO production on day 9 as shown by nitrite/nitrate concentration. N=5 in each group and (*) Indicates significantly different compared with the controls.

Figure 5. Effect of IMD₁₇₋₄₇ infusion on the protein levels of MMP2, MMP9, VEGF and PLGF: Western blot analysis of total protein homogenates of day 9 implantation sites collected from rats infused with IMD₁₇₋₄₇ (200 μ g/day) or vehicle alone from day 3 of pregnancy. Top panel shows the changes in the representative protein bands of; A) MMP2 B) MMP9, C) VEGF and C) PLGF. Bottom panel shows the densitometric analysis of the proteins normalized to respective beta actin protein expression. Bar represents means $\pm$ SEM values from five animals in each group. Asterisk (*) Indicates significantly different compared with the controls.

Figure 6.

Zymogram of serum gelatinolytic activity: Serum MMP2 and MMP9 gelatinolytic activity was assessed on a zymogram in serum collected on day 9 from the rats infused with IMD₁₇₋₄₇(200µg/day) or vehicle alone from day 3 of gestation. Two µg of total serum protein was loaded. The gel picture is shown in the inverted mode for a better view. The bands shown represents clear 72Kd MMP2 and 92Kd MMP9 bands against a blue background (n=5 in each group).