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MODULATION OF AMNIOTIC FLUID ACTIVIN-A AND INHIBIN-A IN WOMEN WITH PRETERM PREMATURE RUPTURE OF THE MEMBRANES AND INFECTION-INDUCED PRETERM BIRTH

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Abstract

PROBLEM—Activins and inhibins are important modulators of inflammatory processes. We explored activation of amniotic fluid (AF) activin-A and inhibin-A system in women with intra-amniotic infection and preterm premature rupture of the membranes (PPROM).

METHOD OF STUDY—We analyzed 78 AF samples: “2nd trimester-control” (n=12), “3rd trimester-control” (n=14), preterm labor with intact membranes [positive-AF-cultures (n=13), negative-AF-cultures (n=13)] and PPRM [positive-AF-cultures (n=13), negative-AF-cultures (n=13)]. Activin-A levels were evaluated *ex-vivo* following incubation of amniochorion and placental villous explants with Gram-negative (LPS) or Gram-positive (Pam3Cys) bacterial mimics. Ability of recombinant activin-A and inhibin-A to modulate inflammatory reactions in fetal membranes was explored through explants’ IL-8 release.

RESULTS—Activin-A and inhibin-A were present in human AF and were gestational age-regulated. Activin-A was significantly upregulated by infection. Lower inhibin-A levels were seen in PPRM. LPS elicited release of activin-A from amniochorion, but not from villous explants. Recombinant activin-A stimulated IL-8 release from amniochorion, an effect that was not reversed by inhibin-A.

CONCLUSION—Human AF activin-A and inhibin-A are involved in biological processes linked to intra-amniotic infection/inflammation induced preterm birth.

Keywords

activin; inhibin; preterm labor; infection; fetal membranes; inflammation; endotoxin

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CONTRIBUTIONS TO AUTHORSHIP

VR, IAB and CSB formulated the hypothesis and designed the study. CSB, IAB and VR drafted the manuscript. VR, ATD, SAR, CD, HL, CMP and CSB recruited patients, collected biological specimens and followed the patients prospectively to the point of delivery. VR, CSB and IAB collected, analyzed, and interpreted the demographic data. VR, ATD, CSB and IAB designed and performed the placental and amnio-chorion explant experiments. ATD, EO and SAR conducted the ELISA assays. All the co-authors participated with aspects of study design, critical interpretation of the data, contributed to writing of the paper and have reviewed and approved the final version.

INTRODUCTION

Activins and inhibins are hormone members of the transforming-growth-factor- β family, and are hetero- or homo- dimers comprised of one α -subunit and one of four possible β -subunit isoforms (β A, β B, β C and β E).¹ Activin-A constitutes a homodimer of two β A subunits (β A β A). The heterodimer of α and β A subunits constitutes inhibin-A ($\alpha\beta$ A). The bioavailability of activin-A is dictated by follistatin, and follistatin-related proteins that function as ligand traps and prevent activin interaction with its own receptors.² Inhibin-A has traditionally been considered a reproductive hormone. Its main function is to abrogate the role of activins through binding to the activin type-II-receptors, ActRIIA and ActRIIB.³ However, the inhibin receptor model suggests that inhibin engages its own receptor, triggering intra-cellular signaling events independent of activin.⁴ While this concept remains to be demonstrated, there is evidence that only the unbound (free) fractions of activin-A and inhibin-A are biologically active.^{4,5}

Activin-A and inhibin-A are important para/autocrine regulators of various biological processes related to steroidogenesis⁶, cell proliferation and differentiation. They act upon a variety of tissues including the prostate, lung, kidney and reproductive tissues such as the uterus and gonads.^{1,4} Of particular interest is their role in modulating innate and adaptive immunity.^{7,8} The versatile role of activins and inhibins in regulating the function of neutrophils, monocytes, macrophages, mast cells, natural killer cells, and type B and T lymphocytes, was demonstrated *in-vitro*.^{7,8,9} In monocyte and macrophage cellular lines, activin-A stimulated the production of IL-1 β , IL-6 and TNF- α .¹⁰ Activin-A inhibited conversion of the IL-1 β precursor to an active cytokine form in human monocytes¹¹, and exhibited a stimulatory or inhibitory effect on IL-6 production by human amniocytes at low and high doses, respectively.¹² In addition, a cross-talk between Toll-like receptor-4 (TLR-4) and activins was demonstrated in animal models of sepsis.¹³ Activin-A levels increased rapidly in response to LPS and amplified the production of pro-inflammatory cytokines.¹³ All of the above support a key role for activin-A, and implicitly, for inhibin-A in inflammatory conditions. Given that both activin-A and inhibin-A modulate the activity and expression levels of several matrix-metalloproteinase (MMPs), important roles in tissue remodeling have been proposed.¹⁴

In human gestation, the placenta, decidua and fetal membranes are important sites of activin-A and inhibin-A synthesis and release.^{15,16} Petraglia et al. reported that spontaneous term and preterm labor are characterized by increased synthesis and release of activin-A in AF and by an augmented expression of ActRIIB in the amniochorion.¹⁷ Patients with intra-amniotic infection were specifically excluded from the study.¹⁷ The presence of inhibin-A in human AF has also been reported.¹⁸ Yet, although they are highly regarded as regulators of inflammation and immunity, little is known about activin-A or inhibin-A in the AF of women with intra-amniotic infection, inflammation or preterm premature rupture of membranes (PPROM).

Herein, we therefore test the hypothesis that AF levels of biologically active forms of activin-A and inhibin-A are upregulated in response to intra-amniotic infection. We found significant gestational age (GA) and inflammation-dependent regulation of AF activin-A levels. Inhibin-A however, varied with membrane status alone. Biological plausibility for our *in-vivo* results was tested *in-vitro* by using an amniochorion and placental explant system of LPS-induced inflammation.

MATERIALS AND METHODS

Patient population, procedures and study design

We analyzed AF samples retrieved by trans-abdominal amniocentesis from 78 women with singleton gestations. In a cross-sectional study design, women were stratified into the following groups: i) “2nd trimester control” [normal karyotype, n=12, GA median, interquartile range [IQR]: 19 [18-20] weeks); ii) “3rd trimester control” (lung maturity, n=14, GA: 36 [36-37] weeks); iii) preterm labor with intact membranes and either positive (n=13, GA: 25 [24-28] weeks), or negative (n=13, GA: 27 [25-31]) AF culture results; iv) PPROM and either positive (n=13, GA: 29 [28-32] weeks) or negative (n=13, GA: 31 [30-32] weeks) AF culture results. Following amniocentesis (February 2004 to September 2008) all women were followed prospectively to the point of delivery at Yale-New Haven Hospital. Women presenting with symptoms of preterm labor or PPROM had a clinically indicated amniocentesis to rule-out infection. All preterm labor women with intact membranes and negative AF cultures delivered a healthy baby at term. Their AF samples were used along with the genetic and lung maturity samples to determine the GA-regulation of AF activin-A and inhibin-A. The Human Investigation Committee of Yale University approved our research protocol. All the participants provided signed informed consent.

Gestational age was established based on last menstrual period and ultrasonographic examination prior to 20 weeks. Details regarding the clinical indications for amniocenteses, exclusion criteria, definitions of preterm labor and PPROM, and clinical protocols employed for clinical care of patients are provided in Supplemental Data.

Evaluation of intra-amniotic infection/inflammation

The biochemical and microbiological studies used for clinical diagnosis of AF infection and inflammation are presented in the Supplemental Data. For research purposes, a diagnosis of intra-amniotic inflammation was established using Surface-Enhanced Laser Desorption Ionization Time-of-Flight (SELDI-TOF) mass spectrometry. Our choice was based on previous studies which determined that the Mass Restricted (MR) score was the most accurate test for diagnosing intra-amniotic inflammation (see Supplemental Data).^{19,40} The MR score ranges from 0 to 4. MR score 0 (zero) indicates “no” intra-amniotic inflammation; MR score 1-2 indicates “minimal” inflammation; MR score 3-4 indicates “severe” inflammation.¹⁹ The intensity of the intra-amniotic inflammatory response to infection was also determined through the levels of the pro-inflammatory cytokine IL-6 (see below).

Histological evaluation of the inflammatory status of the placenta and fetal membranes

Paraffin-embedded tissue sections were available from all cases that delivered preterm with either positive (n=26) or negative (n=13) AF culture results. Details describing the histological criteria used to quantify the degree of histological inflammation are provided in Supplemental Data.

Activin-A, inhibin-A IL-6 and IL-8 immunoassay procedures

ELISA assays for human bioactive activin-A and inhibin-A were performed according to the manufacturer’s instructions (Diagnostic Systems Laboratories, Inc, Webster, TX). ELISA systems for IL-6 (Pierce-Endogen, Rockford, IL) and IL-8 (R&D Systems, Minneapolis, MN) were used to evaluate their levels in AF or explant medium, respectively. For details see Supplemental Data.

Villous trophoblast and amniochorion explant cultures

Our *in-vivo* data determined that infection and inflammation are associated with significant changes in AF activin-A but not inhibin-A. To determine whether specific molecular components of the bacterial wall trigger inflammatory events were associated with the observed changes in the AF levels of activin-A, we conducted *ex-vivo* experiments in placental villous and amniochorion tissue cultures from term elective Cesarean deliveries (GA: 39 [38-40] weeks, n=4). After 1, 4, 18 and 24h of incubation, we quantified activin-A levels in explant medium after exposure to Gram-negative (LPS: 1µg/mL) or Gram-positive (PAM3Cys: 1µg/mL) bacterial mimics.²⁰ Amniochorion explants were also challenged for 18h with recombinant activin-A (5 and 50 ng/mL) alone or combined with recombinant inhibin-A (50 ng/mL). The intensity of the elicited inflammatory response was assessed *in-vitro* through IL-8 release (an NFκB-inducible chemokine involved in inflammatory cell recruitment). Details are presented in the Supplemental Data.

Statistical analysis

To identify the GA point which separates the levels of activin-A and inhibin-A into two clusters (“low” vs. “high”), we used a two-step clustering method complemented by receiver operating curve analysis.²¹ A *P* value of <0.05 was considered statistically significant. Details about other statistical methods used in the current study are provided in the Supplemental Data.

RESULTS

Clinical, laboratory, outcome characteristics, and results of histological examination of the placenta for the study population. Demographic and outcome characteristics, the results of the AF tests (clinical and research) and of the histological examination of the placenta for our study population are presented in Supplemental Data.

Gestational age regulation of AF activin-A, inhibin-A and their ratio

Figure 1 displays the pattern of GA regulation of activin-A, inhibin-A and their ratio in AF of pregnancies with normal outcomes. The best fitted equations corresponded to 3rd order polynomial interpolation of log-transformed values for both activin-A and inhibin-A (Figure 1). As shown in Figure 1A there was an inverse relationship between activin-A and GA (linear regression $R=-0.760$, $P<0.001$) with significantly lower levels of this hormone near term (2nd trimester genetic, median [IQR]: 705 [600-803] vs. preterm labor normal outcome 455 [317-529] vs. 3rd trimester lung maturity: 292 [254-456] pg/mL, Kruskal-Wallis ANOVA $P<0.001$). The transitional point between “high” versus “low” AF activin-A levels occurred at 23 weeks GA. In contrast to activin-A, the AF level of inhibin-A (Figure 1B) increased up to 30 weeks GA followed by a decrease near term (2nd trimester genetic: 660 [275-1,016] vs. preterm labor normal outcome: 1,306 [916-2,410] vs. 3rd trimester lung maturity: 1,102 [378-1,945] pg/mL, $P=0.015$). The ratio of activin-A:inhibin-A varied inversely with GA ($R=-0.491$, $P=0.002$), although the 3rd order polynomial variation was a significantly better fit (Figure 1C). Our data analysis suggests that in early human gestation, there is excess of AF activin-A over inhibin-A, whereas after 20 weeks GA the levels of inhibin A exceed those of activin-A (2nd trimester genetic: 1.28 [0.68-1.99] vs. preterm labor normal outcome: 0.23 [0.18-0.49] vs. 3rd trimester lung maturity: 0.29 [0.18-0.69] pg/mL, $P<0.001$).

Effect of infection and membrane status on amniotic fluid activin-A and inhibin-A

Figure 2A demonstrates that the level of AF activin-A was upregulated in women with intra-amniotic infection independent of membrane status (negative cultures & intact membranes:

455 [316-539], negative cultures & PPROM: 417 [259-689]; positive cultures & intact membranes: 1,806 [1,632-3,393]; positive cultures & PPROM: 1,524 [660-3,730] pg/mL, 2-way ANOVA: infection $P<0.001$; PPROM $P=0.405$; interaction $P=0.286$). Conversely, the levels of inhibin-A varied with the status of the membranes, but not with AF infection (Figure 2B, negative cultures & intact membranes: 1,306 [898-2,442], negative cultures & PPROM: 459 [284-1,198]; positive cultures & intact membranes: 1,030 [477-1,524]; positive cultures & PPROM: 550 [251-1,346] pg/mL, 2-way ANOVA: infection $P=0.400$; PPROM $P=0.006$; interaction $P=0.433$). As shown, the levels of inhibin-A were significantly lower in women with PPROM. Figure 2C shows that the activin-A:inhibin-A ratio varies primarily with AF infection (negative cultures & intact membranes: 0.23 [0.17-0.49], negative cultures & PPROM: 0.64 [0.28-2.69]; positive cultures & intact membranes: 2.27 [1.11-5.26]; positive cultures & PPROM: 1.58 [1.17-10.05], 2-way ANOVA: infection $P<0.001$; PPROM $P=0.101$; interaction $P=0.231$). In the absence of infection, the ratio was significantly increased in women with PPROM driven by the decrease in inhibin-A ($P=0.047$). The type of AF bacteria did not impact on levels of either activin-A or inhibin-A. These relationships were maintained following correction for GA.

Relationship between amniotic fluid activin-A, inhibin-A, intra-amniotic inflammation and histological chorioamnionitis

Figure 3A displays the relationship between severity of intra-amniotic inflammation as determined by the proteomics MR score and the AF level of activin-A. Women with severe inflammation (MR 3-4) had significantly elevated activin-A concentrations compared to women with either mild (MR 1-2) or absent (MR 0) inflammation ($P<0.001$). A direct correlation was seen between activin-A and IL-6 (Figure 3B, Pearson $R=0.755$, $P<0.001$). Among histological variables, the severity of choriodecidual inflammation had the strongest correlation with AF activin-A levels in patients who delivered preterm (Spearman $R=0.595$, $P<0.001$). In multivariate analysis, AF activin-A levels related best with the combination of GA at amniocentesis and AF IL-6 (GA: $P=0.023$, IL-6 $P<0.001$). Variables excluded from the model were the MR score, histological variables, GA at delivery and amniocentesis-to-delivery interval ($P>0.1$ for all). No relationship was found between inhibin-A's concentration and AF inflammatory status as evaluated by the proteomics MR score ($R=-0.086$, $P=0.546$) or IL-6 ($R=-0.195$, $P=0.165$). In contrast to activin-A, AF inhibin-A levels did not correlate with the severity of histological choriodecidualitis ($R=-0.043$, $P=0.796$).

Effect of bacterial mimics on activin-A release ex-vivo

Stimulation of amniochorion explants, but not placental villous tissue, with LPS and PAM3Cys, resulted in significant upregulation of activin-A with maximal effect at 18h (Figure 4A, basal vs. both treatments $P<0.05$). Interestingly, both LPS and PAM3Cys were able to elicit IL-8 release from both placental villous tissue and amniochorion (Figure 4B basal vs. both treatments $P<0.01$) which implies tissue responsiveness. Recombinant activin-A alone was able to stimulate the release of IL-8 from amniochorion at both tested doses (Figure 4C, $P<0.05$). However, at a dose equivalent with that utilized for activin-A, inhibin-A was unable to impact on the release in IL-8 elicited by challenge of the amniochorion with either activin-A or LPS (Figure 4D, $P>0.05$).

DISCUSSION

Previous research has established that in physiologic pregnancy the decidua, placenta and fetal membranes are the main sites for the synthesis and secretion of activin-A and inhibin-A.^{15,22} However, most of our knowledge about human AF activin-A and inhibin-A is based on published reports which focused on their regulation in early pregnancy (8-20 weeks

GA).^{17,18,23,24,25,26,27} A common denominator for two of the referenced studies was that the levels of activin-A were higher in celomic fluid than in AF.^{26,27} At 8-11 weeks, inhibin-A was undetectable in the AF.¹⁸ Muttukrishana *et al.* reported detectable levels of “total” activin-A from 15 to 20 weeks of gestation, but its concentration remained unchanged for the aforementioned GA interval.²⁵ A steady increase in the AF levels of inhibin-A from 15 to 18 weeks, was described by Wallace *et al.*.^{23,24} Petraglia and colleagues determined that between 23 to 40 weeks of gestation, activin-A was assayable in the AF of women with normal pregnancy outcomes.¹⁷ Because their data was not analyzed based on GA intervals, it remained unknown how the levels of AF activin-A and inhibin-A varied across human gestation.¹⁷ The current study intended to fill this gap. By using antibodies designed to detect the bioactive forms of the two hormones, we found that the AF levels of activin-A and inhibin-A follow independent and mostly divergent patterns. These changes impacted on their ratio with excess activin-A over inhibin-A at the beginning of pregnancy. The transition between the “low” vs. “high” AF levels of activin-A and inhibin-A was different for each variable and occurred at a later GA for inhibin-A. Our view is that differences among secretor, metabolic and molecular traffic activities of the placenta, fetal membrane and fetal compartment at various GAs are possible explanations for the current results. Both glycoprotein hormones play roles in cell proliferation and differentiation, erythropoiesis, vasculogenesis, steroidogenesis, prostaglandin synthesis and release of progesterone.¹⁵ Whether or not in normal human pregnancy the AF activin-A and inhibin-A carry any or all of their biological functions remains an open question. A daunting aspect of studying normal AF is the diversity of mediators and sensors, but also the complexity of the pathways they represent. The mechanisms through which activin-A and inhibin-A exercise each of their physiologic actions are for the most part known. Yet, the end result of their coordinated function in the context of multiple points of control and redundancy with other AF metabolic and hormonal networks remains poorly defined, and future studies should provide answer to these questions.

Ascribing the role of activin-A and inhibin-A to a single biological function is impossible.¹⁵ Here we focused our attention on activin and inhibin as components of the complex immune signaling network triggered by infection. The concept that activin-A and inhibin-A are genuine cytokines with important regulatory roles of the immune system²⁸ is supported by the evidence that: 1). activin-A is elevated in patients with Gram-positive and Gram-negative bacterial sepsis;²⁹ 2). in animal models of endotoxin-induced sepsis, LPS administration is followed by an outpouring of activin-A and of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6³⁰; 3). *in-vivo*, engagement of TLR-2 by Gram-positive bacteria or by PAM3Cys augments the systemic levels of activin-A within the first hour after challenge.^{30,31} In human pregnancy, there is a paucity of data in this particular area of research. Petraglia was the first to note that the AF activin-A level in women who delivered preterm was significantly elevated compared to non-laboring controls.¹⁷ As the authors centered their interest on parturition, and in particular on the ability of the activin-A to increase the release of prostaglandins, infection was excluded as a confounding variable.¹⁷ From this perspective our findings are novel because they establish that *in-vivo*, both Gram-negative and Gram-positive bacteria trigger an intra-amniotic inflammatory response characterized by increased levels of activin-A, but not of inhibin-A. By using a mechanistic approach, we confirmed that *in-vitro*, LPS stimulated the production of activin-A by the amniochorion, but not by villous trophoblast. These findings imply that in pregnancies complicated by intra-amniotic infection, fetal membranes are a rich source of activin-A. Notably, in prior *ex-vivo* experiments, Keelan *et al.* established that LPS had a high stimulatory effect on choriodecidual, but not amnion production of activin-A.³² Our analysis found a significant correlation between the severity of choriodecidual inflammation and the AF levels of activin-A, which provides additional support for the premise that inflammatory events occurring in the choriodecidua are linked to an increase synthesis of activin-A.

Furthermore, TLR-2 is expressed by both human amnion and chorion. ³³ In our study, incubation of the fetal membranes, but not placental villous tissue, with the specific TLR-2 agonist PAM3Cys2, significantly enhanced the levels of activin-A in the explant conditioned medium. As such, we provide first hand evidence that Gram-positive microbes have the potential to augment the AF levels of activin-A, at least in part through activation of amniochorion's TLR-2 signaling pathway.

The results we obtained for free activin-A following incubation of the placental tissues with LPS and PAM3Cys2 were provocative. That is, TLR-4 and TLR-2 seemed to be variably expressed by syncytiotrophoblasts and other resident cells of the placenta (i.e. Hofbauer cells, endothelial cells). ³⁴ As shown here, challenging the placental villous tissue with both LPS and PAM3Cys2 promoted synthesis and release of IL-8, arguing that placental TLRs were actively engaged. It is conceivable, although not yet specifically addressed, that the placental cells which express TLRs are primarily designed to mount an inflammatory response to infection. These cells may lack or have, for unknown reasons, suppressed the molecular machinery in charge of increasing the expression and synthesis of activin-A, over the baseline. The divergent response of the fetal membranes over the placenta could be also explained by differences in synthesis and release of follistatin. ² Increased placental production of follistatin over activin-A, may render the latter less detectable by ELISA. Yet, in the light of prior work by Keelan *et. al.* ³², who showed that LPS exerted a limited effect on fetal membranes production of follistatin, this is a less probable explanation. Besides, given that inhibin-A was not upregulated *in-vitro* by an infectious stimulus, Keelan's work ³² was consistent with our data which indicates a lack of association between infection and AF concentration of inhibin-A. So far, we found lower AF levels of inhibin-A and higher activin-A:inhibin-A ratios in PPRM. Increased collagenolytic activity of matrix metalloproteinases in amniochorionic membranes is paramount for the occurrence of PPRM, even in the absence of infection. ³⁵ During this process collagenases cleave the fetal membranes fibrillar collagens which are then further degraded by the gelatinases MMP-2 and MMP-9. ^{36,37} It is known that activin-A stimulates the production and activation of MMP-2 and MMP-9, while inhibin-A opposes these effects. ¹⁴ Given that activin receptors were localized in amnion's epithelial cells, ¹⁷ mesenchyme and chorionic trophoblast, ³⁸ we propose that the inability of the host to synthesize and/or secrete inhibin-A (i.e genetic determinants), or an AF ratio that favors activin-A over inhibin-A, may facilitate PPRM.

We observed that in pregnancies complicated by intra-amniotic infection, the AF concentration of activin-A was highly correlated with the intensity of the inflammatory response as depicted by the proteomics MR score and IL-6. In the setting of intra-amniotic infection, the diversity of the AF anti-microbial peptides, cytokines, chemokines and that of the immune cells populating the gestational sac and its membranes is extremely vast. ^{39,40,41} Our results suggest that activin-A is a dynamic participant of this inflammatory process. As is typical of a host-mediated protective immune response against microbes, neutrophils traffic the decidua, fetal membranes and AF, promoting inflammation. ³⁹ Recent data established that TNF- α stimulated human neutrophils to release preformed activin-A, independent of LPS. ⁹ Thus, further consideration should be given to the possibility that anti-microbial peptides, TNF- α , IL-1 β and IL-8 may on their own contribute to the surge in AF activin-A by facilitating its release from immune cells known to constitutively express this glycoprotein (i.e neutrophils, monocytes, macrophages, mast cells). ⁸ Remarkably, as a multifunctional molecule, activin-A plays a role in modulating the process of cytokine release. ^{1,4,8} Depending on the context of expression, activin-A might have pro- or anti-inflammatory properties. ¹² Given the character of the innate immune response to intra-amniotic infection, here we focused our attention of the pro-inflammatory role of activin-A. Extending the findings of Keelan *et. al.* ¹² we demonstrated that incubation of the

amniochorion with activin-A resulted in a significant upregulation of IL-8, but this effect could not be reversed by inhibin-A at similar doses. Lastly, LPS stimulated the release of IL-8 at a much high order of magnitude compared to activin-A. Again, inhibin-A had no LPS inhibitory effect. First, these results strengthen the argument that both in the AF and fetal membranes inhibin-A may possess biological roles different than abrogation of activin-A's pro-inflammatory function. Second, this data provides proof of concept that engagement of TLRs may lead to a more pronounced pro-inflammatory reaction, compared to activation of activin-A's receptors. Characterizations of the downstream inflammatory events that are secondary to simultaneous activation of the TLR and activin-A pathways require further investigation.

In summary, we demonstrated that across human gestation, the levels of AF activin-A, inhibin-A and their ratio are GA dependent. In our evaluation of pregnancies complicated by PTB, we noted that the AF levels of activin-A changed in relationship to intra-amniotic infection and inflammation, whereas inhibin-A concentrations related primarily to the status of the fetal membranes. Our *ex-vivo* experiments provided support for the view, that activin-A is a dynamic participant of the intra-amniotic inflammatory response triggered by both Gram-negative and Gram-positive pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. AF levels of activin-A, inhibin-A and their ratio in pregnancies with normal outcomes (n=38)

The best fitted equations for both activin-A and inhibin-A corresponded to 3rd order non-linear interpolations of log-transformed AF levels of each of the analytes. Levels of activin-A were inversely related with GA (**A**). Inhibin-A had unimodal variation peaking at ~30 weeks GA (**B**) while their relative ratio decreased steadily until ~30 weeks and then increased toward term (**C**). Thick continuous line: 3rd order regression line; thin continuous lines: 95% confidence intervals; dotted lines: 95% prediction intervals.

Figure 2. Effect of intra-amniotic infection on AF levels of activin-A, inhibin-A and their ratio in women within the rule-out infection group (n=52)

Intra-amniotic infection upregulated the level of activin-A independent of membrane status ($P<0.001$) (A); AF levels of inhibin-A were lower in women with PPROM independent of infection ($P=0.006$) (B); AF activin-A:inhibin-A ratio was elevated in PPROM women and negative cultures and further increased in women with infection ($P<0.001$) (C); The figure displays data in logarithmic format. The ends of the boxes define the 25th and 75th percentiles, the line inside the box defines the median and the whiskers show the largest and smallest values. Data analyzed by 2-way ANOVA followed by Holms-Sidak post-hoc tests. Groups with at least one common letter are not statistically different at $P\geq 0.05$. neg, negative; pos, positive;

Figure 3. Relationship between indicators of intra-amniotic inflammation on AF levels of activin-A in women within the rule-out infection group (n=52)

An AF MR 3-4 was associated with a significant upregulation in the AF levels of activin-A, compared with women with either MR 1-2 or MR 0. Activin-A data is displayed in logarithmic format. The ends of the boxes define the 25th and 75th percentiles, the line inside the box defines the median and the whiskers show the largest and smallest values. Different letters indicate statistical significant difference (Kruskal-Wallis ANOVA followed by Dunn's post-hoc tests) (A); Regression analysis demonstrating a direct relationship between AF activin-A and IL-6 (in logarithmic format). The thick continuous line represents the linear regression line; thin continuous lines: 95% confidence intervals; dotted lines: 95% prediction intervals (B).

Figure 4. Ex-vivo production of activin-A and IL-8 in the placental villous and amniochorion explants

LPS and PAM3Cys stimulated the release of activin-A from amniochorion explants (basal vs. LPS $P=0.032$, basal vs. PAM3Cys $P=0.018$). There was no effect on placental explants ($P>0.05$) (**A**); Both bacterial mimics stimulated the release of IL-8 in both placental villous tissue (basal vs. LPS $P=0.003$, basal vs. PAM3Cys $P=0.001$) and amniochorion explants (basal vs. LPS $P=0.008$, basal vs. PAM3Cys $P=0.006$) (**B**); Incubation of the amniochorion with recombinant activin-A stimulated IL-8 release (basal vs. activin-A 5 ng/mL [ACT5], $P=0.044$ and basal vs. activin-A 50ng/mL [ACT50], $P=0.039$) (**C**); Addition of recombinant inhibin-A (50 ng/mL, INH) did not reverse the stimulatory effect of activin-A (50 ng/mL, ACT) or LPS (1 μ g/mL) on IL-8 release. Data presented as mean+SEM of the % change from basal analyte level at 18 hours and analyzed by one-way ANOVA followed by Holms-Sidak tests. Means with different superscripts are statistically different ($P<0.05$).