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THE ROLE OF TRANSFORMING GROWTH FACTOR β IN CERVICAL REMODELING WITHIN THE RAT CERVIX

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Abstract

Objective—Transforming growth factor β , (TGF β) plays a central role in extracellular matrix remodeling. We hypothesized that TGF β signaling is involved in cervical remodeling. This study evaluates patterns within this signaling pathway.

Study Design—The cervix of non pregnant and timed pregnant rats were obtained. mRNA expression of TGF β 1, TGF β -Receptor 1 (T β R1), T β R2, and T β R3 was evaluated. Four animals were sacrificed for each time point. Western blotting was performed for protein expression. Smad2 and 3 phosphorylation were assessed to evaluate TGF β activation.

Results—TGF β 1 mRNA increased through day 21 and declined on day 22 (ANOVA $p=0.001$). T β R1 expression was unchanged. T β R2 and T β R3 mRNA expression was similar to TGF β 1. T β R3 protein expression was similar to mRNA. Smad2 phosphorylation paralleled changes in T β R3.

Conclusion—Components of the TGF β signaling pathway increase during pregnancy along with Smad2 activation. The decline on day 22 correlates with a transition to the ripening phase supporting a role in cervical remodeling.

Keywords

Cervix; Cervical remodeling; Extracellular Matrix; TGF β

Introduction

Preterm birth is the leading cause of neonatal morbidity and mortality within the United States after exclusion of congenital malformations.¹ Unfortunately, rates of preterm birth have increased by more than 20% since 1990 to a rate of 12.7 in 2005² even though tocolytic agents were commonly used. Although many theories exist, the etiology for preterm birth is multifactorial. Recent studies suggest that identification of early cervical change may provide

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opportunities for earlier intervention.^{3,4} However, our ability to develop new intervention strategies is limited by our understanding of processes involved in cervical remodeling during pregnancy. In other remodeling processes the multifunctional cytokine Transforming Growth Factor β (TGF β) plays an integral role.^{5–10} It is its role in other tissues that has led us to further investigate its potential function within the cervix.

TGF β 1 is the prototypical cytokine in the TGF β superfamily that is involved in regulating cell proliferation, apoptosis, and gene expression.¹¹ It has been shown to specifically regulate the production of extracellular matrix (ECM) molecules such as collagen, proteoglycans, and fibronectin.¹² TGF β activates a variety of signaling pathways, including components of the Smad pathway that can increase or suppress gene expression. Through this pathway increased levels of TGF β have been demonstrated to produce cardiac fibrosis, hepatic fibrosis and keloid formation.^{6,9,10} Animal models have also confirmed that receptor inhibition can blunt the fibrotic response.¹³ In addition to regulating matrix production TGF β regulates tissue turnover by regulating the expression of matrix degrading enzymes including some matrix metalloproteinases (MMP).¹⁴

TGF β signals through a family of transmembrane receptors that activate the Smad family of signal transduction molecules.¹⁵ The specific activity of TGF β is tissue and cell type dependent.¹² TGF β activation of its receptors requires the interaction of Type I and II TGF β receptors (T β R1, T β R2). In addition to T β R1 and T β R2, the Type III receptor (T β R3) regulates T β R1 and T β R2 activity by increasing the affinity of TGF β ligands for T β R1 and T β R2.⁸ Ligand receptor binding results in phosphorylation of Smad signaling proteins. The Smad protein family consist of receptor regulated Smad proteins (R Smads) and inhibitory Smad proteins (I Smads). The primary R Smads include Smad2 and Smad3. The R Smad protein complex translocates to the nucleus to activate gene transcription increasing the production of extracellular matrix proteins.

This study aims to determine if the TGF β signaling pathway is active in the cervix during pregnancy. Considering the known effects of TGF β in other tissue, we hypothesized that TGF β 1 and its receptors are regulated during pregnancy and that this regulation is associated with Smad activation.

Materials and Methods

Tissue harvesting

Timed pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Day 1 was defined as the day after mating when animals were found to be sperm positive. Animals typically deliver on the evening of day 22 or morning of day 23. Four rats were sacrificed on day 12, 16, 18, 20, 21 and 22. In addition, four cervixes were obtained from non pregnant rats. Animals were handled daily until tissue harvest. All procedures were approved by the Joint Institutional Animal Care and Utilization Committee formed by Rhode Island Hospital and Women and Infants' Hospital of Rhode Island. All cervical tissue was harvested under pentobarbital sodium anesthesia. The cervix was separated from the uterus below the bifurcation of the uterine horns; care was taken to exclude the more muscular portion above the mainly fibrous cervix.¹⁶ The tissue was rinsed in normal saline solution and then frozen in liquid nitrogen and stored at -80°C until ready for use.

Cervical mRNA Expression of TGF β and Receptors

The expression of TGF β 1 and its receptors (T β R1, 2, and 3) were evaluated in cervical tissue by quantitative RT-PCR. Total RNA was extracted from cervical tissue using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Total RNA was converted

to cDNA as previously described.¹⁶ mRNA for each target gene was quantified by real-time RT-PCR using commercially available 5' ednonuclease assays (Table 1) with β -actin as an internal reference in a multiplex reaction. All samples were run in triplicate. Relative expression was determined using the standard curve method.

Cervical Protein Expression of TGF β Receptors

Protein expression of T β R1, T β R2, and T β R3 was performed by Western blot using Laemmli technique. Cervical proteins were prepared by homogenization as previously described.¹⁶ Specific proteins were identified by immunoblotting using commercially available antibodies (Table 1). Target proteins were quantified by densitometry. Bands were imaged and captured using a transilluminator and Bio-rad Gel Doc System (Bio-rad, Hercules, CA) in a tiff format and analyzed using the Quantity One software package (Bio-Rad). Differences in loading were normalized to β -actin after reprobing blots.

TGF β Signaling Activation

In order to determine whether Smad signaling pathway was active within the rat cervix, Western blot analysis for total and phosphorylated Smad2 and Smad3 was performed using commercially available antibodies (Table 1). Protein expression was quantified by densitometry. Results were reported relative to β -actin.

Immunohistochemistry was performed on non-pregnant and day 20 cervical specimens. Cervical tissues were excised and fixed with 4% paraformaldehyde and then paraffin embedded. Transverse sections were cut at a thickness of 5 microns. Tissue sections were probed with Smad2 specific antibody (Table 1). Biotinylated secondary antibody followed by ABC (avidin-biotin complex) was used to identify antibody protein interactions. Slides were rinsed with dH₂O. 3,3'-Diaminobenzidine (DAB) staining was used for detection of immune complexes. Counterstaining was performed with methyl green pyronin.

Data Analysis

Data was compiled using Microsoft Excel (Microsoft, Bothel, WA) and analyzed using Sigma Stat statistical software (Jandel Scientific, San Rafael, CA). Descriptive statistics were performed. Means and standard errors were calculated for all treatment groups and controls. Timed pregnant comparisons were performed using ANOVA with appropriate multiple comparison tests when indicated. Statistical significance was defined as a $P < 0.05$.

Results

Cervical TGF β 1 and Receptor mRNA Expression

The expression of TGF β 1 was evaluated by RT-PCR. TGF β 1 is known to be transcriptionally regulated. TGF β 1 expression is depicted in Figure 1. TGF β 1 mRNA expression increased through gestation peaking on day 21 prior to declining on day 22. This trend was found to be significant by ANOVA with $p = 0.001$. Multiple comparisons testing showed the decline on day 22 to be significantly different from the peak mRNA expression observed on day 21. The increase in mRNA expression on day 16 followed by a decrease on day 18 could not be explained.

We next evaluated the expression of the three TGF β receptors. No trend was observed for T β R1 (ANOVA, $p = 0.091$). The mRNA expression of T β R2 and T β R3 increased over gestation (Figure 2 and 3). The mRNA expression of T β R2 was found to increase from day 12 through day 21 prior to declining on day 22 of gestation (Figure 2). This change in mRNA expression was significant across gestational age with an ANOVA of $p = 0.006$. Multiple comparisons testing did not reveal a significant difference between the peak mRNA expression on day 21

and the decline on day 22. The mRNA expression of T β R3 in timed pregnant rat cervixes was similar to that seen for T β R2. T β R3 mRNA expression increased through gestation before reaching a plateau on day 18 then declining on Day 22 (Figure 3). This trend was significant by ANOVA ($p=0.001$). Multiple comparisons testing indicated a significant difference between the peak on day 18 and the nadir on day 22.

Cervical T β R2 and T β R3 Protein Expression

In order to confirm changes in mRNA expression correlated with a change in protein expression, Western blots were performed for T β R2 and T β R3. No change in T β R2 expression was observed (data not shown). T β R3 protein expression mirrored the changes in mRNA (Figure 3). The observed differences were significant by ANOVA ($p=0.016$).

Cervical TGF β Signal Activation

Finally, to determine if TGF β signaling pathway was activated in cervical tissue we evaluated the expression of Smad2 and Smad3 which are known to be activated by TGF β 1. Western blotting was performed for both Smad2 and Smad3. No change in total Smad3 protein expression was observed. To confirm the absence of increased Smad3 activation we performed Western blots for phosphorylated Smad3 and found no changes in phosphorylated Smad3 protein expression.

In contrast, total Smad2 protein expression demonstrated significant changes over gestation. Using phosphorylation specific antibodies for Smad2 we confirmed an increase in phosphorylated Smad2 expression over gestation (Figure 4). The overall trend in phosphorylated Smad2 was similar to what was seen for TGF β 1 as well as T β R2 and T β R3 increasing through gestation then declining on day 22 (ANOVA, $p=0.005$). To localize Smad2 expression within the various cell types in the cervix, immunohistochemistry was performed using Smad2 specific antibodies. Figure 5 depicts Smad2 localization within cervical stromal cells by immunohistochemistry. Smad2 specific antibodies were used to probe tissue sections of cervical tissue from non-pregnant rats and gestational day 20. Smad2 (brown stain) was localized to both the endocervical canal and the cervical stroma. The intensity of staining was greater on day 20 within the cervical stroma and diminished in the endocervical cells. These findings were consistent with quantitative Western analysis for total Smad2.

Discussion

In 2006, the Institute of Medicine recognized the importance of understanding cervical factors and their contribution to preterm birth.¹⁷ In order to better understand the cervical remodeling that occurs throughout gestation, our studies have focused on what factors may be responsible for the changes in cervical resistance that are occurring throughout gestation. It has long been recognized that the extracellular matrix of the cervix undergoes marked transformation during pregnancy.¹⁸ In fact, cervical resistance begins to decline as early as the second trimester.¹⁹ The mechanisms responsible for these changes are not known although it is generally accepted that the changes in cervical structure and resistance predate the onset of labor.

TGF β is involved in extracellular matrix regulation by a variety of mechanisms including induction of transcription. Studies by Westergren-Thorsson described a decrease in cervical TGF β at term compared to non-pregnant cervical samples and a marked increase in the immediate postpartum period²⁰. They suggested that TGF β was involved in postpartum cervical reconstruction.²⁰ Although we found higher expression at term compared to non-pregnant levels, TGF β 1 mRNA expression declined from earlier gestational levels and was only slightly elevated compared to non-pregnant values. This difference may represent relative timing and number of samples that could be obtained in human gestation. The rat model allowed

us to evaluate trends through gestation that would be difficult in human pregnancy. The limited number of sampling points by Westergren-Thorsson limits understanding of the involvement of TGF β in human gestational cervical remodeling during a period where the cervix is undergoing marked changes. Our studies are consistent with our hypothesis. We have shown that the multifunctional cytokine TGF β 1 and its receptors, specifically T β R3, are up regulated during gestation and decrease at term. In addition, we have also shown that the TGF β signaling pathway is activated as demonstrated by increased phosphorylated Smad2 (pSmad2). This suggests that TGF β signaling does play a role in regulating the change in extracellular matrix composition.

The trends in TGF β signaling that were observed are consistent with literature describing increased collagen and matrix production through pregnancy. Rath divided cervical changes in pregnancy into an anabolic and catabolic stage.²¹ Through the majority of pregnancy the cervix produces extracellular matrix components that are associated with an increase in size and mass consistent with an anabolic process. This would be expected to be associated with increased TGF β signaling and Smad2 phosphorylation. The decline on day 22 of both pSmad2 and TGF β signaling components is consistent with a transition from anabolic to catabolic metabolism. We have shown with immunohistochemistry that Smad2 expression is seen within the cervical stroma and appears elevated on day 20 compared to non-pregnant levels consistent with the semi-quantitative Western blot studies. Also, in comparison to the role of TGF β in other tissues, its actions within the cervix appear similar. In other systems, TGF β up regulation is seen during anabolic processes such as wound healing and tissue fibrosis.⁷ Our finding of up regulation during pregnancy and down regulation just prior to parturition, when cervical ripening and loss of cervical resistance is occurring, is an example of the anabolic and catabolic activity of TGF β .

Our study is not however without its limitations. Although we were able to demonstrate an increase in T β R3 expression in conjunction with pSmad2 we did not evaluate transcriptional changes in target genes such as collagen or elastin. We also did not demonstrate increased translocation of pSmad2 to the nucleus. The increase in pSmad2 occurring in the absence of changes in T β R1 and T β R2 expression remains plausible because T β R3 was shown to increase the activity of the TGF β receptor complex.⁸ In addition, increased Smad2 is generally associated with increased production of extracellular matrix components while Smad3 is more commonly associated with regulation of apoptosis.²² The studies do not identify the mechanism responsible for increased expression of TGF β 1 or T β R3. We speculate that these changes are mediated by changes in circulating reproductive hormones such as estrogens and/or progesterone. Reproductive hormones have been shown to modulate TGF β expression in other reproductive tissues such as the ovary and endometrium.^{23–25}

The cervix is a complex organ that is responsible for maintaining the pregnancy through late gestation before allowing for cervical ripening and loss of resistance prior to parturition. Tissue integrity is a function of the interaction occurring between extracellular matrix components such as collagens, elastins, and proteoglycans.^{26, 27} Loss of integrity or cervical ripening is believed to occur as a result of reorganization and degradation of the extracellular matrix.^{18, 28, 29} While we do have some understanding of the biochemical and structural changes that occur within the ECM and suggest a potential role for TGF β in this process, our understanding is incomplete and requires further research.

In summary, the expression of TGF β 1 and T β R3 were modulated during gestation in the rat cervix. These changes were associated with changes in Smad2 signaling confirming the activation of this signaling pathway in the rat cervix during gestation. While our studies do not identify the exact mechanism of TGF β action on the ECM, we do show that increases and decreases in signaling do seem to correlate with the anabolic to catabolic switch associated

with the onset of parturition. Specifically, the expression of TGF β and its receptors decrease in late gestation prior to parturition. Given these findings, TGF β may play a role in regulating cervical remodeling that occurs across gestation. In order to fully understand the role of TGF β 1 and its receptors further studies are certainly needed to better understand how TGF β exerts its effects. Additionally work on human tissue is also needed. Finally, it is our hope that further understanding of this pathway will identify therapeutic targets aimed at reducing the incidence of preterm birth.

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Figure 1. TGF β 1 mRNA Expression in Timed Pregnant Rat Cervix

RT-PCR results representing the relative mRNA expression of TGF β 1 in timed pregnant rat cervixes. Values were normalized to β Actin. Values represent mean values obtained from 4 animals in each group run in triplicate. Error bars represent standard error. The increase in expression was found to be significant by ANOVA with $p = 0.001$. NP-Non Pregnant

Figure 2. T β R2 mRNA Expression in Timed Pregnant Rat Cervix

RT-PCR results representing the relative mRNA expression of T β R2 in timed pregnant rat cervixes. Values were normalized to β Actin. Values represent mean values obtained from 4 animals in each group run in triplicate. Error bars represent standard error. The increase in expression was found to be significant by ANOVA with $p=0.006$. NP-Non Pregnant

Figure 3. TβR3 Expression in Timed Pregnant Rat Cervix

The blue columns represent TβR3 mRNA expression in timed pregnant rats. The maroon columns depict the densitometric analysis of TβR3 protein expression as determined by Western blot. Values were normalized to β Actin for both protein and mRNA studies. Values represent mean values obtained from 4 animals in each group. Error bars represent standard error. Amounts are relative to β Actin. NP-Non Pregnant

Figure 4. Phosphorylated Smad 2 Expression

Densitometric analysis of phosphorylated Smad 2 protein expression as determined by Western blotting. Values were normalized to β Actin. Values represent mean values obtained from 4 animals in each group. Error bars represent the standard error. NP-Non Pregnant

Smad2 Expression in Cervical Stromal Cells

Figure 5. Smad 2 Expression in Cervical Tissue

Smad2 expression in cervical tissue. Smad2 expression is depicted in non pregnant (left panel) and gestational day 20 rat cervical tissue. Brown DAB staining intensity is qualitatively increased in day 20 compared to non-pregnant stromal tissue. Staining was done in parallel to limit variability in technique. There is decreased staining in endocervical epithelial cells on day 20 vs non pregnant tissue. Tissue counterstained with methyl green pyronin.

Table 1

Probe and Antibody Information

	RT-PCR	Western Blot
	Taqman Probes Catalog Number Applied Biosystems	Primary Antibodies Catalog Number, Vendor
TGFβ1	Rn00572010	
TβR1	Rn00562811	sc-9048, Santa Cruz Biotechnology, Santa Cruz, CA
TβR2	Rn00579682	sc-220, Santa Cruz Biotechnology
TβR3	Rn00568482	sc-28975, Santa Cruz Biotechnology
β-Actin	4352340E	AAN02, Cytoskeleton, Denver, CO
Smad2		Ab40855, Abcam, Cambridge, MA
pSmad2		#3101, Cell signaling, Danvers, MA
Smad3		#9523, Cell signaling
pSmad3		#9520, Cell signaling