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Transforming Growth Factor- β 1 Modulates Insulin-Like Growth Factor Binding Protein-4 Expression and Proteolysis in Cultured Periosteal Explants

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Abstract

Objective: Periosteum is involved in bone growth and fracture healing and has been used as a cell source and tissue graft for tissue engineering and orthopedic reconstruction including joint resurfacing. Periosteum can be induced by transforming growth factor beta (TGF- β) or insulin-like growth factor-I (IGF-I) alone or in combination to form cartilage. However, little is known about the interaction between IGF and TGF- β signaling during periosteal chondrogenesis. The purpose of this study was to determine the effect of TGF- β 1 on IGF binding protein-4 (IGFBP-4) and the IGFBP-4 protease pregnancy-associated plasma protein-A (PAPP-A) expression in cultured periosteal explants.

Design: Periosteal explants from rabbits were cultured with or without TGF- β 1. IGFBP-4 and PAPP-A mRNA levels were determined by real-time quantitative PCR. Conditioned medium was analyzed for IGFBP-4 and PAPP-A protein levels and IGFBP-4 protease activity.

Results: TGF- β 1-treated explants contained lower IGFBP-4 mRNA levels throughout the culture period with a maximum reduction of 70 % on day 5 of culture. Lower levels of IGFBP-4 protein were also detected in the conditioned medium from TGF- β 1-treated explants. PAPP-A mRNA levels were increased 1.6 fold, PAPP-A protein levels were increased 3 fold, and IGFBP-4 protease activity was increased 8.5 fold between 7 and 10 days of culture (the onset of cartilage formation in this model) in conditioned medium from TGF- β 1-treated explants.

Conclusions: This study demonstrates that TGF- β 1 modulates the expression of IGFBP-4 and PAPP-A in cultured periosteal explants.

Keywords

Cartilage; Chondrocyte; Insulin Like Growth Factor I; Periosteum; Transforming Growth Factor Beta; insulin-like growth factor binding protein-4; pregnancy-associated plasma protein-A

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1. INTRODUCTION

Periosteum, the connective tissue that surrounds bones, is a versatile tissue that can be used as a whole tissue graft or a cell source for tissue engineering or regeneration of bone and cartilage¹⁻¹⁴. The regenerative capacity of periosteum is due to the presence of mesenchymal stem cells capable of differentiating into chondrocytes, osteoblasts, adipocytes, and skeletal myocytes¹⁵. This unique tissue contains two discrete layers: the inner cambium, which contains the mesenchymal stem cells, and an outer fibrous layer^{16, 17}. As a whole tissue graft, periosteum can be used to resurface a joint without the need for *in vitro* culture. One obstacle regarding the use of periosteum, however, is the decrease in the number of mesenchymal stem cells in the cambium that occurs with age¹⁸. Using an *in vitro* periosteal organ culture model, we have demonstrated that periosteal cell proliferation and chondrogenesis can be induced by exogenous treatment with TGF- β 1 or IGF-I alone or in combination^{9, 19-22}. Recently we also demonstrated that local injection of TGF- β 1 alone or in combination with IGF-I can be used to rejuvenate aged periosteum for the purpose of cartilage regeneration²³. However, in order to further exploit the potential benefits of TGF- β 1 and IGF-I in cartilage regeneration we need to better understand the interactions between these signaling systems in periosteum.

Previous studies suggest that TGF- β can regulate the bioavailability of IGF-I in chondrocytes and osteoblasts through modulation of IGFBP levels²⁴⁻²⁸. IGFBP-4, which is expressed in cartilage and bone, is a negative regulator of local IGF action²⁹⁻³¹. In bone, IGFBP-4 binds and sequesters IGF-I from its receptors, thereby inhibiting osteoblast proliferation^{32, 33}. IGFBP-4 bioavailability is determined by gene expression and proteolysis^{31, 34, 35}. PAPP-A is an IGF-dependent-IGFBP-4 protease expressed by human fibroblasts and osteoblasts in culture³⁶. Cleavage of IGFBP-4 at Met¹³⁵-Lys¹³⁶ reduces its binding affinity for IGF allowing for greater receptor stimulation and subsequent growth response in cultured cells^{34, 35, 37, 38}. Ortiz et al. demonstrated that TGF- β regulates IGFBP-4 and increases the expression of the IGFBP-4 protease PAPP-A in cultured osteoblasts²⁷. We hypothesized that TGF- β regulates IGF-I bioavailability in periosteum by modulating the expression of IGFBP-4 and its protease PAPP-A. Thus, the objective of this study was to determine the effect of TGF- β 1 treatment on IGFBP-4 and PAPP-A expression and IGFBP-4 proteolysis in cultured periosteal explants.

2. MATERIALS AND METHODS

2.1. Periosteal explant harvest and culture

For the gene expression experiments, 352 explants ($2 \times 3 \text{ mm}^2$) were harvested from the medial proximal tibiae of 44 two-month old New Zealand white rabbits using sharp elevation. The explants were cultured in a 0.5 % agarose suspension in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, N.Y.) with 1 mM L-proline (Sigma, St. Louis, MO), 50 $\mu\text{g}/\text{mL}$ L-ascorbic acid (BDH Chemicals, Toronto, Canada), penicillin-streptomycin (Sigma, St. Louis, MO) (10% fetal bovine serum (FBS) with or without 10 ng/mL TGF- β 1 (R&D Systems, Minneapolis, MN) during the first two days of culture as previously described⁹. The periosteal explants were cultured for 3 to 42 days. At harvest, explants were washed in phosphate buffered saline (PBS) and pooled (2 explants/group) flash frozen in liquid nitrogen and stored at -70°C until RNA isolation.

For conditioned media experiments, 184 explants were cultured as described above for 7 days. After 7 days, the serum containing medium was removed and replaced with serum-free DMEM containing 0.1% BSA for 48, 72, or 96 h. The conditioned medium was collected and centrifuged at 2000 g at 4°C for 15 minutes and stored at -70°C .

2.2. RNA isolation and cDNA synthesis

Cultured periosteal explants were pulverized in liquid nitrogen, homogenized using a QIA shredder (Qiagen, Inc., Valencia, CA, USA), incubated for 20 min. at 55°C in 20 mg/mL with proteinase K (Qiagen, Inc., Valencia, CA, USA) and extracted using the Rneasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) including the “on-column” DNase digestion protocol. A second “off-column” DNase treatment using RQ1 Rnase-Free DNase (Promega, Madison, WI, USA) followed by re-purification with the Rneasy Mini Kit was used to eliminate residual genomic DNA contamination. The total RNA yield from two cultured periosteal explants ranged from 1 to 3 µg. Approximately 500 ng of total RNA was reverse transcribed with random hexamer primers at 37°C for 60 min.

2.3. Quantitative real-time PCR

Quantitative real-time PCR was performed using the ABI PRISM 7700 Sequence Detection System and software (PE Applied Biosystems, Foster City, CA). Rabbit specific cDNA sequences were obtained using a gene digging technique previously described³⁹. These rabbit sequences were used to generate primer and probe sequences using the Primer Express™ (PE Applied Biosystems, Foster City, CA) software (Table 1). Standard curves were generated from synthetic oligonucleotides of the experimental amplicons to obtain copy number data. All samples (n=6) were run in duplicate and quantitated by normalizing the target signal with the GAPDH signal.

2.4. PAPP-A protein levels

PAPP-A protein levels in periosteal conditioned medium samples were measured using an Ultra-sensitive PAPP-A ELISA kindly provided by Diagnostic Systems Laboratories, Inc. (Webster, TX). The minimum sensitivity of the PAPP-A ELISA is 0.24 mIU/L with intra- and inter-assay coefficients of variation of 4.7% and 4.2% respectively.

2.5. IGFBP-4 protease activity

Fifty microliters of periosteal explant conditioned medium were incubated in a microcentrifuge tube containing ¹²⁵I-IGFBP-4 with or without 5 nM IGF-II at 37 °C for 24 hr, as previously described^{36, 37}. The IGF-II is added as a cofactor to the cell-free reaction because IGFBP-4 protease activity is dependent on IGF-II binding to IGFBP-4⁴⁰. Reaction products from three different samples for control and TGF-β groups were separated by SDS-PAGE, 7.5-15% gradient and visualized by autoradiography.

2.6. Western ligand blot

Fifty microliters of 48 and 72 h conditioned medium samples were separated by SDS-PAGE using a 7.5-15% linear gradient. Separated proteins were blotted onto nitrocellulose filters, blocked, labeled with ¹²⁵I-IGF-I overnight at 4°C, visualized by autoradiography^{37, 41, 42} and quantitated using NIH Image™ analysis software.

2.7. Statistical analysis

Quantitative real-time PCR data were analyzed using a 1 or 2-factor ANOVA to determine the effect of time and/or TGF-β1 dosage on the measured mRNA levels. Where appropriate, *post-hoc* testing using Duncan's Multiple Range test was performed to determine significance ($p < 0.05$) between specific time points for a given TGF-β1 dosage (i.e. 0 or 10 ng/mL). Significance between TGF-β1 dosage groups for specific time points were determined using means contrast comparisons. PAPP-A protein levels (ELISA) and activity (protease assay) results were analyzed using student *t*-tests.

3. RESULTS

3.1. IGFBP-4 and PAPP-A mRNA

Periosteal explants were cultured for up to 42 days with or without 10 ng/mL TGF- β 1 (for the first 48h) and gene expression was analyzed using Real Time PCR with rabbit-specific primers and probes for IGFBP-4, PAPP-A and GAPDH. As shown in Figure 1A, IGFBP-4 mRNA was detected in all periosteal explants. IGFBP-4 mRNA levels increased during the first week of culture in both the TGF- β 1-treated and control explants followed by a decrease after peaks on days 10 and 7 respectively. The relative levels of IGFBP-4 mRNA were significantly reduced ($p<0.05$) in the TGF- β 1-treated explants at all time points except day 42. The greatest effect of TGF- β 1 was observed on day 5 where IGFBP-4 mRNA levels were 70 % lower (TGF- β 1: $0.35 \pm .15$ vs. control: 0.71 ± 0.19 copies IGFBP-4/copies GAPDH) in the TGF- β 1-treated group compared to controls.

As shown in Figure 1B, PAPP-A mRNA was also detected in periosteal explants throughout the culture period. PAPP-A mRNA levels also increased early during the culture period with peak levels on day 5 in the control group and on days 7 and 10 in the TGF- β 1 treated explants. TGF- β 1 treated explants also contained 1.7 and 1.6 fold higher levels of PAPP-A mRNA on days 7 and 10 respectively ($p<0.001$) and 50 % lower levels on day 14 ($p<0.001$). In order to verify that the explants used in this experiment responded in the expected chondrogenic manner to TGF- β 1 treatment, aggrecan mRNA levels were analyzed in the same pool of periosteal explants. As seen in Figure 4, relative aggrecan mRNA levels were significantly increased in the TGF- β 1 treated explants compared to controls on days 21-42 of culture ($p<0.008$).

3.2. IGFBP-4 and PAPP-A protein levels

In order to measure IGFBP-4 protein levels, periosteal explants were cultured in serum containing medium until day 7 (with or without 10 ng/mL TGF- β 1 for the first 48h). The medium was then changed to serum free medium and conditioned medium was collected 48h later. The conditioned medium was then analyzed by Western ligand blot. As shown in Figure 2A, conditioned medium from control explants contained detectable amounts of intact IGFBP-4 (24 kDa) whereas no IGFBP-4 was detectable in the conditioned medium from TGF- β 1-treated explants. A second aliquot of conditioned medium was harvested 24h after the initial sample and similar results were observed in these samples (data not shown).

The levels of PAPP-A protein between days 7 and 10 of culture were also determined in periosteal conditioned medium by ELISA. Periosteal explants were again cultured in serum containing medium until day 7 (with or without 10 ng/mL TGF- β 1 for the first 48h). The medium was then changed to serum free medium and conditioned medium was collected 96h later. As shown in Figure 2B, conditioned medium from TGF- β 1 treated explants contained 3 fold more PAPP-A protein ($p<0.0003$) than conditioned medium from control explants.

3.3. IGFBP-4 proteolysis

IGFBP-4 proteolysis in conditioned medium from cultured periosteal explants was also determined (based on IGFBP-4 fragment production). In this experiment, aliquots of the same conditioned medium used in the PAPP-A ELISA were analyzed using the IGFBP-4 protease assay as described in the Methods. As shown in Figure 3, addition of 5 nM IGF-II significantly increased IGFBP-4 protease activity in both the control ($p<0.0001$) and TGF- β 1 treated ($p<0.024$) samples. In addition, TGF- β 1 treatment enhanced IGFBP-4 protease activity 8.5 fold in the control ($p<0.0001$) and 2.5 fold in the IGF-II treated ($p<0.0005$) samples compared to the respective controls.

4. DISCUSSION

In this study we demonstrate that TGF- β 1 modulates IGFBP-4 and PAPP-A expression in cultured periosteal explants. To the best of our knowledge, this is the first study to demonstrate the expression of PAPP-A and its regulation during the process of chondrogenesis. Because IGF-I is an important chondrogenic growth factor, regulation of IGF-I bioavailability may be an essential aspect of TGF- β 1 action on periosteum. In these experiments a decrease in IGFBP-4 mRNA levels was observed throughout the six week culture after treatment with 10 ng/mL TGF- β 1. In TGF- β 1 treated periosteal explants, mRNA levels of the IGFBP-4 protease, PAPP-A, were also elevated on days 7 and 10 of culture compared to untreated explants. These results were confirmed by protein analysis, as a 3-fold increase in PAPP-A protein was detected in conditioned medium from TGF- β 1 treated explants compared to controls. Furthermore, the secreted PAPP-A protein was functional as evident by a significant increase in IGFBP-4 proteolysis in CM from TGF- β 1 treated explants. Together these data demonstrate that TGF- β 1 increased the level of functional PAPP-A protein between 7 and 11 days of culture. This observation is of particular interest because it occurs during the transition from the proliferation to differentiation stage in this *in vitro* periosteal chondrogenesis model⁵. At this time cartilage formation is initiated as chondrocyte precursors begin to differentiate into mature chondrocytes that are capable of matrix synthesis⁷. Therefore, because IGFBP-4 plays a fundamental role in regulating IGF-I bioavailability, alterations in PAPP-A activity during this period may have a functional role in TGF- β 1 induced periosteal chondrogenesis.

The results from the present study are similar to previously reported findings in cultured human osteoblasts^{27, 43}. In these studies, TGF- β treatment also increases PAPP-A expression and IGFBP-4 proteolysis^{27, 43}. In addition, recent studies revealed that fracture healing was delayed, and cartilage formation in the fracture callus was reduced in PAPP-A knock-out mice compared to wild-type mice⁴⁴. These findings suggest that PAPP-A is needed for optimal cartilage formation from periosteum and support a potential role for PAPP-A in the chondrogenic response of cultured periosteal explants to TGF- β 1.

Interestingly, alterations in the IGF-I axis have been reported in synovial fluid from arthritic joints and in osteoarthritic cartilage⁴⁵⁻⁴⁷. Osteoarthritic chondrocytes have been shown to be hyporesponsive to IGF-I despite having increased binding sites⁴⁸. The response of chondrocytes to IGF-I also decreases with age, and this may be due to increased levels of IGFBPs^{46, 49}. Therefore, IGFBP degradation pathways may be suitable targets for articular cartilage repair especially in patients with elevated synovial IGFBP levels as in older patients or those with RA or OA. Therefore, if even short-term TGF- β 1 treatment can produce a sustained inhibition of IGFBP production during cartilage repair, this may provide additional benefit to the initial quality and homeostasis of the repair tissue.

In summary, these studies demonstrate that short-term TGF- β 1 treatment of cultured periosteal explants results in modulation of the IGF-I axis, including some sustained affects. These findings are likely to be relevant to the use of periosteal grafts or periosteal cells for the regeneration of musculoskeletal tissues such as articular cartilage especially if short-term TGF- β 1 and/or IGF treatment is used to augment growth and cartilage formation²³.

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Fig. 1.

A) IGFBP-4 and B) PAPP-A mRNA levels in cultured periosteal explants (12 explants/group). Periosteal explants were cultured for up to 42 days with (blue line) or without (black line) 10 ng/mL TGF- β 1 (for the first two days). RNA was extracted from the explants (pooled 2 explants/group) after 3, 5, 7, 10, 14, 21, 28, 35 and 42 days of culture, reverse transcribed and analyzed using Real Time PCR with rabbit-specific primers and probes for IGFBP-4, PAPP-A and GAPDH. Standard curves from specific oligonucleotides containing primers and probe sequences were generated to obtain copy number data. All samples ($n=6$) were run in duplicate and quantitated by normalizing the target signal with the GAPDH signal. The data are presented as means $\pm$ standard error. *Post-hoc* testing using Duncan's Multiple Range test was performed to determine significance between specific times $\pm$ TGF- β 1.

Fig. 2.

A) Western ligand blot analysis of IGFBP-4 in conditioned medium from cultured periosteal explants. Periosteal explants were cultured in serum containing medium until day 7 (with or without 10 ng/mL TGF- β 1 for the first 48h). The medium was then changed to serum free medium and conditioned medium was collected 48h later. The conditioned medium was then analyzed by Western ligand blot. B) PAPP-A secretion from cultured periosteal explants. Periosteal explants were cultured in serum containing medium until day 7 (with or without 10 ng/mL TGF- β 1 for the first 48h). The medium was then changed to serum free medium and conditioned medium was collected 96h later. The conditioned medium was then analyzed by ELISA. The data are presented as means $\pm$ standard deviation. Two-tailed students t-test was used to determine significance.

Fig. 3. IGFBP-4 proteolysis in conditioned medium from cultured periosteal explants. Periosteal explants were cultured in serum containing medium until day 7 (with or without 10 ng/mL TGF- β 1 for the first 48h). The medium was then changed to serum free medium and conditioned medium was collected 96h later. Fifty microliters of periosteal explant conditioned medium were incubated in a microcentrifuge tube containing 125 I-IGFBP-4 with or without 5 nM IGF-II at 37 °C for 24 hr, as previously described 36, 37. A) Reaction products from three different samples for control and TGF- β groups were separated by SDS-PAGE, 7.5-15% gradient and visualized by autoradiography. B) Densitometry analysis of the IGFBP-4 fragment bands. The results are presented as means $\pm$ standard error. Two-tailed students t-test was used to determine significance.

Fig. 4.

Aggrecan mRNA levels in cultured periosteal explants (12 explants/group). Periosteal explants were cultured for up to 42 days with (blue line) or without (black line) 10 ng/mL TGF- β 1 (for the first two days). RNA was extracted from the explants (pooled 2 explants/group) after 3, 5, 7, 10, 14, 21, 28, 35 and 42 days of culture, reverse transcribed and analyzed using Real Time PCR with rabbit-specific primers and probes for aggrecan and GAPDH. Standard curves from specific oligonucleotides containing primer and probe sequences were generated to obtain copy number data. All samples (n=6) were run in duplicate and quantitated by normalizing the target signal with the GAPDH signal. The data are presented as means $\pm$ standard error. *Post-hoc* testing using Duncan's Multiple Range test was performed to determine significance between specific times $\pm$ TGF- β 1.

Table 1

Primers, probes and amplicons used in the real-time PCR analyses.

Gene	Primers	Probe	Amplicon
IGFBP-4	Forward: GCACCCACGAGGACCTCTT Reverse: GGGCTGGGTGACACTGCTT	6FAM- CGACCGCAACGGCAACTTCCAC- MGBNFQ	GCACCCACGAGGACCTCTTC ATCATCCCCATCCCCAACTGC GACCGCAACGGCAACTTCCAC CCCAAGCAGTGTACCCAGCCCTG
PAPP-A	Forward: GACTTGCTTTGATCCCGACTCT Reverse: ATGTGTTGATCCATCCAATTTCAG	6FAM- CTCACAGAGCTTATCTG-MGBNFQ	GACTTGCTTTGATCCCGACTCT CCTCACAGAGCTTATCTGGATG TTAATGAGCTGAAGAACATTCTG AAATTGGATGGATCAACACAT
Aggrecan	Forward: CTGCTACGGAGACAAGGATGAGT Reverse: CTGCGAAGCAGTACACGTCATAG	6FAM- CCCTGGCGTGAGAACCTACGGCA- MGBNFQ	CTGCTACGGAGACAAGGATGAGT CCCTGGCGTGAGAACCTACGGCAT CCGGGACACCAACGAGA CCTATGACGTGTACTGCTTCGCAG
GAPDH	Forward: GAGACACGATGGTGAAGGTCG Reverse: CTGGTGACCAGGCGCC	6FAM- CCAATGCGGCCAAATCCGTTCA- MGBNFQ	GAGACACGATGGTGAAGGTCG GAGTGAACGGATTTGCGCCGA TTGGGCGCCTGGTCACCAG