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Role of Gender in Burn-Induced Heterotopic Ossification and Mesenchymal Cell Osteogenic Differentiation

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Abstract

BACKGROUND—Heterotopic ossification (HO) most commonly occurs after burn injury, joint arthroplasty, and trauma. Male gender has been identified as a risk factor for the development of HO. It remains unclear why adult males are more predisposed to this pathology than adult females. In this study, we explore differences in heterotopic ossification between male and female mice using an in vivo burn/tenotomy model.

METHODS—Our Achilles tenotomy and burn model was used to evaluate the osteogenic potential of tissue-derived mesenchymal stem cells (MSCs) of male and female mice in injured and non-injured mice. Groups consisted of injured male (n=3), injured female (n=3), non-injured male (n=3), and non-injured female (n=3). The osteogenic potential of cells harvested from each group was assessed through RNA and protein levels and quantified using micro-CT scan. Histomorphometry was used to verify micro-CT findings, and immunohistochemistry was used to assess osteogenic signaling at the site of HO.

RESULTS—MSCs of male mice demonstrated greater osteogenic gene and protein expression than female MSCs ($p < .05$). Male mice in the burn group formed 35% more bone as compared to female mice in the burn group. This bone formation correlated with increased pSmad and IGF-1 signaling at the HO site in male mice. Differences were also seen between the non-injured male and female groups.

CONCLUSIONS—We demonstrate that male mice form quantitatively more bone as compared to female mice using our burn/tenotomy model. These findings can be explained at least in part by differences in BMP and IGF-1 signaling.

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Keywords

Heterotopic ossification; gender; mesenchymal stem cells

1: Introduction

Heterotopic ossification (HO) is the pathologic formation of ectopic bone that commonly occurs in the setting of extensive burn injuries, traumatic amputations, and joint arthroplasties.¹⁻⁵ The clinical and pathophysiologic factors contributing to HO remain unclear, but trends exist that allow us to explore reasons why specific groups of adult patients are more likely to develop HO than others. Gender is one such variable that appears to influence the predisposition towards developing HO.⁶

The hormonal influence of androgens and estrogens results in variable manifestations of many disease processes linked to dysregulated calcification such as osteoporosis, coronary artery disease, and rheumatologic conditions.⁷⁻⁹ In addition to systemic effects, gender can also affect local tissue health. Local differences in wound healing potential and bone remodeling have been documented between males and females through the differential modulation of various inflammatory pathways.¹⁰⁻¹² On a molecular level, this altered inflammatory response can result in differences in neutrophil function and adhesion molecule expression.^{11,13,14,15} Ozveri et al found that castration, estrogen, and anti-androgen medications decreased the extent of systemic inflammation and neutrophil infiltration after burn injury, suggesting that pharmacologic agents targeting these hormone pathways may minimize post-burn sequelae caused by heightened inflammatory states.¹⁶ Higher testosterone levels have been shown to increase the number of chondrocytes with the potential to form bone through stimulation of insulin growth factor-1 (IGF-1).^{17,18,19} IGF-1 has also been shown to stimulate osteogenic signaling and osteogenic differentiation within mesenchymal stem cells.²⁰ In growth plates, testosterone stimulated IGF-1 signaling has been shown to enhance condylar growth and ossification.¹⁷

Differences exist between adult male and female mesenchymal stem cells (MSCs) and the production of osteogenic mediators.²¹⁻²⁸ Zanotti et al identify that female mice exhibit 70% lower alkaline phosphatase activity and undergo less osteoblastic differentiation than male mice.²⁹ Levels of osteocalcin, a protein secreted by osteoblasts responsible for bone mineralization and calcium regulation, are also significantly greater in adult male mice than in adult female mice.³⁰ Some also propose that differences in stem cell differentiation are attributable to variations in cell surface marker expression.^{21,31}

Protein-protein interactions are affected by the presence of sex steroid hormones. Interactions between kinase-mediated transcription factors, nuclear factor kappa B proteins, and canonical Smad complexes are affected by the presence of androgens and estrogens.⁶ These relationships are important in examining the overlap between hormonal pathways and the BMP cycle as it relates to HO formation. The goal of the current study is to examine gender differences that exist in the formation of heterotopic bone, and to identify factors that explain why males and females respond differently to burn injury and trauma leading to HO.

2: Methods

2.1: Animals

Eight-week old male and female mice were used for all studies. Four groups of mice were used as follows: 1. male burn + tenotomy (n=3), 2. female burn + tenotomy (n=3), male tenotomy only (n=3), female tenotomy only (n=3). All animal procedures were carried out in accordance with the guidelines provided in the *Guide for the Use and Care of Laboratory Animals* from the Institute for Laboratory Animal Research (ILAR, 2011) and were approved by the Institutional Animal Care and Use Committee (PRO0001553).

2.2: Tenotomy and burn models

Mice were anesthetized according to protocol using a 50mg/kg intraperitoneal injection of sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL.) Dorsal hair was trimmed using an automatic clipper. A custom-made rectangular metal block was preheated in a 60 degree Celsius water bath. The block was applied to the dorsum of the mouse and held in place for 18 seconds to create a 30% total body surface area partial thickness scald burn as confirmed by histological analysis.³² The block was placed at the midpoint of the distance between the base of the cervical spine and the lumbosacral spine. Sham animals underwent the same treatment but the mold was placed in a 30 degree Celsius water bath instead. After the site was dried, a Tegaderm HP (3M HealthCare, St. Paul, MN) dressing was placed over the operative site to prevent contamination. Each burn mouse was resuscitated with 1 mL of saline solution through an intraperitoneal injection and 0.5 mL of normal saline in a subcutaneous injection. Buprenorphine (0.01 mg/kg, Buprenex; Reckitt Benckiser Pharmaceuticals Inc, Richmond, VA) was administered by subcutaneous injection every 12 hours for the first 72 hours after burn injury.

All mice then received an Achilles tenotomy of the left leg as previously described.³³ Briefly, a sterile iris scissors was used to make a 1 cm incision along the lateral aspect of the Achilles tendon. The Achilles tendon was aseptically exposed from the distal portion of the gastrocnemius muscle to the insertion on the calcaneus. The tendon was divided sharply at its midpoint and the incision was then closed with absorbable suture.

2.3: Isolation and culture of primary adipose derived mesenchymal cells

Adipose-derived MSCs were harvested from the inguinal fat pads of mice (n=3 per group) at two hours post tenotomy injury as previously described.³³ When first establishing our model, we performed a timepoint analysis of 2, 6, 24, 48 hours and 5 days after burn injury to see the maximal change in ASC biology and found 2 hours to be the most significant. We used the inguinal fat pad as the source of MSCs as it is the population of ASCs closest to the Achilles' tenotomy site; ASCs from this site provide a reliable, robust population of cells that are affected by the same cell signaling pathways that affect the tenotomy site itself based on anatomic proximity. The inguinal fat pads were dissected, finely minced, and enzymatically digested in a 0.075% type 1 collagenase solution (Sigma-Aldrich, St. Louis, MO) and the MSCs were then isolated by adherent cell culture using standard procedures as described.³³ Adherent MSCs were cultured and propagated in standard growth medium containing Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, and 100

IU/ml penicillin/streptomycin, and passaged on confluence by trypsinization. Cells were passaged 3 times before being used for the following assays.

2.4: In vitro osteogenic differentiation

MSCs were seeded onto six-well plates at a density of 100,000 cells/well and onto a 12-well plate at a density of 35,000 cells/well.³⁴ After MSCs achieved 80% confluence, the cells were treated with osteogenic differentiation medium containing DMEM, 10% fetal bovine serum, 100 g/ml ascorbic acid, 10mM Beta-glycerophosphate, and 100 IU/ml penicillin/streptomycin.¹⁶ Early osteogenic differentiation was assessed by alkaline phosphatase (ALP) stain and quantification of ALP enzymatic activity after 7 days in osteogenic differentiation medium (ODM) as previously described.³⁴ Alizarin red staining for bone mineral deposition and photometric quantification was completed at 2 weeks as previously described.³⁴

2.5: Quantitative PCR

RNA was harvested from cells after 7 days in ODM using RNeasy Mini Kit (Qiagen, Germantown, MD) according to manufacturer's specifications. Reverse transcription was performed with 1 μ g RNA using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was carried out using the Applied Biosystems Prism 7900HT Sequence Detection System and Sybr Green PCR Master Mix (Applied Biosystems) as previously described.^{36,37} (Table 1)

2.6: Western Blot analysis

Cells were lysed and protein collected after 7 days of differentiation in ODM, separated on polyacrylamide gels, transferred to polyvinylidene fluoride membranes, and assayed with standard immunoblotting technique as previously described.³⁹ Antibodies against pSmad1/5/8, IGF-1, alpha tubulin, and Smad5 were selected. Immunoblotted products were visualized by enhanced chemiluminescent substrate (Thermo Scientific, Rockford, IL). All bands were normalized with the loading controls (α -tubulin) and quantified by densitometry.³⁶

2.7: μ CT analysis

In-vivo development of bone formation was assessed with longitudinal μ CT scans at 5 weeks, 7 weeks, 9 weeks, and 15 weeks post defect (μ CT: GE Healthcare Biosciences, using 80kVp, 80mA and 1100 ms exposure). Using the reconstructed images, bone volume formation was analyzed using a calibrated imaging protocol as previously described.³⁸

2.8: Histologic Processing and Analyses

At 5 days (n=3 per group) and 3 weeks postoperatively (n=3 per group) animals were euthanized for histology. Skin was removed from the tenotomy site and the area of heterotopic bone formation was then fixed in buffered formalin solution for 24 hours at 4°C. Decalcification of the sample was completed with 19% ethylenediaminetetracetic acid (EDTA) solution for 28–42 days at 4°C. Decalcified tissues were dehydrated through graded ethanol, and paraffin embedded. Sagittal or transverse sections were completed with a width

of 5 microns, mounted on Superfrost plus slides (Fisher Scientific, Pittsburg, PA), and dried overnight at 37°C. Histology was performed as previously described, including H&E, and Pentachrome staining and immunohistochemistry for pSmad 1/5/8 and IGF-1.³⁹ Images were taken with a Nikon E-800 upright microscope (Nikon, Melville, NY) utilizing 4x and 10x bright field microscopy. Tenotomy sites including localized foci of heterotopic ossification were visually analyzed using NIS-Elements (Nikon, Melville, NY).

2.7: Statistical analysis

Data were analyzed using SPSS software (IBM). Means and standard deviations were calculated from numerical data and statistical analysis was performed using an appropriate ANOVA. Equivariance was assessed with Levene's test and a Welch correction was applied when indicated. Post-hoc analysis for more than 2 datasets was completed with Tukey's test with equivariant data or Games-Howell if Levene's test failed. In figures, bar graphs represent means, whereas error bars represent one standard deviation. For all assays, significance was defined as a $p < 0.05$.

3: Results

3.1: Trauma-induced heterotopic bone formation is greater in males than females

Using micro-CT, Radiographic evidence of HO formation at the tenotomy site was detected between three and five weeks post-injury. At 5–7 weeks, there was more bone formation at the tenotomy site in the male mice compared to the female mice after burn injury ($6.6 \pm 1.1 \text{ mm}^3$ vs. $4.1 \pm .6 \text{ mm}^3$; $p < 0.05$). (Fig. 1) These differences remained consistent throughout observation up to 15 weeks post-injury ($11.8 \pm 1.7 \text{ mm}^3$ vs. $5.7 \pm 1.1 \text{ mm}^3$; $p < .05$). Male mice in the group that underwent sham injury formed more bone than the female mice in the sham injury group at 15 weeks ($6.1 \pm .8 \text{ mm}^3$ vs. $4.3 \pm .7 \text{ mm}^3$, $p < .05$).

As endochondral ossification is one of the major mechanisms leading to HO, we performed histological analysis to determine the extent of cartilage deposition at the tenotomy site. (Fig. 2) At one week post-injury, H&E staining demonstrated more inflammation within the soft tissues at the tenotomy site of the injured male mice compared to the injured female mice. At 3 weeks, pentachrome staining identified more cartilage deposition in both the male and female injured mice than in the sham-treated mice from either gender group, with the greatest amount of cartilage deposition evident in the injured male mice. (Fig. 3)

3.2: MSCs derived from male mice after burn injury are more osteogenic than MSCs derived from female mice

After burn injury, adipose-derived MSCs from male mice exhibited significantly greater alkaline phosphatase (ALK) staining and enzymatic activity compared to MSCs derived from injured female mice. MSCs derived from male sham-treated mice also exhibited ten times more alkaline phosphatase activity than MSCs derived from either injured or sham-treated female mice ($p < .05$). There was no difference in ALK activity between female mice in the injured and sham-treated groups. Similar results were found using alizarin red staining to assess the degree of calcium deposition, an early marker of matrix mineralization (Fig. 4).

3.3: MSCs derived from male mice express greater pSmad1/5/8 and IGF-1 than females

To evaluate/explore potential differences in gene expression of osteogenic factors, western blot and PCR were performed to assess gene targets previously demonstrated to account for differences in osteogenesis between males and females; BMP-mediated canonical Smad signaling and IGF-1 protein expression were assessed in MSCs harvested from the inguinal fat pads of the mice.⁴⁴ We have focused on downstream BMP signaling via the pSmad 1/5/8 western blot analysis as this is a direct effect of BMP binding to BMP receptors 1 and 2 and subsequent phosphorylation of pSmad 1/5/8 by the BMP receptor kinase domain. We feel that looking at BMP ligand expression is not representative of the actual signaling which is what is central to prove a difference in downstream effects. In comparison to MSCs derived from female injured mice, MSCs derived from male injured mice demonstrated significantly higher levels of pSmad 1/5/8 activity ($p<.05$). Significantly greater IGF-1 expression in the male injured mice was seen as compared to the female injured mice. (Fig. 5)

3.4: Increased IGF-1 and Smad signaling in male mice following early trauma-induced HO formation

To verify that differences in IGF-1 and pSmad signaling contribute to the osteogenic capacity of MSCs of male and female mice, we stained the tenotomy site and surrounding tissues to identify the presence of these two mediators at the site of HO formation. At three weeks, tissue obtained from the site of HO formation qualitatively stained for canonical Smad and IGF-1 more in injured male mice compared to injured female mice after burn injury. (Fig. 6)

4: Discussion

Clinical outcomes studies often report a larger number of male patients suffering from heterotopic ossification than females. Most clinical studies, however, are retrospective, making it difficult to discern whether the increased incidence in males is due to intrinsic differences related to gender or to the use of a predominantly male cohort of trauma patients that have been exposed to high-energy injuries.⁴⁰ We demonstrate that gender affects the predisposition to form HO following trauma in mice; more specifically, we demonstrate that MSCs derived from injured male mice are more osteogenic than those from injured female mice as manifested by greater bone deposition and higher pSmad and IGF-1 activity.

The local niche of growth factors and cytokines strongly influences the differentiation potential of MSCs and plays an important role in cell maturation and construct formation. MSCs harvested from the inguinal fat pads of both sham-treated and injured male mice demonstrated greater osteogenic potential than MSCs isolated from female mice. It is now important to understand the exact reason behind such differences.

Over-activation of the BMP signaling pathway is thought to result in HO due to increased cell differentiation and transcription of osteogenic protein mediators. In our model, male mice had significantly greater levels of pSmad 1/5/8 compared to the female injured mice after burn injury with tenotomy. Our results are consistent with those of a study performed by Maor et al which demonstrated increased chondrocyte differentiation and bone deposition

in mandibular condyle cells treated with testosterone as compared to treatment with estrogen or no medication.¹⁷

Through histological and micro-CT analysis, we demonstrated greater cartilage and ectopic bone formation in male mice after tenotomy plus burn injury compared to identically injured female mice. With this finding, however, we must be cautious in attributing greater ectopic bone formation in males to alterations in the BMP cascade alone given that we also demonstrated increased expression of IGF-1, a factor whose expression is affected by testosterone.

While over-activation of the BMP pathway could be responsible for increased bone formation in males, it is also important to consider that females may experience suppression of osteogenic potential. Zanotti et al demonstrated that osteoblasts isolated from female mice had less osteogenic potential than those obtained from male mice based on alkaline phosphatase levels, findings that may be related to sex-hormone specific pathways or additional mediators of bone formation.²⁹ Although not statistically significant, female mice after burn injury formed a slightly greater amount of bone compared to the female mice that did not suffer burn injury. The reason behind this may be due to a decrease in the amount of osteogenic suppression in the female mice as a result of burn injury. By clearly delineating these mechanisms, we may more effectively utilize currently available treatments and create new pharmacologic agents in the therapy or prophylaxis of heterotopic ossification.⁵

There are important limitations to consider. There are many factors that affect bone formation. To account for these, we have chosen young mice of 8 weeks to avoid differences seen after menopause and in a state of estrogen deficiency in older mice. Additionally, the formation of bone in any clinical setting depends on various factors including local stress, amount of load present, and tissue metabolism. While it is possible that females form less bone due to differences in body habitus or type of trauma rather than variations in BMP or hormonal pathways, the males and females underwent identical treatments and manifested differential levels of pSmad 1/5/8 and IGF-1 levels. Although the increased expression of BMP-mediated canonical Smad signaling and IGF-1 signaling in male mice does not verify causality in terms of eventual levels of bone formation, we do believe these findings are noteworthy and have an impact on the biochemical pathways that lead to HO formation. Specifically, by understanding why males respond differently to burn injury than females, we can target these specific mechanisms to create novel therapeutic options in the future. Future studies that specifically stimulate and block these pathways in this model are needed for additional analysis. It is also important to evaluate other tissue-derived subsets of MSCs related to muscle and connective tissue that may also affect ectopic bone formation.

5: Conclusions

In this study, we demonstrate that gender differences are an important factor to consider in HO. These findings may be attributable to distinct levels of osteogenic mediators and differential stimulation of both hormonal and BMP related cascades. These results justify the need for additional studies in larger animal models of post traumatic HO. By identifying these gender differences, we can better understand the pathophysiology behind HO and

develop targeted pharmacologic interventions to improve the care of this growing patient population.

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Figure 1. Assessment and quantification of HO at 5, 7, 9 and 15 weeks post-injury
A. Micro-CT images of ectopic bone formation in burned and unburned mice. Green indicates heterotopic bone. White indicates the normal bone. **B.** Qualitative analysis of HO volume by week. Threshold set at 1250 HU. Values represent mean amount of HO formed ± standard deviation. Error bars represent standard deviation.

Figure 2. Histological assessment of inflammation and soft tissue swelling one week post-burn +tenotomy injury

Tenotomy site at one week post-burn. Arrows indicate inflammation around cut end of the Achilles tendon. Sample stained via H&E. All images at 10× (**right**) and 20× (**left**) magnification.

Figure 3. Histological assessment of cartilage formation, soft tissue mineralization, and early evidence of ectopic bone formation at three weeks post-burn+tenotomy injury
Arrows indicate sites of cartilage formation. Slides stained with pentachrome. All images at 4× and 10× magnification.

Figure 4. Alkaline phosphatase (ALP) and Alizarin Red staining in MSC derived from injured and sham-treated male and female mice

A. Qualitative and quantitative analysis of alkaline phosphatase activity; representative images collected from each experimental subset. **B.** Qualitative and quantitative analysis of alizarin red staining demonstrating in vitro mineralization potential; representative images collected from each experimental subset. Quantification of alizarin red staining in each experimental subset. Asterisk indicates significance $p < 0.05$. Error bars indicate standard deviation (SD).

Figure 5. pSmad 1/5/8 and IGF-1 protein expression in MSCs derived from injured and sham-treated male and female mice

A. Quantification and representative immunoblots of pSmad 1/5/8 normalized via densitometry. Normalization via alpha-tubulin. Asterisk indicates significance $p < 0.05$. **B.** Quantification and representative immunoblots of IGF-1 normalized via densitometry. Normalization via alpha-tubulin. Error bars indicate SD. Male mice after burn injury respond with greater activation of the BMP cascade as manifested by higher levels of pSmad and IGF-1 compared to females after burn injury.

Figure 6. IHC staining for pSmad or IGF-1 in the healing soft tissue of male and female mice at 3 weeks post-burn + tenotomy injury

A. IHC staining for pSmad. Samples stained via IHC for pSmad. **B.** IHC staining for IGF-1. Samples stained via IHC for IGF-1. Representative images of early heterotopic ossification at the tenotomy site 3 weeks post-burn injury in male (**left**) and female (**right**) mice. All images at 10× magnification. Arrows indicate positivity for pSmad or IGF-1.

Table 1

PCR primers Table 1

Gene target	Forward	Reverse
<i>Alp</i>	TGAGCGACACGGACAAGA	GGCCTGGTAGTTGTTGTGAG
<i>BMP-2</i>	TCTTCCGGGAACAGATACAGG	TGGTGTCCAATAGTCTGGTCA
<i>IGF-1</i>	GGGCTGAGTTGGTGGATG	CTCCAGCCTCCTCAGATCAC
<i>Ocn</i>	CTGACAAAGCCTTCATGTCCAA	GCGGGCGAGTCTGTTCATA
<i>Runx2</i>	GTGCGGTGCAAACCTTTCTCC	AATGACTCGGTTGGTCTCGG
<i>TNFα</i>	CCTGTAGCCACGTCGTAG	GGGAGTAGACAAGGTACAACCC
<i>Gapdh</i>	CAAGGTCATCCATGACAACTTTG	GGCCATCCACAGTCTTCTGG