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CLINICAL RESEARCH

Osteogenic Gene Expression Correlates With Development of Heterotopic Ossification in War Wounds

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Abstract

Background Heterotopic ossification (HO) is a frequent complication of modern wartime extremity injuries. The biological mechanisms responsible for the development of HO in traumatic wounds remain elusive.

Question/purposes The aims of our study were to (1) characterize the expression profile of osteogenesis-related gene transcripts in traumatic war wounds in which HO developed; and (2) determine whether expression at the mRNA level correlated with functional protein expression and HO formation.

Methods Biopsy specimens from 54 high-energy penetrating extremity wounds obtained at the initial and final surgical débridements were evaluated. The levels of selected osteogenic-related gene transcripts from RNA extracts were assessed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. As a result of its key role in osteogenesis, the concentration of BMP-2 in the effluent of 29 wounds also was determined.

Results The transcripts of 13 genes (ALPL [$p = 0.006$], BMP-2 [$p < 0.001$], BMP-3 [$p = 0.06$], COL2A1 [$p < 0.001$], COLL10A1 [$p < 0.001$], COL11A1 [$p = 0.006$], COMP [$p = 0.02$], CSF2 [$p = 0.003$], CSF3 [$p = 0.012$], MMP8 [$p < 0.001$], MMP9 [$p = 0.014$], SMAD1 [$p = 0.024$], and VEGFA [$p = 0.017$]) were upregulated greater than twofold in wounds in which HO developed compared with wounds in which it did not develop. Gene transcript expression of BMP-2 also correlated directly with functional protein expression in the wounds that formed HO ($p = 0.029$).

Conclusions Important differences exist in the osteogenic gene expression profile of wounds in which HO developed compared with wounds in which it did not develop. The upregulation of multiple osteogenesis-related gene transcripts indicates the presence of a proosteogenic environment necessary for ectopic bone formation in traumatic wounds.

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Clinical Relevance Understanding the osteogenic environment associated with war wounds may allow for the development of novel therapeutic strategies for HO.

Introduction

Heterotopic ossification (HO) is the formation of mature lamellar bone in soft tissue. Familial forms of the condition include progressive osseous heteroplasia and Albright hereditary osteodystrophy [48]. Recurrent mutations in the BMP type-1 receptor, activin receptor IA (ACVR1), and local changes in the expression of BMP-4 and its receptor (BMPR1A) have been linked to the rare genetic disorder fibrodysplasia ossificans progressiva [11, 13, 18, 20, 26]. The more common acquired forms of HO frequently occur as a complication of THA, elbow or acetabular fractures requiring surgical treatment, soft tissue injury secondary to trauma or deep muscle dissection, and traumatic brain or spinal cord injuries [3, 7, 8, 25, 49].

Regardless of etiology, it is theorized that the pathogenesis of HO requires several factors, the first being an inducing agent or event. This can be the result of closed or penetrating trauma resulting in damage to the bone or surrounding musculature. The concomitant hematoma may then deliver the mesenchymal progenitor cells or committed osteoblast precursor cells from the surrounding tissue or systemic circulation. Finally, an environment conducive to osteogenesis primed by local and systemic mediators is required [13]. As such, injured soft tissues in the setting of a dysregulated posttraumatic inflammatory response may represent an ideal environment for the dysplastic differentiation of mesenchymal stem cells toward osteoblastic lineage and ectopic bone formation [37].

Heterotopic ossification has proven to be a common and problematic complication of modern wartime extremity injuries, and it causes patient morbidity and loss of function [16, 39]. Studies suggest the rate of ectopic bone formation in combat-related injuries is substantially higher than prior estimates reported in civilian trauma populations [16, 39]. Despite several studies investigating systemic factors and the in vitro measurement of osteoblastic activity of cells from heterotopic bone [2, 27, 45], the biological mechanisms responsible for HO as a result of traumatic injury have not been defined.

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Advances in molecular profiling technologies have identified effector molecules, transcription factors, and regulatory genes involved in the tightly regulated process of bone metabolism [1]. A similar understanding of the molecular and cellular mechanisms associated with ectopic bone formation may lead to targeted therapies aimed at effective prevention, diagnosis, and treatment for patients with or at risk of HO. Given the high prevalence of HO in combat-related wounds, we asked whether expression at the mRNA level would correlate with the development of radiographically apparent HO.

The aims of our study were (1) to characterize the expression profile of osteogenesis-related gene transcripts in traumatic war wounds in which HO developed; and (2) determine whether expression at the mRNA level correlated with functional protein expression and HO formation.

Patients and Methods

To determine the expression level of gene transcripts involved in the development of HO in traumatic wounds, 34 wounded US service members with 54 wounds were enrolled in this prospective observational study. We included patients with high-energy, penetrating extremity wounds (< 75 cm^2) to one or more extremities, which were evacuated to the National Naval Medical Center from combat zones within Iraq and Afghanistan. Those with confounding premorbid systemic conditions including diabetes, immune disorders, connective tissue disorders, or any conditions requiring immunosuppressive agents and patients with prior extremity surgery complicated by infection were excluded and not enrolled. All patients were male with a mean age of 23.4 (range, 18–42 years) who presented to our institution a mean 5.6 (SD 2.6) days after having undergone intercontinental aeromedical evacuation from theater through Landstuhl Regional Medical Center in Germany. Up to three extremity wounds per patient were studied. In patients with more than three wounds, the largest three wounds were chosen for analysis based on wound volume (cm^3) from clinical and radiographic measurements and calculations from digital photographs using PictZar® planimetry software (BioVisual Technologies, Elmwood Park, NJ, USA). All patients underwent surgical débridement every 48 to 72 hours after arrival at our institution until definitive wound closure or coverage. Wound effluent from the vacuum-assisted wound closure device without a gel-pack (V.A.C.®; Kinetic Concepts, Inc, San Antonio, TX, USA) and a 1-cm^3 tissue biopsy specimen from the center of the wound bed were obtained prospectively at each surgical débridement. Tissue biopsy specimens were placed in an RNA preservation medium (RNAlater™; Ambion Inc, Austin, TX, USA) and all

samples stored at −80°C until analysis. Bacterial colonization and eventual wound outcome also were defined as previously described [22]. Colonization was defined as greater than 10^4 total bacteria on tissue-based quantitative bacteriology using standard techniques. The study was approved by the institutional review boards of the National Naval Medical Center and the Naval Medical Research Center. All patients signed informed consent to participate in this study and one patient died and thus was lost to followup.

Biopsy specimens from the 54 wounds obtained at the initial and final surgical débridements before definitive closure or soft tissue coverage were used for analysis to explore the temporal pattern of expression. Our power analysis indicated that 10 wounds per group were required to have an 80% power to detect a twofold threshold of differential gene expression (SAS, Cary, NC, USA). Total RNA was extracted and purified according to the manufacturer's instructions using the RNeasy® Fibrous Tissue Mini Kit (Qiagen Inc, Valencia, CA, USA) from 30 mg of tissue sample after homogenization. The samples were incubated in the presence of DNase1 to minimize genomic DNA contamination. RNA quantity and purity were assessed spectroscopically using the NanoDrop Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA) and RNA integrity assessed by microcapillary electrophoresis (Agilent 2100 BioAnalyzer; Agilent Technologies Inc, Santa Clara, CA, USA). RNA integrity values for all samples in this study were 7.0 or greater. Using the RT^2 First Strand Kit (SABiosciences, Frederick, MD, USA), 1 μg of total RNA was reverse-transcribed into cDNA for quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). A commercially available low-density RT-PCR array of 96 primer sets (including respective forward and reverse primers) for 84 osteogenesis-focused, five housekeeping, and seven quality control genes (SABiosciences, Gaithersburg, MD, USA) was used to assess gene expression in duplicate. Quantitative RT-PCR and dissociation curve analyses were performed using the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR® Green (SABiosciences) detection. Amplification parameters were as follows: one cycle of 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds and 60°C for 1 minute. RT-PCR data were analyzed using the Sequence Detection System Version 2.1 included with the ABI Prism 7900HT SDS and Microsoft Excel. The threshold was manually set and the baseline was set automatically to get the threshold cycle (Ct) value for each target. GAPDH was used as an endogenous housekeeping control gene for normalization. HO and non-HO samples were run in duplicate wherein Ct measurements per samples were normalized using GAPDH. Relative expression between HO and non-HO tissue was determined using the comparative Ct method (2-ΔΔCt) [31]. Results are expressed as the mean ± SD difference in relative expression. Transcription of a particular gene transcript in BMMSCs was considered to be differentially up- or downregulated if it was differentially expressed by at least twofold when compared with the expression level in HSPCs and vice versa for the reverse analysis. Assays with Ct values greater than 35 cycles were excluded from analysis.

HO was defined as the formation of ectopic matrix mineralization in soft tissues evident on routine AP and lateral radiographic views of the involved extremity at a minimum of 2 months after injury, as previously described [17, 39]. The minimum time for radiographic followup was selected based on previous studies from this and other surgical patient populations showing that HO is reliably evident radiographically within 2 months of the inciting event [4, 16, 34, 35, 39]. A two-author blinded independent review of radiographs was performed to determine the presence of ectopic bone with complete agreement between reviewers. HO developed in 47% (16 of 34) of the study patients (Table 1).

To investigate the correlation of gene transcript expression at the mRNA level with functional protein expression and biologic relevance, the presence and concentration of BMP-2 in the effluent of a cohort of 29 wounds (selected based on sample availability; 14 HO, 15 non-HO wounds) were determined according to the manufacturer's instructions using the Human BMP-2 ELISA Kit (R&D Systems, Minneapolis, MN, USA). Data were analyzed on a "per wound" basis, because not every wound in every patient formed HO. BMP-2 was selected for this subset analysis because it is a key regulator of bone formation and its ability to act as a potent osteoinductive agent when targeting osteoprogenitor cell populations has been shown repeatedly in vivo and in vitro [9, 32, 38].

Statistical differences between continuous variables were evaluated using Student's t-test. Equality of variance for continuous variables was determined using Levene's test. Associations between categorical variables were studied with Fisher's exact test or chi-square analysis as appropriate. Individual samples were compared with an average of control expression values of wounds in which HO did not develop. Transcript quantification was derived using the comparative threshold cycle method and reported as an n-fold difference in the experimental to the control samples [31]. Differential gene expression was considered significant when there was a twofold or greater difference in expression between the two groups and a two-tailed p value < 0.05. Differences in gene expression and nonparametric means were evaluated using the Mann-Whitney U test. The coefficient of determination was used to assess

Table 1. Patient demographics

Demographic information	Number of patients (N = 34)	Heterotopic ossification (N = 16; 47%)	No heterotopic ossification (N = 18; 53%)	p value
Age (years; mean ± SD)		25.4 ± 6.8	21.8 ± 4.3	0.086†
Sex				
Male	34	16 (100%)	18 (100%)	Not applicable
Female	0	0	0	
Combat theater				
Iraq	26	12 (75%)	14 (78%)	1.0*
Afghanistan	8	4 (25%)	4 (22%)	
Evacuation time from theater in days (mean ± SD)		6.4 ± 2.9	4.8 ± 2.2	0.082†
BMI (kg/m^2; mean ± SD)		25.3 ± 4.3	24.9 ± 3.6	0.74†
Tobacco use				
No	24	13 (81%)	11 (61%)	0.27*
Yes	8	3 (19%)	7 (39%)	
Injury Severity Score (mean ± SD)		25.7 ± 13.4	14.5 ± 6.8	0.006†
Traumatic brain injury				
No	6	1 (6%)	5 (28%)	0.18*
Yes	28	15 (94%)	13 (72%)	

* Fisher's exact test; †Student's t-test; BMI = body mass index.

the correlation between mRNA and functional protein levels. Statistical analysis was performed using SPSS Version 16.0 (SPSS Inc, Chicago, IL, USA). Data are represented as mean ± SEM unless otherwise specified.

Results

The mean duration of clinical and radiographic followup was 12.4 months (range, 5–17 months). There was no difference in followup between the study cohorts with and without HO (11.3 months versus 12.5 months; $p = 0.17$). There also were no differences with respect to patient demographics between the two groups. The mean Injury Severity Score (ISS) was significantly higher ($p = 0.006$) in the HO cohort. Of the 54 total wounds investigated, 24 (44%) showed radiographic evidence of HO (Table 2). There were significant differences between the two cohorts in terms of wound healing, size of the wound, presence of a traumatic amputation, and bacterial colonization ($p < 0.05$).

Several key genes involved in osteogenesis were upregulated at the transcript level in wounds in which HO developed. Of the 84 osteogenesis-focused gene transcripts analyzed, 13 (ALPL, BMP-2, BMP-3, COL2A1, COLL10A1, COL11A1, COMP, CSF2, CSF3, MMP8, MMP9, SMAD1, VEGFA) were upregulated greater than twofold at the initial débridement in wounds in which HO developed compared with wounds in which HO did not develop (Table 3). When analyzing transcript levels at the final débridement, transcripts of the same 13 genes were upregulated in wounds in which HO developed but showed a greater fold change when compared with the initial débridement. Several genes (ANXA5, BGN, COL1A, COL3A1, COL5A1, COL12A1, COL14A1, COL15A1, CTSK, ITGB1, MMP2, SERPINH1) involved in the development and maintenance of the integrity of the extracellular matrix during wound healing were highly expressed in wounds at the initial and final débridements from both cohorts ($C_t \leq 25$). Because these genes were highly expressed in both groups at the initial and final débridements, we observed no significant change.

Gene expression at the mRNA level correlated directly with functional protein expression. The coefficient of determination (r^2) for the expression of BMP-2 transcripts in relation to observed concentrations in the wound effluent was 0.76. Additionally, the effluent of wounds in which HO developed expressed higher levels of BMP-2 compared with wounds in which HO did not develop. There was no difference in the concentration of BMP-2 in the effluent of both groups at the initial débridement ($p = 0.22$; Fig. 1). However, there was a 2.5-fold increase in the concentration of BMP-2 in the effluent of wounds in which HO developed at the final débridement ($p = 0.029$; Fig. 1). This suggests that BMP-2 may play a critical role in regulating the development of combat-related HO.

Discussion

Heterotopic ossification is exceedingly common after combat-related extremity trauma. Regardless of etiology,

Table 2. Wound demographics

Wound demographic information	Number of wounds (N = 54)	Heterotopic ossification (N = 24; 44%)	Number with heterotopic ossification (N = 30; 56%)	p value
Mechanism of injury				
Gunshot wound	3	1 (4%)	2 (7%)	0.496‡
Blast	50	22 (92%)	28 (93%)	
Crush	1	1 (4%)	0	
Wound outcome				
Normal healing	40	12 (50%)	28 (93%)	< 0.001*
Impaired healing (delayed closure or dehiscence)	14	12 (50%)	2 (7%)	
Number of surgical débridements (mean ± SD)		4.5 ± 3.3	3.2 ± 2.3	0.135†
Wound location				
Upper extremity	12	2 (8%)	10 (33%)	0.167‡
Lower extremity	42	22 (92%)	20 (67%)	
Traumatic amputation				
No	30	9 (37%)	21 (70%)	0.027*
Yes	24	15 (63%)	9 (30%)	
Size of wound (cm^3) (mean ± SD)		613.4 ± 615.4	173.7 ± 252.9	0.003†
Associated vascular injury				
No	44	17 (71%)	27 (90%)	0.089*
Yes	10	7 (29%)	3 (10%)	
Wound colonization				
No	19	2 (8%)	17 (57%)	< 0.001*
Yes	35	22 (92%)	13 (43%)	
Wound closure method				
Suture	41	18 (75%)	23 (77%)	0.741*
Skin graft	13	6 (25%)	7 (23%)	

* Fisher's exact test; †Student's t-test; ‡chi-square test.

the pathogenesis of HO is multifactorial and requires the interaction of several biologic mediators and cell types. Efforts to identify the molecular mechanisms associated with its development have focused on the conditions permissive to osteogenesis in the cellular microenvironment [10, 15, 29]. An understanding of these mechanisms could lead to better means of risk stratification and prophylaxis. In this study, we investigated the expression pattern of genes related to osteogenesis in traumatic wounds, compared gene expression levels between cohorts with and without HO, and confirmed that a correlation exists between transcript levels and functional protein expression for BMP-2, a key regulator of bone formation.

The findings of our study must be interpreted in the context of its limitations. First, analysis of transcript expression was performed in a relatively homogeneous population with specific injury patterns and the findings presented may not be applicable to all populations at risk for HO. Second, analysis of selected mRNA transcripts was determined at initial and final débridements and the exact pattern of temporal expression related to the pathogenesis of ectopic bone formation was not determined nor was an analysis of systemic mediators. However, analysis of traumatic wounds at these times provides an accurate assessment of the posttraumatic environment conducive to osteogenesis because in vivo studies have shown fibroproliferative chondrogenesis by 7 days and heterotopic osteogenesis by 14 days after injury [19, 44].

Gene Upregulation in Patients Who Developed Heterotopic Ossification

The development of HO is theorized to require differentiation of mesenchymal progenitor cells toward chondrocyte and osteoblastic lineage, necessary steps for endochondral ossification [25]. As seen in our study, the expression of transcripts necessary for synthesis of cartilaginous matrix (COL2A1, COL10A1, COL11A1, COMP) and tissue remodeling (MMP8, MMP9) were upregulated in wounds in which HO developed. Specifically, COL2A1 encodes type II collagen, the chief component of cartilaginous

Table 3. Gene expression analysis

Gene	Initial dèbridement			Final dèbridement		
	Fold change (normalized to GAPDH)	95% CI	p value	Fold change (normalized to GAPDH)	95% CI	p value
ALPL	4.1	3.5–4.8	0.006	6.2	5.7–6.8	0.054
BMP-2	4.8	4.3–5.4	< 0.001	8.2	7.7–8.8	< 0.001
BMP-3	2.1	1.6–2.5	0.06	2.9	2.4–3.3	0.083
COL2A1	4.7	4.2–5.3	< 0.001	8.4	8.1–8.9	0.004
COL10A1	4.3	3.6–5.0	< 0.001	8.5	7.8–9.1	0.001
COL11A1	6.0	5.1–6.8	0.006	10.8	9.2–10.4	0.010
COMP	4.2	3.8–4.6	0.02	7.9	7.3–8.4	0.032
CSF2	4.8	4.2–5.3	0.003	8.7	8.2–9.5	0.003
CSF3	5.8	5.2–6.3	0.012	9.2	8.5–9.9	0.024
MMP8	5.8	5.4–6.5	< 0.001	9.3	8.7–9.6	< 0.001
MMP9	4.5	4.1–5.1	0.014	9.1	8.8–9.5	0.03
SMAD1	4.4	3.9–5.2	0.024	8.1	7.5–9.0	0.028
VEGF-A	4.2	3.6–4.8	0.017	8.3	7.8–8.9	0.001

Expression values represented as fold change in wounds in which heterotopic ossification (HO) developed compared with control expression values of wounds in which HO did not develop at initial and final surgical débridements. Differences in gene expression between groups evaluated using the Mann-Whitney U test; ALPL = alkaline phosphatase, COL = collagen alpha, COMP = collagen oligometric matrix protein, CSF = colony stimulating factor, MMP = matrix metalloproteinase; SMAD1 = SMAD family member.

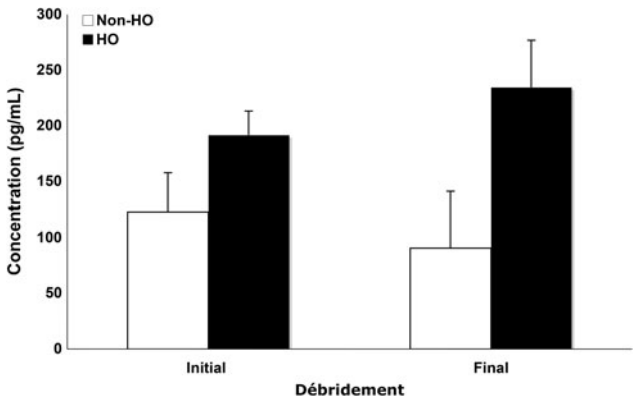

Fig. 1 BMP-2 concentration in the wound effluent is depicted at the initial (n = 14 HO wounds, n = 15 non-HO wounds) and final débridement (n = 14 HO wounds, n = 15 non-HO wounds). The débridements are further stratified by wounds in which HO developed (HO) and wounds in which HO did not develop (non-HO). Data are depicted as mean ± SEM. * $p < 0.05$.

matrix, whereas COL10A1 encodes type X collagen, a marker for hypertrophic chondrocytes intimately associated with calcification of the cartilaginous matrix [40, 43].

Transcript levels of genes necessary for the growth, remodeling, and maintenance of the extracellular matrix in wound healing were highly expressed in wounds from both groups, suggesting their importance in the pathogenesis of HO. In addition, transcript levels of genes encoding type I collagen (COL1A1, COL1A2), the predominant structural component of bone, also were highly expressed in both groups [43]. Significant differences between the two cohorts, however, were found regarding systemic injury severity and the local inflammatory response to injury. Patients who had HO develop sustained more severe systemic injuries as evident by elevated ISS (Table 1). Furthermore, the increased expression of genes encoding inflammatory cytokines (CSF2/GM-CSF, CSF3/G-CSF) suggests that a more pronounced inflammatory microenvironment is present in wounds in which HO developed. The increased bioburden imparted by bacterial colonization also may contribute to the proinflammatory microenvironment, and we noted a significantly increased rate of critical bacterial colonization in the HO cohort, similar to that previously described in combat casualties [15]. The expression of mitogenic growth factors and proinflammatory cytokines has also been shown to be upregulated during bacterial colonization and specifically after exposure to lipopolysaccharide [6].

Functional Protein Expression and Heterotopic Ossification

Soft tissue injury in the setting of a hyperinflammatory response has been shown to contribute substantially to ectopic skeletogenesis [16, 25, 32]. Specifically, the expression of BMP-2 and related osteoinductive morphogens by tissue macrophages and other inflammatory mediators is highly

upregulated at sites of muscular damage and soft tissue injury [6, 25, 41]. Overactive BMP signaling has been implicated in the development of HO [25, 46, 48]. Notably, the expression of BMP-2 transcripts was upregulated in wounds that had HO develop at the initial and final débridements. Our findings are consistent with those of previous reports in which muscle injury and associated inflammatory changes were reported as sufficient to trigger the development of ectopic bone in the setting of increased BMP expression [25, 32]. Several human and animal studies have further implicated the role of BMPs and their downstream signaling pathways in the pathogenesis of HO [21, 24, 53]. The upregulation of SMAD1, a gene encoding protein that serves as a signal transducer and transcriptional modulator of BMP signaling, further supports this observation [47].

Angiogenesis is an absolute requirement for endochondral ossification, successful fracture union, and the formation of ectopic bone [1, 5]. Thus, it was not surprising that VEGFA expression was upregulated in wounds in which HO developed. A hypoxic environment during the early stages of endochondral bone formation is necessary for differentiation of progenitor cells toward chondrocyte lineage [14, 38, 50]. However, the final event in endochondral ossification and ectopic bone growth is replacement of the avascular cartilage template by highly vascularized bone. Hypoxic induction of VEGFA expression necessary for angiogenesis appears to be a ubiquitous response mediated by hypoxia inducible factor-1α [30, 33].

The expression of BMP-2 in wounds in which HO developed also plays a critical role in the expression of VEGFA. The ability of BMPs to increase the levels of VEGFA mRNA in a concentration and time-dependent fashion has been shown [12, 23, 51]. Additional synergy between diffusible osteogenic morphogens and proangiogenic factors has been shown to enhance the rate of ectopic bone growth through modulation of angiogenesis, recruitment and induction of local progenitor cells, and enhancement of cell survival [28, 42]. Increased expression of BMP-2 and VEGFA transcripts in wounds that had ectopic bone develop further emphasizes their importance in the pathogenesis of HO. The ability of BMP antagonists and antiangiogenic therapy to slow or inhibit ectopic bone growth by altering the microenvironment conducive for osteogenesis holds promise for future mechanistic and gene-directed therapy [36, 44, 52].

Conclusions

Our data suggest the process of ectopic bone formation is initiated shortly after a traumatic insult, findings consistent with those of others [4, 44]. Wounds in which HO developed exhibited an elevated, protracted inflammatory state and may account for the increased levels of mRNA transcripts observed at the final débridement. A similar trend was noted in the temporal pattern of BMP-2 expression at the protein level. The interactions among the inflammatory response to injury, overexpression of genes related to osteogenesis, and expression of BMPs in the microenvironment parallel the clinical observation of more severe forms of HO occurring in patients with more severe injuries. Therefore, these results offer the opportunity to use the identified genes as markers to predict the development of HO in severely injured patients.

We also showed that significantly different osteopromotive gene expression profiles are present in combat-related wounds that have HO develop as compared with similar wounds in which HO does not form and that mRNA expression correlated directly with functional protein expression in wound effluent for BMP-2. The overexpression of selected gene transcripts related to ectopic matrix mineralization and endochondral bone formation indicates the presence of a microenvironment conducive to osteogenesis in traumatic wounds with BMP-2 potentially serving as a critical regulatory modulator. Continued research into the mechanisms responsible for the formation of HO is needed to further identify patients at risk and potential therapeutic targets for this and other patient populations.

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