Investigation into differential RNA expression between diabetic and non-diabetic patients in acute ankle fractures.

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Background:
• The time to fracture union and the complication rates of treating ankle fractures is significantly higher in diabetic patients. The current hypothesis is that differences in RNA expression exist between diabetic and non-diabetic patients at the site of the fracture during the acute phase of healing.

Study Design:
• Patients with an acute Weber B fibular fracture were enrolled in the study at presentation. At the time of surgery, fracture hematoma/soft callus was collected from the fibular fracture site. The sample was processed for RNA and the RNA expression levels of the genome (expressome) were analyzed for variation compared to controls using a gene chip array. Results were verified by qRT-PCR.

Results:
• Demographically, both diabetic (n=4) and non-diabetic (n=5) groups were comparable. The top 5% of genes whose mean expression differed from the control group were identified. These genes were then clustered for analysis. This qualitative analysis revealed genes involved in the cell signaling pathways of osteoblasts namely the BMP pathway, SMAD pathway, and TGF-Beta pathway. We analyzed the same RNA with an osteoblast specific qRT-PCR array to verify these differences. Specifically, the genes with increased expression included COL2A, COL4A3, CSF3, AMELY and MMP10. Those that were under-expressed included COL1A1&2, COL3A1, TGFBR2, MMP2, ITGB1, ITGA1, FN1, CTSK, ANXA5.

Discussion:
• We found differences in gene expression related to bone formation during the acute phase of healing in diabetic patients. Specifically, COL1 genes were under-expressed while COL2 genes were over-expressed suggesting that the activity of the chondrocytic cells was greater than osteoblastic cells. Alternately, it could reflect a delay in the transition from a chondrocyte dense soft callus to an osteoblastic driven hard callus. This is less likely as one would also expect a difference in the relative density of osteoblastic cell markers. Markers of chondroblasts (SOX9 and COLX) and osteoblasts (RUNX2) were not different. Cell surface receptors such as TGF-Beta 2 also demonstrated under-expression in diabetic patients. The relative under-expression of TGFBR2 suggests that this pathway maybe less responsive to signaling along the BMP/TGFBR/SMAD axis. This maybe a contributory factor in the clinically apparent diminished healing of diabetic fractures.

• Disclosure Statement: Our disclosure is in the Final AOFAS Program Book. We have no potential conflicts with this presentation.
## Background:

### Ankle Fractures

- Among the most common fractures treated by Orthopedic surgeons
  - Approximately 260,000 per annum
  - 4% lifetime actuarial risk of ankle fracture
- Frequency of ankle fractures have been increasing from 57/100,000 in 1970 to 147/100,000 in 2000
  - The rate is estimated to triple by 2030
- 4-6% of all ankle fractures are complicated by DM

### Ankle Fracture in DM Patients

- DM is second only to Open Fractures in Short- and Intermediate-Term Complication Rates
- The overall complication rate in treating DM ankle fractures operatively is 30%
  - Complication rate >40% in those with peripheral neuropathy
  - Non-op complication rate has been reported even higher
- Time to fracture union is significantly longer in DM patients at an average of 83 days
  - 187% longer in DM1
  - 186% longer in DM2
• Hypothesis:
  • Local differences in RNA expression exist between diabetic and non-diabetic patients at the site of fracture during the acute phase of healing.

Why during the acute phase of healing?
  • Differences identified in the acute phase would be the most efficacious for future pharmacologic therapies.
  • Addressing healing deviations early allows for possible therapeutic interventions that prevent short and intermediate complications

Overall Study Design
  • Patient introduced to Study at time of injury presentation to clinician
  • Fracture hematoma/soft callus collected at time of surgery
  • Sample processed for RNA/cDNA
  • RNA expression levels of expressome simultaneously analyzed for variation compared to controls

  • Qualitative Analysis:
    • Top 5% of genes whose mean expression differed in the DM patients from the controls were selected for further analysis – approximately 1500 genes

  • Quantitative Analysis
    • qRT-PCR using Osteoblast specific array
Results

Summary:
Demographically, both diabetic (n=4) and non-diabetic (n=5) groups were comparable. The top 5% of genes whose mean expression differed from the control group were identified. These genes were then clustered for analysis. This qualitative analysis revealed genes involved in the cell signaling pathways of osteoblasts namely the BMP pathway, SMAD pathway, and TGF-Beta pathway. We analyzed the same RNA with an osteoblast specific qRT-PCR array to verify these differences. Specifically, the genes with increased expression included COL2A, COL4A3, CSF3, AMELY and MMP10. Those that were under-expressed included COL1A1&2, COL3A1, TGFBR2, MMP2, ITGB1, ITGA1, FN1, CTSK, ANXA5.
Qualitative Analysis: Part 1
K Clustering of Calculated Means using Pearson Correlation by MeV
Qualitative Analysis: Part 2
Clustering using Term-Centric Enrichment analysis by Database for Annotation, Visualization, and Integrated Discovery (DAVID v6.7)
David.abcc.ncifcrf.gov/home.jsp

• DAVID’s Medium Classification Stringency

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• DAVID clustering overlay in Kegg pathways
Quantitative Analysis

qRT-PCR Results

Fold Regulation of DM compared to Controls

Normalized to GAPDH and RPL13a
Scatter Plot of qRT-PCR results for DM vs Controls

Significantly Over-Expressed

Significantly Under-Expressed
There are differences in the expression of genes related to bone formation and fracture healing during the acute phase of healing in diabetic patients compared to our control group. Specifically, TGFBR2 was identified by clustering analysis and verified by qRT-PCR as being significantly under-expressed in diabetic fracture hematoma. Interestingly, although COL1 genes were seen to be under-expressed both by gene array clustering analysis and qRT-PCR analysis, COL2 genes demonstrated a relative over-expression. This suggests that the activity of the chondrocytic cells was greater than osteoblastic cells. Alternately, it could reflect a delay in the transition from a chondrocyte dense soft callus to an osteoblastic driven hard callus. This is less likely as one would also expect a difference in the relative density of osteoblastic cell markers. Other markers of chondroblasts (SOX9 and COLX) and osteoblasts (RUNX2) where not found to be different between the two groups by qRT-PCR. The relative under-expression of receptors such as TGFBR2 suggests that this pathway is less able to be stimulated even in the presence of sufficient TGF-Beta and this maybe a contributory factor in the clinically apparent diminished healing potential of diabetic ankle fractures.

**Conclusion**

Therapeutic relevance:
The results suggest that patients with diabetes have a different RNA expression at the time point investigated. Specifically, during the acute phase of healing, the cells in the fracture callus express fewer markers of osteoblast and bone formation and a relatively greater amount of genes related to chondrogenesis. Importantly, cell surface receptors such as TGF-Beta 2, and possibly BMP2R, demonstrate under-expression in diabetic patients. This suggests that cells active during the acute phase of healing maybe less able to respond to signals along the BMP/TGFBR/SMAD axis thus leading to a damped response and slowing healing.

Future Work:
The current study suggests that there are differences in the expression profile of the cells present at the site of fracture healing in diabetic patients compared to the control group. A larger cohort of patients would be needed to fully elucidate the magnitude of the difference between these patient populations. Our results suggest that stimulation of BMP and TGF-B pathways may not be effective as the receptors for these pathways are less available to respond. Further characterization of cell surface markers, such as IGF receptor, FGF receptors, and the WNT/FRIZZLED pathway may shed light on alternate pathways active during this phase of healing. This could allow directed treatment, specific to the altered biology of diabetic fracture healing, to stimulate fracture healing. A greater number of time points would also demonstrate either differences in the rate of progression in the healing process or an absolute difference in the process itself.
References

- Carrol PV et al. IGF-I Treatment in Adults with Type 1 Diabetes Effects on Glucose and Protein Metabolism in the Fasting State and During a Hyperinsulinemic-Euglycemic Amino Acid Clamp. Diabetes. 2000. 49:789-796.