Osteogenic Effects of Preparations of Rat Pulmonary Alveolar Macrophages Challenged with *Staphylococcus Aureus*

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Introduction

Infection of a surgical or traumatic wound is one of the most common complications associated with elective and trauma surgery because of the inherent bacterial skin colonization that may inoculate deep bone and tissue when there is a break in the skin. Under the right conditions normally docile bacterial whose growth are normally kept in check may proliferate until there may be damage to the local soft tissues and bone (even expedite death). This damage can be aided with the right metabolic requirements for bacterial growth, the host's impaired ability to fight infections, impaired vascularity to deliver antibiotics, and the lack of skin coverage allowing continued colonization. An infection with normal skin flora, occurring at either a fracture or orthopedic prosthesis, may prevent or impair healing leaving the prosthesis loose and or the fracture ends atrophic.[1] In either case, the remaining bone will resorb due to a variety of mechanical and physiologic causes expediting its failure.

However, in some instances, and for an unknown reason, extra skeletal osseous formation and remodeling may occur. This change in skeletal geometry is typically a hallmark of a deep infection or osteomyelitis. Therefore the same organism in on instance will cause new bone to be formed and in the other old bone will resorb. Though reports demonstrated that there is a macrophage mediated osteogenic effect which ceases to exist upon proinflammatory stimulus by way of introduction of bacterial Lipopolysaccharide (LPS), a molecule typically found in the wall of gram negative bacteria.[2] Lipopolysaccharide has limited clinical significance since most of the infections do not come from gram negative bacteria, but from gram positive bacteria and mainly the Staph and Strep species. Because of the questionable utility of previous studies, further validation is necessary to validate the earlier reports by bacterial strains that are relatively more clinically relevant.

The purpose of this study was to measure the effect of a clinically important infectious agent (*staphylococcus aureus*) on in vitro ossification by using rat pulmonary alveolar macrophages and then applying the products to rat marrow stromal cell cultures (rMSCs) and assessing the osteogenic response. To do this we measured Von cossa staining and alkaline phosphatase at 6 and 72 hours after a mixture of staph and inoculation a culture of macrophage and added variables of nutrition and cell wall.

Methods

Staphylococcus aureus obtained from ATCC (Bacteria: ATCC 6538PNA *Staphylococcus aureus*, Manassas, VA) and Macrophages (ATCC CRL-2192, Manassas, VA). Bacteria were cultured overnight (tryptic soy broth 12114-05, Mobio, Carlsbad, CA)), collected at a concentration of 10^8 cells/ml, heat killed at 65˚C, cooled to room temperature and centrifuged at 3000g for 5 minutes. rMSC (ATCC 30-2004 F-12K Medium [Kaighn's Modification of Ham's F-12 Medium], St. Louis, MO) media was added onto the pellet, sonicated and vortexed 5 cycles, centrifuged, fresh rMSC media was added onto the pellet and resuspended preserving the initial concentration. The macrophages were seeded onto 24-well plates supplemented with modified Ham's F12K medium and 15% heat inactivated fetal bovine serum. Half of the macrophage cultures were supplemented with 50µl/well of the bacterial preparation explained above and retained for 6 and 72 hours (Bac/Mac). The other half was not supplemented with bacterial preparation (Mac). After 6 and 72 hours, the media was removed from the wells, centrifuged at 125g for 5 minutes and the supernatant was collected and stored at -80˚C before being applied to the rMSC cultures. rMSCs were obtained from marrow aspirated from the femurs and tibiae of 60 days old male Long-Evans rats (Harlan, Indianapolis, IN) and subcultured 4 to 7 times before being used in osteogenic assay. MSC media was composed of α-MEM supplemented with 10% FBS, 2 mM L-Glutamine, 100 U/ml Pen-strep, 1.5µg/ml Fungizone, 10 mM β-glycerophosphate and 50µg/ml ascorbic acid. A total of five groups were employed: 1) rMSCs without any factors, 2&3) rMSC media supplemented with 10% (v/v) Bac/Mac preparation collected after 6 hours or 72 hours, and 4&5) rMSC media supplemented with 10% (v/v) Mac preparation collected after 6 hours or 72 hours. Alkaline phosphatase assay and Von Kossa (von Kossa: 2% silver nitrate [sigma 239139] in 1x PBS, St. Louis, MO) mineralization assay were performed in order to test the osteogenic differentiation of rMSCs and to assess the endpoint mineralization, respectively. Significance of differences was assessed by Kruskal Wallis followed by *post hoc* Mann Whitney U test at ($p < 0.050$).

Results

Alkaline phosphatase assay at day 14 indicated that MSCs supplemented with the MAC preparation had a sig-

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nificantly greater activity than the control group both at 6 hr and 72 hr time points. (Figure 1) Of the two Bac/Mac groups, 72h group had greater amount of alkaline phosphatase activity. Von Kossa mineralization assay (day 21) indicated that MSCs supplemented with Mac preparations at both time points had significantly greater mineralization than the control group. (Figures 2 & 3) The MSC cells supplemented with Bac/Mac preparation did not induce a statistically significant osteogenic response on the MSCs as per Von Kossa mineralization assay on day 21. (Figure 2)

Discussion

The results of this preliminary study suggest that rat pulmonary alveolar macrophages induce osteogenic response on the rat MSCs. However, this osteogenic effect on rat MSCs is inhibited with the addition of *staphylococcus aureus*. These findings validate the previous work by Champagne et al.[2] The results also establish a time table for inhibition such that the osteogenic effect of macrophages emerges as soon as 6 hours and persists for up to 72 hours. While there was a trend for a temporal reduction in osteogenicity by macrophages, the number of samples per group did not provide sufficient power; therefore, current results need to be supplemented with further samples. It was also observed that Bac/Mac-72hr group had greater alkaline phosphatase activity than controls, which suggest that the inflammatory effect subsides by 3 days. It is also likely that Bac/Mac-72hr group had undergone osteoblastic differentiation in a delayed manner and it may be possible that a time point later than 72hr may display increased mineralization. Future studies will assess longer time durations to assess full recovery from this osteogenic inhibition.

Conclusion

From the results included here we were able to conclude that rat pulmonary alveolar macrophages induce osteogenic response on the rat MSCs. Also, when rat pulmonary alveolar macrophages are challenged by *Staphylococcus Aureus*, they fail to induce a statistically significant osteogenic response on rat MSC. Lastly, *Staphylococcus aureus* inhibits macrophagemediated osteogenic effects in a time-dependent fashion.

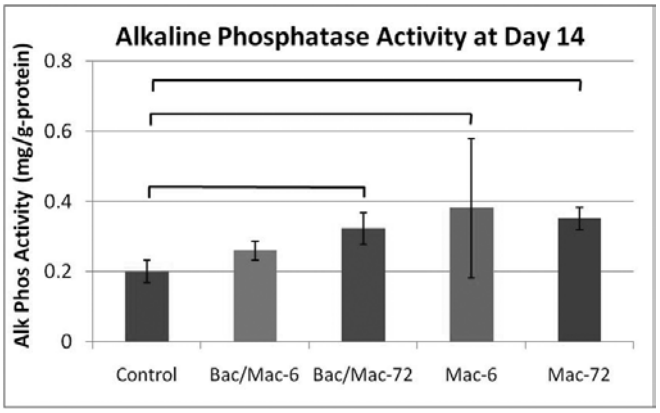

Figure 1. *Alkaline phosphatase assay on the 14th day.*

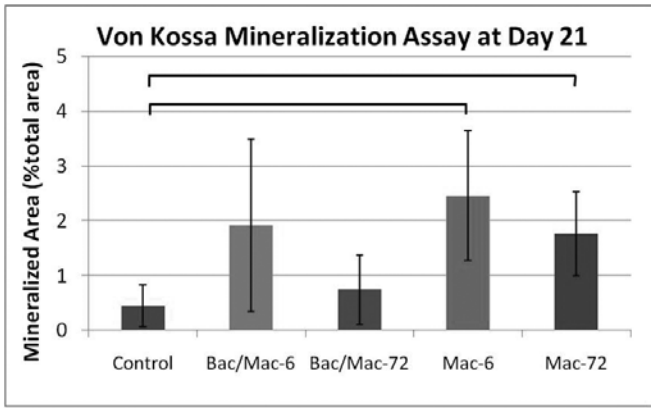

Figure 2. *Von Kossa mineralization assay on the 21st day. (Brackets connecting individual groups indicate statistical significance less than 0.05, n=4)*

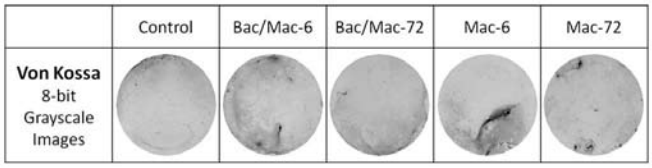

Figure 3. *Typical wells with mineralized rat marrow cells.*

References

1. **Zych GA, Hutson JJ Jr.** Diagnosis and management of infection after tibial intramedullary nailing. *Clin Orthop.* 1995; 315:153-62.
2. **Champagne CM, Takebe J, Offenbacher S, Cooper LF.** Macrophage cell lines produce osteoconductive signals that include bone morphogenetic protein-2. *Bone.* 2002; 30:26-31.