Local Environment Changes Driving Progression to Nonunion

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INTRODUCTION:

The aim of this study was to investigate the osteogenic potential and functions of mesenchymal stromal cells (MSCs) isolated from patients with established atrophic, aseptic nonunions, having no other risk factors for nonunion. Consequently, we compared proliferation, osteogenesis, gene expression, and protein secretion of MSCs isolated from the nonunion site, with MSCs isolated from bone away from the nonunion site. To investigate the effect of circulating cytokines on the functions of MSCs, we compared the proliferation and osteogenic potential of MSCs cultured with standard medium, compared to autologous serum medium. Furthermore, we assessed the serum concentrations of the cytokines found to be under- or over- regulated in nonunion tissue. The knowledge from this study can help us enhance our understanding of the function of MSCs and pathways involved in progression nonunion, but most importantly of the effect of the local biological environment to functional changes of the MSCs. Identifying these mechanisms can help us target molecules and biological pathways in regenerative therapies.

METHODS:

Following informed written consent, samples (peripheral blood, nonunion tissue, bone away from the nonunion site) were collected from 10 patients. Inclusion criteria were adult patients (18 to 65 years old) having revision surgery for an atrophic nonunion of the femur or tibia, with no evidence of infection or having any risk factors for developing a nonunion.

For the determination of cell proliferation, viability, and metabolic activity, a non-radioactive colorimetric assay (XTT based) was used, in triplicates. Additionally, the CFU-F (Colony Forming Units – Fibroblast) assay was performed to enumerate highly-proliferative (i.e., colony-forming) MSCs in cultures, in duplicates. Osteogenic differentiation was assessed with calcium and ALP assays, in triplicates.

To investigate potential differences in osteogenesis of the different MSCs groups (1. NU MCSs; 2. uninvolved bone MCSs; 3. NU MCSs cultured with osteogenic medium, harvested on day 21; 4. uninvolved bone MCSs cultured with osteogenic medium, harvested on day 21), a pathway-focused gene expression analysis was used. Finally, all the molecules that were identified to be up- or under- regulated on the PCR array described above, were quantified from supernatants collected from each culture condition (standard and osteogenic media).

For all assays, P3 (Passage 3) cells were used to ensure cell aging was not accelerated and cell senescence was not increased. All assays were also closely monitored to ensure that there was no evidence of change in shape, increased apoptosis, or arrest of proliferation.

RESULTS:

Ten patients (8 males; age: mean 46.2 y.o., SD 12.1 years, median 48.9 years, range 23.8 years to 60.5 years) were recruited in this study. The right side was involved in four cases. The femur was involved in six cases and the tibia in the remaining four cases. The mechanism of injury included road traffic accidents in five cases, whereas the remaining five were secondary to direct injuries or twisting injuries resulting to a fracture. As anticipated with high energy injuries, presence of comminution was common during the original injury (seven patients). The average duration of nonunion was 18.7 months (SD 12.3 months, median 14.9 months).

No differences were found in terms of MSC content and proliferation between NU and uninvolved bone regardless of medium used (commercial serum - XTT assay: $p=0.407$, CFU-F assay: $p=0.846$; patient's own serum - XTT assay: $p=0.448$, CFU-F assay: $p=0.899$), but culture with medium containing patient's own serum led to superior proliferation both in NU and uninvolved bone MSCs ($p=0.016$ and $p=0.006$ respectively). The osteogenic differentiation of P3 cells was comparable in the cells isolated from uninvolved bone and those isolated from nonunion tissue (calcium assay: $p=0.446$; ALP activity: $p=0.963$).

Comparing the mRNA expression of samples at baseline, the expression of ICAM1, MMP10, and GLI1 was significantly higher in nonunion derived cells compared to bone derived cells at baseline, even though other four genes were over-expressed more than two-fold, but did not reach statistical significance due to donor variation (COL15A1, FLT1, GDF10, TNF). On the contrary, the expression of EGF, IGF2, MMP8, and COL14A1 was significantly lower in nonunion derived cells, while another seven genes were under-expressed more than two-fold, but did not reach statistical significance (BMP4, CD36, DLX5, FGFR2, TGFB2, TNFSF11, VCAM1).

Following osteogenic stimulation, the expression of IGF1 and CALCR was higher in nonunion derived MSCs compared to uninvolved bone derived MSCs (more than two-fold), but did not reach statistical significance. On the contrary, seven genes were under-expressed more than two-fold (IGF2, EGF, FGFR2, AHSG, CSF2, MMP10, MMP9), but only IGF2 and EGF genes reached statistical significance.

Investigating the above molecules on a protein level, MMP-8 and IGF-2 were reduced in the nonunion tissue ($p<0.001$). On the contrary, the concentration of Dkk-1 was found to be increased in the nonunion derived MSCs ($p=0.011$). The remaining of the molecules examined (ICAM-1, MMP-9, MMP-10, and EGF) did not reach statistical significance ($p>0.05$).

DISCUSSION AND CONCLUSION:

Nonunion tissue and unaffected bone contain MSCs with comparable proliferation and osteogenic potential. Comparing the expression of nonunion and bone MSCs at baseline suggest that an inflammatory environment may be the cause of their downregulation, a finding which may support that the cause of the nonunion may be local and not systemic. In other words, the patients have otherwise ‘normal’ MSCs which can proliferate and differentiate, but at the site of the nonunion their functions are impaired.