

CORS Paper Session 1: Cartilage and Muscle •

Moderators Mark Hurtig, ON, and Nadr M. Jomha, AB

1 –

Inflammation Causes Muscle Injury in Compartment Syndrome: An Experimental Study

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Purpose: Compartment syndrome is a limb-threatening complication of skeletal trauma. Both ischemia and inflammation may be responsible for tissue necrosis in compartment syndrome (CS). In this study, normal rodents were compared with neutropenic animals to determine the importance of inflammation as a mechanism of cellular damage using techniques of intravital videomicroscopy (IVVM) and histochemical staining. **Method:** Forty Wistar rats were randomised. Twenty animals served as a control (group C). Twenty rats were rendered neutropenic using cyclophosphamide (250mg/kg) (group N). Animals were anaesthetised with 5 % isoflurane. Elevated intracompartmental pressure was induced by saline infusion into the anterior hindlimb compartment and maintained at 30–40 mmHg for 0, 15, 45 or 90 minute time intervals. Following fasciotomy, the EDL muscle was analyzed using IVVM to quantify tissue injury, capillary perfusion, and inflammatory response. **Results:** The proportion of injured cells decreased in group N compared to group C at all time intervals of EICP ($p < 0.05$). The proportion of injured cells in group N was 8 % after 0 minutes EICP, and 12, 15, and 10 % at 15, 45, and 90 min of EICP. In group C injured cells increased from 8 % to 20, 22, and 21 % at 15, 45, and 90 minutes EICP respectively. Groups N and C both demonstrated a time-dependent reduction in capillary perfusion. In group N continuously-perfused capillaries decreased from 79 ± 4 / mm with 0 min of EICP, to 48 ± 11 / mm (15min), 36 ± 7 / mm (45min), and 24 ± 10 / mm (90min) ($p < 0.05$). Overall, There was no difference between groups N and C with regards to perfusion ($p > 0.05$). **Conclusion:** This study demonstrates the importance of inflammation as a cause of injury in compartment syndrome. There was a 50% decrease in injury in neutropenic animals compared to controls after 90 minutes of elevated intracompartmental pressure. Microvascular perfusion analysis demonstrated a time-dependent decrease in capillary perfusion in both neutropenic and control animals. Blocking of the inflammatory response via neutropenia was protective against tissue injury. These results provide evidence toward a potential therapeutic benefit for anti-inflammatory treatment of elevated intracompartmental pressure.

2 –

Chondroitinase ABC and Acute Electrical Stimulation are Beneficial for Muscle Reinnervation After a Sciatic Nerve Transection in Rat

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Purpose: Nerve re-generation and functional recovery are often incomplete after a peripheral nerve lesion. The aim of this study was to determine if the injection of chondroitinase ABC at the lesion site, one hour of electrical stimulation, and the combination of these treatments at the time of repair are effective in promoting nerve regeneration and muscle re-innervation. **Method:** A complete right sciatic nerve section was done on 32 female Sprague-Dawley rats. End-to-end microsuture repair was performed and fibrin glue was added. Five groups were studied: 1- Sutures and Fibrine glue (S+F), 2- S+F and chondroitinase ABC, 3- S+F and electrical stimulation, 4- S+F and chondroitinase and electrical stimulation, 5 uninjured nerve. Video kymematic, EMG, muscle strength and axonal count were used to assess nerve recovery at 150 days post-repair. **Results:** Side video kinematics was performed and a larger excursion of the hip-ankle-toe angle during walking was showed in groups 2, 3, and 4. ($p < 0.05$) At 150 days, in-vivo EMg activity and maximal muscle force were similar in group 2, 3, 4, 5 and all of them were higher compared to group 1 ($p < 0.05$). Histological study revealed equivalent number of axone in all group and pore correlation with nerve function. **Conclusion:** In conclusion, five months after nerve transection, the recovery is incomplete when using suture and fibrine glue only. Moreover, an injection of chondroitinase ABC at the lesion site and/or one hour of electrical stimulation of the proximal nerve stump is beneficial in promoting nerve regeneration and functional muscle re-innervation.

3 –

The Mast Cell Stabilizer Ketotifen, Significantly Reduces Contracture Severity and Molecular Manifestations of Joint Capsule Fibrosis in a Rabbit Model of Posttraumatic Joint Contractures

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Purpose: To determine if mast cell activity is vital to the induction of joint capsule fibrosis and contracture formation in a rabbit model of posttraumatic joint contracture. **Method:** To reproducibly induce joint contractures, we used a model of surgical injury and immobilization of the knee in skeletally mature New Zealand white rabbits. Four animals groups were studied: a non-operative control group (CON), an operative contracture group (ORC) and two-operative groups treated with a mast cell stabilizer, Ketotifen fumarate at doses of 0.5mg/kg (KF0.5) and 1.0mg/kg (KF1.0) twice daily subcutaneously, respectively. Animals were sacrificed after 8 weeks of immobilization. Flexion contractures (biomechanics), cellular counts of myofibroblasts and mast cells within the joint capsule (immunohistochemistry) and the joint capsule protein expression of TGF- β 1, collagen I and III were quantified (western blots). Biomechanical data was interpreted using a linear regression analysis of repeated measures and an ANOVA analysis of variance was used for molecular data. Significance was

defined at $p < 0.05$ for all statistical tests. **Results:** Flexion contractures were most severe in the ORC group and treatment with Ketotifen (both KF0.5 and KF1.0) significantly reduced contracture severity by 52% and 42%, respectively ($p < 0.03$). Joint capsule myofibroblast and mast cell hyperplasia was a prominent feature of the more severely contracted ORC group and myofibroblast and mast cell numbers were dramatically reduced in both Ketotifen groups ($p < 0.001$). The expression of TGF- β 1 and collagen I was also increased in the ORC group and significantly reduced in both Ketotifen groups ($p < 0.01$). **Conclusion:** Joint capsule fibrosis, characterized by hyperplasia of myofibroblasts and mast cells and enhanced collagen deposition, is a prominent feature of posttraumatic joint contractures in this animal model. Treatment with a mast cell stabilizer reduced the molecular markers of joint capsule fibrosis and the resultant biomechanical severity of contracture formation. These results suggest mast cell activity may be an important process in the development of posttraumatic contractures and future work is needed to determine if pharmacological inhibition of mast cell activity has a preventative or therapeutic role in humans.

4 –

Isolation of a Subpopulation of Human Mesenchymal Stem Cells with Enhanced Chondrogenic Potential

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Purpose: The introduction of supplementary cells into a region of diseased or damaged tissue is becoming a viable treatment strategy in many areas of medicine. Mesenchymal stem cells (MSCs) are attractive for this purpose because they represent an autologous, multipotent cell source. However, it has been recognized that populations of MSCs represent a heterogeneous group of cells with each cell subpopulation possessing unique terminal differential capacity. The CD44 cell surface receptor has previously been identified on some of the cells within the MSC population. It is also present on chondrocytes and is thought to play a critical role in cartilage matrix generation and homeostasis. We hypothesized that a CD44⁺ purified subpopulation of MSCs will possess enhanced chondrogenic potential and be more suitable for articular cartilage regeneration. **Method:** Bone marrow aspirates were collected from orthopaedic patients undergoing iliac crest bone grafting. Human MSCs were isolated and cultured using standard techniques. Flow cytometry was utilized to identify the cell surface antigens characteristic of the MSC population. FACS was utilized to isolate the CD44 positive cells based on antigenic recognition, generating a CD44 positive population and a CD44 negative population. To confirm the multilineage potential of the isolates, defined media and culture conditions were utilized to differentiate both groups into osteocytes, adipocytes and chondrocytes. Real time polymerase chain reaction was utilized to quantify and compare the essential markers, collagen II, collagen I and aggrecan, in the stem cell derived chondrocytes. The CD44 enriched and CD44 depleted populations were compared. **Results:** The cells isolated possessed a cell morphology and surface antigen profile consistent with a MSC population. In addition,

both experimental groups demonstrated multipotent ability. Real time PCR analysis of the chondrogenic cells demonstrated that the CD44 positive population expressed collagen II and aggrecan at a significantly higher level than the CD44 negative population. **Conclusion:** To date no group has successfully identified a relationship between a MSC subpopulation and the multipotent progenitors responsible for generating cartilage. This work demonstrated that there are MSC subpopulations with different potential for chondrogenic expression and represents an important step towards identifying MSC subpopulations with enhanced cartilage formation potential.

5 –

Mechanism of Decreased Expression of Type X Collagen in Human Mesenchymal Stem Cells Cultured on Nitrogen-rich Plasma Polymers: Implication of Cyclooxygenase-1

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Purpose: Recent evidence indicates that a major drawback of current cartilage and intervertebral disc (IVD) tissue engineering is that human mesenchymal stem cells (MSCs) from osteoarthritic patients rapidly express type X collagen (COL10A1), a marker of late-stage chondrocyte hypertrophy associated with endochondral ossification. We recently discovered that a novel atmospheric-pressure plasma-polymerized thin film substrate, named “nitrogen-rich plasma-polymerized ethylene” (PPE:N), is able to inhibit COL10A1 expression in committed MSCs. However, the cellular mechanisms implicated in the inhibition of COL10A1 expression by PPE:N surfaces are unknown. **Method:** Human mesenchymal stem cells (MSCs) were obtained from aspirates from the intramedullary canal of donors (60–80 years of age) undergoing total hip replacement for osteoarthritis. Bone marrow aspirates were processed and MSCs were cultured on commercial polystyrene (PS control) and on PPE:N surfaces in the presence of different kinases and cyclooxygenase inhibitors for 3 days. Total RNA was extracted with TRIzol reagent (Invitrogen, Burlington, ON) and the expression of COL10A1, cyclooxygenase-1 (COX-1), and 5-lipoxygenase (5-LOX) genes was measured by real-time quantitative RT-PCR. **Results:** Results showed that a non-specific inhibitor of cyclooxygenases reduced the expression of COL10A1. In contrast, inhibitors of protein kinases stimulated the expression of COL10A1. Furthermore, potent and selective inhibitors of COX-1 and 5-LOX also reduced the expression of COL10A1. However, COX-2 and 12-LOX inhibitors had no significant effect on the expression of COL10A1. COX-1 gene expression was also decreased when MSCs were incubated on “S5” PPE:N surfaces. Interestingly, MSCs did not express 5-LOX. **Conclusion:** PPE:N surfaces suppress COL10A1 expression through the inhibition of COX-1 which is directly implicated in the synthesis of prostaglandins. The decreased expression of COX-1 and COL10A1 in human MSCs cultured on PPE:N is therefore in agreement with the induction of the osteogenic capacity of rat bone marrow and bone formation by systemic or local

injection of PGE2 in rats. However, PGE2 and other prostaglandins inhibited COL10A1 expression in chick growth plate chondrocytes. This suggests that the effect of prostaglandins on COL10A1 expression may be cell-specific or may be dependent on pre-existing patho-physiological conditions.

6 –

Hypoxic Regulation of Chondrocyte Differentiation and its Application to Cartilage Repair

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Purpose: Chondral injuries of the knee are commonly seen at arthroscopy, yet there is no consensus on the most appropriate treatment method. However, untreated cartilage injury predisposes to osteoarthritis contributing to pain and disability. For cell-based cartilage repair strategies, an ex-vivo expansion phase is required to obtain sufficient numbers of cells needed for therapy. Although recent reports demonstrated the central role of oxygen for the function and differentiation of chondrocytes, little is known of the effect of physiological low oxygen concentrations during the expansion of the cells and whether this alters their chondrogenic capacity. **Method:** Initial studies of chondrocyte expansion were performed in mature mice, with cells expanded at either atmospheric oxygen tension (21%) or 5% O₂ in monolayer cultures. Chondrogenic differentiation was subsequently assessed via micromass culture. Having determined that oxygen tension influences murine chondrocyte expansion and differentiation, similar studies were conducted using adult human chondrocytes taken from knee arthroplasty off-cuts, with mRNA expression of select genes involved in the chondrogenic program analyzed by q-PCR. **Results:** Cellular morphology was improved in hypoxic culture, with a markedly more fibroblastic appearance seen after greater than 2 passages in 21% O₂. Micromass cultures maintained in hypoxic conditions demonstrated stronger staining with Alcian blue, indicating stronger expression of cartilaginous glycosaminoglycans. Collagen type II mRNA expression was two-fold higher in cells expanded at 5% as compared to expansion at 21% O₂. Micromass cultures grown at 21% O₂ showed up to a twofold increase in the tissue content of glycosaminoglycans when formed with cells expanded at 5% instead of 21% O₂. However, no differences in the mRNA expression or staining for collagen type II protein were observed in these micromass cultures. Hypoxia (5% O₂) applied during micromass cultures gave rise to tissues with low contents of glycosaminoglycans. **Conclusion:** In-vivo, chondrocytes are adapted to a hypoxic environment. Taking this into account, applying 5% O₂ in the expansion phase in the course of cell-based cartilage repair strategies, may result in a repair tissue with higher quality by increasing the content of glycosaminoglycans.

7 –

The Molecular Mechanisms of Compartment Syndrome

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Purpose: Compartment syndrome is a severe complication of skeletal trauma. Intravital microscopy (IVVM) has demonstrated an inflammatory response to compartment syndrome (CS). The molecular mechanisms underlying this inflammatory response are unknown. The purpose of this study was threefold. First, a broad inflammatory cytokine profile was examined to determine the molecules responsible for white cell recruitment. As well, skeletal muscle expression of white cell adhesion molecules including P-Selectin, E-Selectin, Mac-1 and ICAM-1 were examined to assess the extent of white cell activation in target tissues. Finally, skeletal muscle apoptosis was measured to determine the magnitude of cell death.

Method: Normal and neutropenic rats were randomised to either compartment syndrome or control groups. CS Animals were treated with 45 minutes of elevated intracompartmental pressure (EICP) of the hindlimb. Fasciotomy was then performed, followed by 60 minutes of reperfusion. Control animals experienced no EICP. Blood was collected from carotid arterial lines used for pressure monitoring. Skeletal muscle tissue samples were collected from the EDL following reperfusion. Blood samples were obtained from carotid arterial lines and skeletal muscle was collected following reperfusion. A Multiplex assay was used to examine serum levels of 24 proinflammatory cytokines/chemokines. Skeletal muscle mRNA levels of P-Selectin, E-Selectin, Mac-1 and ICAM-1 were evaluated using real-time PCR. Finally, skeletal muscle apoptosis was measured by DNA laddering and a caspase-3 assay. **Results:** Neutropenic CS animals demonstrated a continuous increase in TNF-alpha levels, peaking at 700+/-350pg/ml by 60 minutes of reperfusion. TNF-alpha values for other groups did not increase. A 104-fold increase in ICAM-1 mRNA levels was observed in neutropenic CS rats while other groups showed no significant increase. There was no significant increase in any group for P-Selectin, E-Selectin, or Mac-1.

Conclusion: This study is the first to attempt to describe the molecular inflammatory response in CS. Neutropenic CS animals demonstrated an upregulation in TNF-alpha and ICAM-1 mRNA levels. This likely represents an attempt to generate an inflammatory response in the neutropenic animals. Additional data at incremental timepoints is necessary to further characterize the molecular mechanisms. However, both TNF-alpha and ICAM-1 appear to be important in the mechanism of inflammatory activation in compartment syndrome.

8 –

Insights into Mesenchymal Stem Cell Differentiation to Adult Chondrocytes -Human Facilitative Glucose Transporters (SLC2A proteins)? A New Generation of Physiological Markers

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Purpose: Differentiation of BM-MSCs into adult chondrocytes represents a complex physiological mechanism and full characterization of each individual stage through which the BM-MSC differentiate into adult chondrocytes is not yet understood. The physiological micro-environment of the chondrocytes is intensely hypoxic which triggers over-expression SLC2A proteins (GLUTs) in their membranes as a compensatory mechanism for energy production within the glycolytic cycle. **Method:** We cultured and differentiated BM-MSC, and adult chondrocytes in hypoxic (5% O₂ tension) and normoxic (20% O₂) conditions. Within this cell populations we screened for the presence of the 12 GLUT genes as well as quantification of the variation of the 12 GLUTs gene translation by simple pcr and rt-pcr. The expression profile of the GLUT proteins was investigated using western blot analysis and immunohistochemistry. Functional characterization of the GLUTs expressed in the different cell populations was carried out by the means of radio-isotope labeled hexose fluxes done accordingly to the substrate specificity and kinetic properties particular to each SLC2A isoforms. **Results:** Our data showed that the functional genotype and phenotype of the adult chondrocyte and hypoxic BM-MSC comprised an extensive expression of fructose-transporting GLUTs as opposed to the glucose-only transporting isoforms expression in normoxic BM-MSC. The flux data showed clear similarities in functional GLUT profiles between BM-MSC cultured in hypoxic conditions, adult chondrocytes. Investigation of the uptake of a panel of five individual sugars (glucose, fructose, 2-deoxy-glucose, 3-orthomethyl-glucose and galactose) in these cellular populations under both hypoxic and normoxic conditions and in the presence and absence of Cytochalasin B (a GLUT1-specific inhibitor) showed that SLC2A class II transporters (GLUTs 5, 7, 9 and 11) play a more important role in the uptake of sugars by the normal hypoxic chondrocytes when compared to the ubiquitously-expressed GLUT1. **Conclusion:** Use of this approach allows the correct culturing conditions to be identified that would select for those chondrocyte precursors from the total BM-MSC population that would have the best potential for producing viable articular cartilage. In addition, specific substrates for GLUTs isoforms could be used for physiologic, non-invasive and real time imaging of cartilage, BM-MSC and cartilage autograft by means of Positron Emission Tomography.

9 –

The Effect of Remaining at Confluence on the Chondrocytic Phenotype
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Purpose: Current techniques for articular cartilage repair remain sub-optimal. The best technique involves the introduction of cultured chondrocytes into the injury site. Experimental results of current chondrocyte culture and expansion techniques (passaging) have shown phenotypic alteration resulting in fibroblast-like cells. Therefore, treatment methods that propose the transplantation of cultured chondrocytes might be transplanting

fibroblast-like cells instead of chondrocytes. This experiment explored the difference in genetic expression of chondrocytes left at confluence compared to chondrocytes that were passaged as performed in current culture techniques. It was hypothesized that chondrocytes left at confluence would maintain their collagen I and collagen II gene expression over time. **Method:** Fresh normal human articular cartilage was collected from deceased donor patients. The matrix was digested and the chondrocytes were plated in monolayer to create two groups. The first group was cultured and passaged 2? at confluence seven times. The second group was cultured at confluence and left for seven weeks, with medium changes every 3-4 days without passaging. At weekly intervals RNA was extracted from cells in both groups and analyzed with real time PCR, probing specifically for the genes responsible for the production of collagen I, collagen II, aggrecan, and GAPDH. This was done in duplicate. **Results:** Collagen II gene expression was maintained over seven weeks in cells left at confluence but was decreased in passaged cells. Collagen I gene expression decreased over seven weeks in cells left at confluence, but remained the same in passaged cells. Aggrecan gene expression remained the same in both groups. **Conclusion:** Current culture and expansion techniques that employ passaging (as used in clinical scenarios) result in significant alterations in gene expression that are inconsistent with the current definition of a "chondrocyte". Culturing chondrocytes at confluence can produce gene expression more similar to native chondrocytes but even these cells have expression of collagen type I that should not be present in chondrocytes. The results of this study suggest that further investigation is required to develop chondrocyte culture and expansion techniques that minimize the de-differentiation of chondrocytes by maintaining collagen II gene expression and eliminating/preventing collagen I gene expression.