



Session: Others

25 APPLICATION OF ULTRASOUND SHORTENED THE DECALCIFICATION DURATION OF HUMAN CORTICAL BONE SAMPLE

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Introduction: Calcium hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ accounts for 60% to 70% of the bone tissue and contributes to the hardness of bone [1]. Decalcification is a process to dissolution the hydroxyapatite complex in bone matrix to soften the bone. After decalcification, the bone samples would be compatible with routine paraffin histologic preparation. The traditional decalcification method requires long incubation time and can lead to tissue swelling and hydrolysis of the bone matrix [2]. Ultrasound (US) is believed to enhance the decalcification by a cavitation mechanism [3]. We hypothesized that the application of US during bone decalcification would accelerate the decalcification compare with the traditional decalcification method without damaging bone tissue for histological assessments.

Methods: A human femur was obtained from Science Care (Arizona, USA) and was sectioned into 5mm thick transverse sections. The bone slices (n=6) were fixed in 4% phosphate buffered paraformaldehyde for 24 hours and were divided into two groups: Ultrasound Decalcification group (US DeCal) and Normal Decalcification group (Normal DeCal). For US DeCal, the bone sections were place in the US decalcifier (DeCa DX100, Pro-Cure Medical Technology Co. Ltd, Hong Kong, 50W at a frequency of 40kHz) with 300mL of 0.5M EDTA solution. The temperature of the EDTA solution was maintained at 30–45°C. For the Normal DeCal, bone slices were placed in a container with 300mL of 0.5M EDTA and maintained at 37°C. All EDTA solution was refreshed daily. The mineral content of the bone slices was measured by micro-CT and Dual-energy X-ray absorptiometry (DXA) at different time points. Calcium concentration of EDTA was measured by inductively coupled plasma optical emission spectrometry (ICP-OES). After decalcification had been completed, the samples were processed, embedded in paraffin, sectioned at 5µm, and stained with hematoxylin and eosin for analysis.

Results: For the US DeCal, the bone samples retained less than 7% of the mineral content at Day 6 and they were completely decalcified by Day 8. For Normal DeCal, the mineral content of these bone samples was 36.32±5.09% and 24.30±4.80% at Day 6 and Day 8 respectively. In addition, Normal DeCal samples took over 50 Days to complete decalcification. The concentration of calcium ions in the EDTA solution of the US DeCal group was 80% higher than the Normal DeCal (p<0.05). Histological analysis showed that there was no significant difference between the sections of the two groups.

Discussion and Conclusion: Ultrasound decalcification accelerated the decalcification process of human femoral bone slices compared with the traditional decalcification method. Our results suggested that decalcification by US shorten the duration by six times compare with the normal decalcification. Others reported that US shortened the decalcification process by 4–12 times depending on the thickness of the sample and the type of tissues [2]. The application of ultrasound technology would be suitable for the routine sample decalcification process for bone histology in both basic and clinical research.

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51 A PLATELET AGGREGATION-INDUCING FACTOR PODOPLANIN IS HIGHLY EXPRESSED IN METASTATIC LESIONS OF OSTEOSARCOMA

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Introduction: Osteosarcoma is the most common primary malignant bone tumor and has a high rate of systemic spread especially to the lungs. Primary metastasis is one of the risk factors, and increases mortality rate. Podoplanin (PDPN/Aggrus), a platelet aggregation-inducing type I transmembrane sialoglycoprotein, is involved in tumor invasion and metastasis. Several studies have reported that osteosarcoma expresses high levels of podoplanin. Moreover, podoplanin expression was reported to be involved in poor prognosis of osteosarcoma patients. However, the association between podoplanin expression and metastasis of osteosarcoma remains to be clarified because of the lack of high-sensitive anti-podoplanin monoclonal antibodies (mAbs), although many anti-podoplanin mAbs such as NZ-1 and D2-40 have been established. In this study, we established a novel anti-podoplanin mAb, LpMab-7, which possesses high sensitivity against podoplanin. Using LpMab-7, we investigated podoplanin expression in primary and metastatic lesions of osteosarcomas.

Methods: Hybridoma production

BALB/c mice were immunized by i.p. injection of LN229/hPDPN cells. After several additional immunizations with LN229/hPDPN cells, a booster injection was given i.p. 2 days before spleen cells were harvested. The spleen cells were fused with mouse myeloma P3U1 cells. The hybridomas were grown in RPMI medium with hypoxanthine, aminopterin, and thymidine selection medium supplement. The culture supernatants were screened using ELISA for binding to recombinant human podoplanin purified from LN229/hPDPN cells.

Epitope mapping using ELISA, Western blot and flow cytometry

To determine the epitope of LpMab-7, ELISA was performed for synthetic peptides of podoplanin. Several point mutants of podoplanin were produced and detected using Western blotting and flow cytometry.

Immunohistochemistry (IHC) against osteosarcoma tissues

Tissue specimens from 16 osteosarcoma patients, who underwent surgery at University Hospital of our institute, were used for IHC using LpMab-7, NZ-1, and D2-40. Four pulmonary metastatic specimens were used for additional studies. The study was approved by the institutional ethical committee. Informed consent for obtaining samples and for subsequent data analyses was obtained from each patient or the patient's guardian.

Results: We first established a novel anti-podoplanin mAb, LpMab-7, by immunizing mice with LN229/hPDPN. We next identified minimum epitope of LpMab-7, and identified it as RIEDL, which corresponds to Arg79-Leu83 of human

podoplanin using ELISA, Western-blot, and flow cytometry. Using IHC analysis, LpMab-7 showed high reactivity against osteosarcoma tissues compared with NZ-1 mAb. Furthermore, LpMab-7 detected podoplanin expressed in metastatic lesions of osteosarcomas. Of interest, podoplanin expression at metastatic lesions was higher compared with primarily lesions in 3 of 4 cases with lung metastasis.

Discussion: We investigated podoplanin expression by IHC using LpMab-7 mAb against 16 osteosarcoma tissues, four of which have pulmonary metastatic lesions. Although 3 of 4 metastatic lesions showed higher podoplanin expression than primary ones, more cases should be examined to conclude the association between podoplanin expression and osteosarcoma metastasis. Because LpMab-7 has high sensitivity against podoplanin, LpMab-7 mAb is expected to be useful for molecular targeting therapy and a metastatic marker for osteosarcomas.

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88

INTRODUCING A NOVEL SURGICAL TOOL TO FACILITATE IM NAILING – FEMORAL ANTEGRADE STARTING TOOL (FAST)

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Introduction: The current standard of care in lower extremity long bone fracture stabilization is closed intramedullary nailing (IMN). The surgical protocol associated with this surgery is well defined. Yet, challenges arise that impede the surgical workflow and lead to frustration in the operating room. Specifically, two surgical steps of entry point selection and reduction have been identified in the literature as the most challenging steps. Both of these steps utilize 2D fluoroscopic imaging to guide 3D alignment. Challenges arise when the alignment in one plane is lost while adjusting the alignment in the perpendicular plane. This leads to unpredictable repetition of activities which can be time consuming and frustrating. The primary aim of this study is to develop an innovative surgical instrument to facilitate the entry point selection.

Methods: Design requirements were identified by shadowing three surgeons performing eight IMN procedures in the operating room and conducting semi-structured interviews. Once a 3D model of the device was developed, a team, consisting of an experienced staff surgeon, a junior orthopaedic surgeon, and a mechanical engineer was consulted. Upon addressing the team comments, a potential device design was finalized and prototyped. The prototype was shown to the team to ensure the ease-of-use of the device as well as its functionality. However, multiple design improvements were made to optimize the ease-of-use of the device. The prototype of the new design was manufactured and tested on a synthetic bone with surrounding foam to simulate soft tissue. Three surgeons conducted the surgery under standard operating room conditions and provided feedback. The above process allowed the inventors to finalize the device's patent-pending design and establish the device use protocol.

Results: The proposed design consists of a fixed frame and a rotatable multicannulated arm to assist in guide wire insertion in femoral IMN. The device is initially placed at the approximate IMN entry point location on the femoral head under fluoroscopic image guidance. An anterior-posterior (AP) image is taken to align the rotatable arm with the intramedullary canal of the femur. Once the 2D alignment is satisfactory, the device is temporarily fixed to the femoral head via two pins. Based on the AP image a K-wire is placed into one of the three cannulated entry point AP positions. A subsequent lateral (or oblique-lateral) image is then taken to identify the correct 3D trajectory for accessing the intramedullary canal. The device can then be adjusted within the selected plane to match the correct trajectory via fixed rotation of the arm. The entry point can also be adjusted via selection of an alternate lateral cannula. The K-wire is then advanced through the cannulated guide into the intramedullary canal.

Conclusion: This simple device represents a novel surgical tool for use in IMN. In contrast to the current entry point selection activity cycle (which may include the acquisition of multiple AP and lateral images, patient repositioning and inaccurate drilling), use of the device ensures maintenance of the 2D alignment obtained in the AP plane while adjusting positioning in the perpendicular (lateral) plane.

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160

THE EFFECT OF ANTI-TNF INHIBITORS ON INNATE IMMUNE SYSTEM IN SYNOVIAL TISSUES IN RHEUMATOID ARTHRITIS PATIENTS

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Background: Potential biologic therapies have been developed for the prevention of joint destruction in rheumatoid arthritis (RA) patients. However, 20–30% of RA patients using biologics including anti-TNF inhibitors are non-responders or only minor improvement. Residual inflammation suggests a risk for progression of joint destruction. Furthermore, recent evidence has strongly suggested that the onset

and progression of RA depend on many different factors including innate immune sensors, such as Toll-like receptors (TLRs), participate in the induction of innate inflammatory response, and also following adaptive and/or autoimmune responses play an important role in RA inflammation.

Objectives: The aim of this study was to investigate the immunoinflammatory cells, including Toll-like receptor (TLR)-equipped cells, in synovial tissue samples from RA patients on anti-TNF inhibitors compared to patients with treatment of conventional synthetic disease-modifying antirheumatic drug (csDMARD).

Methods: Immune-inflammatory cells were evaluated in RA synovitis in patients with anti-TNF group [n=20 (etanercept 14, infliximab 6)] or csDMARD group (n=20) by immunohistochemical and immunofluorescence study. Mean duration of affection by RA of anti-TNF group and csDMARD-group was 8.3 years and 11.5 years, respectively. Period of anti-TNF group was 14 months. Mean CRP level of anti-TNF group and csDMARD-group was 15 g/dl and 22 g/dl and that of DAS28-CRP score (4) of anti-TNF group and csDMARD-group was 4.0 and 4.6 at collecting their samples, respectively. CD3 (T cells), CD20 (B cells), CD68 (macrophages), S-100 (dendritic cells:DC) and TLR1 to 9 immunoreactive cells were counted in at least five ×200 light microscope fields in larger lymphoid infiltrates. The intensity of the inflammation was estimated using the Krenn histopathological grading system (grade 0–3).

Results: The grading scores of synovitis was both 1.7 in each group and correlated best with the T and B cells in the both groups (p<0.05). Interestingly, both T and B cell counts were lower in the anti-TNF than in the csDMARD group (p<0.05). In contrast, the C-reactive protein (CRP) and disease activity score DAS28-CRP did not show clear-cut correlations with the inflammatory grade of the synovitis. Similar numbers of cells immunoreactive for TLR-1 to TLR-9 were found in synovitis in both groups.

Conclusion: Patients clinically responding to anti-TNF inhibitor might still have the potential of moderate/severe local joint inflammation, composed in particular of and possibly driven by the autoinflammatory TLR+ cells.

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274

USE OF THE COLLABORATIVE CROSS GENE MINE MOUSE PHENOTYPE LIBRARY TO IDENTIFY NOVEL GENES REGULATING BONE MASS AND BONE ARCHITECTURE

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Background: It is well established that there is a strong genetic effect on bone mass, bone loss and fracture risk; however, the vast majority of genetic variance for osteoporosis-related phenotypes remains unexplained. Novel approaches are needed to better identify the genetic underpinning of osteoporosis and to develop an understanding of the physiological and pathological roles of genes identified in this process.

Subjects and Methods: We utilised the Collaborative Cross (CC) Gene Mine mice to identify genes associated with bone volume. We employed μ CT to scan hindlimbs of 940 CC mice across 56 strains incorporating multiple ages and genders where available for each strain, and generated data on variables including BV/TV, Tb.N, Tb.Sp, Tb.Th, SMI, DA and Ct.Th from reconstructed femur images using CTAn software. Genomapping was then performed to identify candidate genes responsible for bone volume. We also correlated the candidate genes with femoral neck BMD in human cohorts.

Results: Dozens of candidate genes associated with these variables in mice were identified based on our analyses. Based on linkage analyses of BV/TV, peak loci were found at chromosome 13 in female mice and chromosome 3 in male mice, respectively. The former locus harbours candidate genes like Epha5, Itga1, Pelo and Itga2, and the latter harbours candidate genes like Snx27 and Txnip. With comparison of young and old mice, peak loci were found at chromosome 11 and 17 in female mice, suggesting potential genes like Fam83g and Rnf112; whereas peak loci were found at chromosomes 2 and 12 in male mice, suggesting potential genes such as Apob, Slc7a15, Laptm4a and Matn3. DA analyses in female mice showed peak locus on chromosome 18, which harbours genes like Ska1, Myo5b, Lipg, Epg5, Setbp1 and Nfatc.

Discussion and Conclusion: Among these candidate genes, five potential genes, namely Txnip, Nfatc1, Setbp1, Apob and Itga1, have been reported to be associated with femoral neck BMD in human subjects. Nfatc1 has been extensively explored in its role in the osteoclast fusion process. Txnip, which is linked to glucocorticoid-induced bone loss, is thought to be a strong candidate gene for bone mass. Setbp1, which is closely related to Schinzel Giedion syndrome and atypical CML, is hypothesised to produce a gain-of-function mutation which may result in reduced PP2A and subsequently enhanced osteoblastogenesis. Itga1 is highly expressed in osteoblasts and its loss has been identified to be correlated with impaired fracture healing, accelerated knee osteoarthritis and reduced BMD in humans. Apob is mainly responsible for carrying lipids. Mutations in this gene