INTRODUCTION:

In contrast to tens of microns long linear microcracks, present between lamellae and osteon-matrix interfaces, diffuse damage, containing sub-lamellar cracks, contributes superiorly to bone toughness [1]. Moreover the faces of the submicroscopic cracks in diffuse damage patches are linked by the strained elements of bone’s organic matrix having glue like properties [2]. Thus, it seems likely that bone’s extracellular matrix proteins such as osteopontin (OPN) and osteocalcin (OC) that are intimately associated with one another and bone collagen and mineral [3,4] may influence microdamage formation and bone fragility. Here, using a new experimental approach that permits isolation and quantification of proteins of interest in laser-microdissected bone tissue, we demonstrate difference in select non-collagenous matrix proteins (NCPs) associated with bone microdamage and tissue age.

METHODS:

Laser Microdissection of Bone Tissue. Human cortical bone beams, fatigued to 50% stiffness loss and stained en bloc in basic fuchsin were sectioned transversely (i.e., perpendicular to its long axis) into 5 um thick sections. Sections were bonded to a membrane slide (Molecular Machines and Industries (MMI), Florida, USA), pre-imaged, and laser-cut directly using the Olympus IX71 Inverted Microscope (Olympus America Inc., Pennsylvania, USA) equipped with MMI CellTools (MMI, Eching, Germany). The sample was automatically collected into an eppendorf tube with an adhesive cap (MMI, Florida, USA) and used immediately for protein isolation or stored at -80°C until use. Images of the sections that were used to cut the selected areas of interest (e.g. circular areas enclosing damaged, osteonal or interstitial bone and their spatial controls) are shown in Figure 1.

Protein Isolation. Extraction buffer (0.05 M EDTA, 4M Guanidine chloride, 30 mM Tris-HCl, 1 mg/ml BSA, 10 ul/ml Halt Protease Inhibitor from “Pierce”; pH 7.4) was added directly into the tubes containing microdissected samples. Subsequently, the tubes were inverted and incubated overnight at 4°C to facilitate the detachment of the samples from the adhesive cap and protein extraction. In the next step, each sample was transferred into an eppendorf tube that had a whole melted in the lid and was covered with a dialysis membrane (SpectraPor3 3 Dialysis Membrane, Spectrum Laboratories, Inc., CA, USA). The dialysis was conducted at 4°C against several changes of the PBS buffer pH 7.4. The samples were either used directly for OPN and OCN ELISA analyses or freeze-dried and stored at -80°C until use.

Enzyme-Linked Immunosorbent Assays. Detection and quantification of OCN and OPN were performed according to the protocols included with the hOST-EASIA and Human Osteopontin Assay kits (ARP American Research Products, Inc., MA, USA), respectively.

RESULTS:

There was 20 times or more OCN present in osteonal bone (293x10^3 ng/mm^3 bone) than OPN (16.1x10^3 ng/mm^3 bone) but the amounts of OPN and OC were similar in the interstitial bone (OC = 7.07x10^3 ng/mm^3 bone, OPN = 7.54x10^3 ng/mm^3 bone) (Fig. 2). Consequently, osteonal bone had higher OCN contents than its spatially weight-matched interstitial bone. The qualitative differences in the OC and OPN content within and between osteonal and interstitial bone were maintained whether we analyzed three discs or one disc per section. More importantly, when diffuse damage areas and their spatially-matched non damaged tissue was analyzed, the diffuse damage area had almost 25 times more OCN content (76.36x10^3 ng/mm^3 bone) than non-damaged area surrounding it (3.18 x 10^3 ng/mm^3 bone). OPN content in the damaged area (44 x 10^3 ng/mm^3 bone) was also greater than its non-damaged control (32 x 10^3 ng/mm^3 bone).

DISCUSSION:

We developed a new technique facilitating isolation and quantification of major non-collagenous proteins in targeted micro-regions of cortical bone that allowed us to explore how such proteins may influence microdamage formation and morphology.

When investigating protein contents in younger (osteonal) versus more matured (interstitial) bone tissue, we found that there was a greater prevalence of OPN and OC in osteonal bone. This may indicate that OC and OPN could play a structural role in determining the morphology of microdamage since these two tissue types have different tendencies to form microdamage. Diffuse damage formation, which makes bone more ductile, is preferentially associated with osteonal bone while linear microcracks are associated with interstitial bone [1].

More importantly, we also found that damaged bone had more OC and OPN than undamaged bone even within similarly aged cortical bone tissue. As OC is considered an inhibitor of bone formation (3) while OPN is a trigger of bone resorption [5], the combination of these proteins may further act to alter bone remodeling and microdamage accumulation. In conclusion, this study found that select NCPs including OPN and OC are present in greater amount in bone areas containing damage morphology beneficial to bone toughness.

REFERENCES:


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Figure 1. Upper panel: Location (a) and extraction of diffuse damage area (b) and its spatial control (c). Lower panel: Location (d) and extraction of osteonal (e) and its surrounding interstitial (f) tissue.

Figure 2. Contents of OCN (A, B) and OPN (C, D) in osteonal (O) and interstitial (I) as well as damaged (DD) and surrounding interstitial (SIc, control) undamaged tissue of cortical bone.

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