Abstract 33: Orthopaedic Research Society; 1999.

QUANTITATIVE MICROPROBE ANALYSES SUGGEST THAT CEMENT LINES OF SECONDARY OSTEONAL BONE ARE NOT MINERAL DEFICIENT

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INTRODUCTION Local variation in mineralization caused by cement lines (reversal lines) is considered important in bone micro-mechanics, including microcrack propagation and arrest. Controversy over the mineralization at the cement line has been the motivation for this study. Using a 60 kV electron beam and energy dispersive analysis of X-rays (EDX), recent investigators have concluded that cement lines of secondary osteons are *mineral deficient*, and hence form a relatively low modulus, viscous interface with respect to the surrounding bone (1). This is an important conclusion since it challenges conventional wisdom and large bodies of experimental data and observations suggesting that cement lines are relatively *highly mineralized* interfaces (2). EDX techniques that use high energy electrons, however, are known to sample relatively large volumes of tissue and can readily cause specimen degradation, making it uncertain if this technique is acceptable for determining mineral content variations in minute volumes on the order of cement lines (<5 μm wide). The present investigation used two advanced techniques to test the hypothesis that cement lines in osteonal bone are mineral deficient.

MATERIALS AND METHODS Cortical bone samples were obtained from transverse segments of the anterior cortex of mid-shafts from 13 human femora and 5 radii. Donors were between the ages of 18 and 87 (7 males and 11 females); most were trauma victims, and all were free of bone diseases that may affect bone tissue. With minimal modification, samples were prepared in accordance with methods used in a previous study where cement lines were shown to be mineral deficient (1). Details of the specimen preparation can be found in published methods on the following two techniques: 1) quantitative backscattered (BSE) microscopy (3,4), and 2) quantitative EDX (5). Bone specimens were both dried and embedded in methylmethacrylate, and all specimens were undecalcified.

BSE ANALYSIS Analysis was performed in the BSE mode of the scanning electron microscope (SEM). The SEM was configured with a four-quadrant BSE detector, the Link eXL analysis system and the following settings: 0.75 nA probe current, 30 kV, and 15 mm working distance (3). Digitized images were captured randomly at 1500X after increasing the signal to noise ratio with 9 Kalman scans. Eight radial sections (3 unembedded, 5 embedded) and 11 femoral sections (3 unembedded and 8 embedded) were used in the BSE analysis. Relative differences in the atomic number contrast (i.e. ash content) (3) were expressed as regional differences in weighted mean graylevel (WMGL) using custom modification to public domain software (3). Figure 1 show the position of the sites analyzed: 1) within extraosteonal bone ~10 μm from cement line (S1), 2) within the cement line (i.e., crenulated interface)(S2), 3) 0.7 μm inside the cement line (S3), 4) 1.4 μm inside the cement line (S4), and 5) within secondary osteonal bone ~10 μm from the cement line (S5). Sampling at these five sites was done 3 times within each osteon, and 5 osteons were analyzed in each bone specimen. Since BSE/WMGL analysis is done using digitized images, these five sites were analyzed at the same juxta-position. Data from femora and radii from each microprobe technique were combined and analyzed in all possible paired combinations. A one-way ANOVA design and Fisher's LSD was used to assess paired comparisons for statistical significance ($p<0.05$).

EDX ANALYSIS The SEM was configured with the Link ISIS X-Ray analysis system, and the following settings: 2.5 nA probe current, 20 kV, 15 mm working distance, and 100 second livetime (5). $CaCO_3$ and InP were used as reference standards for ZAF corrections. Digitized images were collected at 4000X to ensure proper beam placement. Nine radial sections (4 unembedded, 5 embedded) and 22 femoral sections (10 unembedded, 12 embedded) were used in the EDX analysis. Osteons and the various analysis locations were selected using a random number generator and grid system. Spectra were obtained from the above mutually exclusive sites (less site 3, the measurement 0.7 μm inside the crenulated interface) of each osteon and analyzed for weight percent calcium and phosphorus. Sampling at these four sites was done twice within each osteon, and 5 osteons were analyzed in each bone specimen.

RESULTS During the EDX analysis, recorded observations made with the unaided eye showed that at low magnification all images revealed a relatively white line coincident with the historic location of the cement line. All BSE images showed a bright white line (Figure 2). As shown in Table 1, EDX data did not reveal any significant differences in wt % Ca, wt % P, or Ca/P ratios among the four sampled sites for unembedded bone. For embedded bone, EDX data showed no significant difference between S4 and S5 for wt.% Ca or wt.% P. Wt.% Ca at S2 was significantly ($p<0.05$) greater than S5. Minor regional differences in Ca/P molar ratios were detected between three paired comparisons (S2>S1, $p=0.02$, S2>S5, $p<0.01$; S5<S4, $p=0.5$). Ca/P ratios typically exceeded that of stoichiometric hydroxyapatite (1.67). In contrast to the EDX data, BSE/WMGL data revealed that S2 (the traditionally defined cement line) was notably brighter (at least 37.5%>WMGL, S2 vs. S1, $p<0.0001$) than each of the other four sampled sites in both embedded and unembedded bone. S3 was significantly brighter than S5 ($P<0.0001$) in unembedded bone. Only S4 in embedded bone showed any signs of hypomineralization ($p<0.001$) with respect to osteonal bone (S5).

Table 1 Unembedded (u) and Embedded (e) Data

Site	Wt % Ca	Wt % P	Ca/P	WMGL
1 (u)	23.68 (3.56)	8.99 (2.32)	2.09 (0.24)	92.89 (22.43)†
2 (u)	24.18 (4.64)	9.02 (2.98)	2.17 (0.34)	127.81(24.47)*†
3 (u)	----------	----------	----------	87.53 (22.77)†
4 (u)	22.88 (3.73)	8.61 (2.23)	2.11 (0.22)	76.52 (22.04)*
5 (u)	22.34 (3.76)	8.59 (2.04)	2.04 (0.17)	77.85 (21.12)*
1 (e)	25.11 (3.12)	9.69 (2.17)	2.05 (0.24)	74.23 (22.23)†
2 (e)	25.13 (2.67)†	9.29 (2.12)	2.16(0.31)*†	109.10 (20.74)*†
3 (e)	----------	----------	----------	65.71 (20.00)*
4 (e)	23.82 (3.19)*	8.78 (2.02)*	2.14 (0.24)†	56.79 (21.76)*†
5 (e)	24.13 (2.49)	9.29 (1.79)	2.05 (0.22)	64.69 (21.25)*

*significantly different than site 1; significantly different than site 5

DISCUSSION Two plausible conclusions can be drawn from the present study. The EDX data clearly reveal the absence of regional wt.% Ca differences. Hence, there is no hyper- or hypo-mineralized zone at the periphery of secondary osteons. In contrast, BSE data clearly indicates a zone of highly mineralized (or alternatively, collagen deficient) tissue. There may also be a region of hypomineralized tissue 1.4 μm from the osteonal periphery, indicating a two-phase mineralization zone. This hypomineralized zone was, however, only statistically significant in embedded bone tissue. The discrepancy between the two analysis techniques may indicate that the uniform EDX data are artifactual, reflecting the sampling of relatively large interaction volumes which extend well beyond the narrow limits of the target material. Although we used a 20 kV beam and random sampling of relatively distant regions (in contrast with previous studies (1)), large sampling volumes are inevitable in EDX (5). Consequently, the greater resolution and stability of BSE image analysis (3,5) may be the only accurate microprobe technique used in the present study. Using either technique, however, the data contradicts previous observations of hypomineralized cement lines.

REFERENCES **1)** Burr et al., 1988 J Biomech. 21:939-45; **2)** Currey, 1984 The Mech. Adaptations of Bones, Princeton Univ Press; **3)** Bloebaum et al., 1997 Bone 20:485-90; **4)** Skedros et al., 1993a,b J Biomed Mat Res 27:47-56; **5)** Vajda et al., 1996 Cells & Materials 6:79-92.

ACKNOWLEDGEMENTS This project was funded by the Department of Veterans Affairs Medical Research Fund.

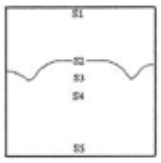

Fig. 1 Position


Fig. 2 BSE image

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