Human Skeletal Biology: Insights from Surgical Biospecimens

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INTRODUCTION

Information about mechanisms of skeletal physiology and pathophysiology often comes from research with animal tissues or from mice with specific genes that have been knocked out or constitutively upregulated. In biomedical research, "connectivity" is the relationship between a clinical condition and the research model (animal, cell culture, mathematical), with "low connectivity" to indicate a weak relationship between the data from the model and the human condition. Isolation of human cells may introduce artifacts that are not related to the *in vivo* setting, and expansion of the cells *in vitro* may introduce additional artifacts. Nevertheless, it has been our experience that research with freshly isolated or minimally expanded marrow and cartilage that is discarded during the course of arthroplasty provides important insights into the biology of human bone and cartilage cells. A critical aspect of this approach is understanding the impact on the findings of co-morbid conditions and drugs taken at the time of tissue procurement. Conclusions can be confounded if attention is not paid to such factors.

With review and approval of the institutional board that oversees compliance with ethical, safety, and privacy matters related to clinical investigations, it is possible to conduct different levels of studies with discarded tissue that are referred to as Research-Related Uses of Excess Human Material (Table I). In level 2, the identity of the subject may not be maintained, but relevant clinical information can be scanned to determine suitability for the study; after procurement of excess discarded tissue, the sample is deidentified and clinical records cannot be re-examined. As shown by the findings summarized in this article, it is important to determine potentially relevant clinical information (age, gender, diagnosis, medications, etc.). In level 4, subjects can be recruited with informed consent for additional tests or questionnaires that are not part of their clinical care, with those data to be linked to the discarded tissue.

Levels of Research/ Review	Excess Materials: Tissue, Blood	Human Subject Research	Consent	Access to Clinical Information
1	Yes	No	No	No
2	Yes	Exempt from IRB review (Exemption #4)	No	Limited access; deidentified; can never go back to the record
3	Yes	Yes Annual IRB review	Waiver of Consent (8 criteria must be met)	Can access, record, and reaccess medical data; coded to protect identification
4	Yes	Yes Annual IRB review	Informed consent	Interact with patient: questionnaires, additional tests, follow-up

Table I. Levels of Research with Excess Human Materials, IRB Requirements, and Research Limitations[a]

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HUMAN CHONDROCYTES

When surgical management for *pectus excavatum* was common, discarded rib segments from growing children were available for research on the problem of *in vitro* de-differentiation of chondrocytes, a problem that compromised investigations of chondrocyte biology [1]. This research showed that the chondrocyte phenotype could be maintained by growing the isolated chondrocytes on a substratum that prevented cell spreading. Cultures with the roundest configuration were associated with greater production of cartilage-specific extracellular matrix. The flattest cells and those with intermediate shape showed a range of phenotypic functional loss.

In a more recent study with discarded articular cartilage, it was our goal to determine whether sufficient RNA could be obtained for profiling of expressed genes and whether the gene profile was related to disease severity [2]. Excess cartilage was

obtained from subjects undergoing knee replacement. It was critical to this study that the tissue was subdivided into homogeneous portions by the surgeon according to the Outerbridge classification. For some subjects, it was possible to separate the tissue into distinct fractions with different disease severity. Portions of each sample were prepared either for histological analysis and ranking according to the Mankin system or for RNA extraction. We found that clinical grading was correlated with histological score (Spearman $r=0.60$, $p=0.043$). Quantitative, competitive RT-PCR assays were used for measurement of mRNA for cartilage-specific genes. The average weight of the graded cartilage samples was 3.7 g ± 2.2 g. An average of 34 ± 13 million cells was isolated by enzymatic digestion. After cell lysis, an average of 9.4 µg mRNA was obtained (range 3.0 to 23.8 µg). For samples that weighed more than 2 g, peak yield of cells and mRNA were achieved. Thus, 2 g is the smallest sample that will provide optimal recovery of cells and mRNA; samples smaller than this may not provide representative data. This study revealed an inverse relationship between regional disease severity in osteoarthritis and expression of aggrecan. Use of quantitative, competitive RT-PCR is practical for assessment of chondrocyte gene signatures, and clinical grading of the portion that is tested influences gene expression.

THE BONE MARROW MICROENVIRONMENT

On gross examination, adult bone marrow may be red or yellow. Red marrow contains all the cell lines normally associated with hematopoiesis. The major cell type in yellow marrow is fat. In 1882, Neumann noted that at birth all bones that contain marrow, have red marrow. With advancing age, there is regression of hematopoiesis such that in adulthood, yellow, or fatty, marrow predominates in peripheral locations. The fat cells appear to serve as storage of energy and may be replaced in states of stimulated hematopoiesis.

Red bone marrow is known as the seedbed of the blood that throughout adult life has pluripotent stem cells that renew, proliferate, and differentiate into each of the many hematopoietic and lymphocytic cell lines. Red marrow contains immature, maturing, and mature hematopoietic cells, along with a stromal fraction that supports hematopoiesis with secreted and cell-surface growth and differentiation factors. Hematopoietic stem cells can be isolated and can differentiate *in vitro* to different blood lineages if the specific growth and differentiation factors are provided. For example, erythropoietin stimulates red blood cell differentiation of immature cells that possess its receptor. Among the committed macrophage progenitor cells are cells that give rise to bone-resorbing osteoclasts.

The term hematopoietic microenvironment describes different microgeographical niches having specific interactions between the stromal cells and their products with the hematopoietic stem cells. Lineage in the microenvironment is determined by the medley of factors produced by the stromal cells. Likewise, homing and repopulation of damaged marrow is directed by the stromal cells [3].

In addition to supporting hematopoiesis and osteoclast differentiation, marrow stromal cells include a fraction of cells capable of differentiation into osteoblasts, chondrocytes, adipocytes, tendon, and other connective tissue cells. Although they do not display the self-renewal shown by hematopoietic stem cells, the pluripotential stromal cells have been termed mesenchymal stem cells, but more precise terms are marrow stromal cells or marrow-derived osteoblast progenitor/precursor cells.

Marrow can be obtained as excess discarded tissue from patients undergoing hip arthroplasty for non-inflammatory osteoarthritis. Because marrow contains many maturing and mature blood cells, it is useful to separate them from stem and progenitor cells by density centrifugation. Upon centrifugation over a solution with density of 1.077, low-density cells, with a high ratio of nucleus to cytoplasm, are concentrated at the top, whereas heavy cells with differentiated cytoplasmic organelles collect at the bottom and can be discarded. The yield of low-density cells from the femoral head and marrow discarded during hip arthroplasty ranges between 1 and 800 million. The yield from shoulder arthroplasty ranges between 2 to 220 million. The low-density population includes hematopoietic stem cells and immature marrow stromal cells. Interactions between these two fractions can be studied *in vitro*. They can be separated most easily by the fact that the latter are adherent to tissue culture plastic dishes. Removal of the non-adherent (hematopoietic) cells after 24 hours leaves a dish enriched with marrow stromal cells for studies on osteoblast and chondrocyte differentiation.

MARROW-DERIVED PROGENITORS OF OSTEOCLASTS

Osteoclasts are derived from hematopoietic cells upon stimulation with Macrophage-Colony Stimulating Factor (M-CSF) and RANKL (Receptor Activator of NF-κB Ligand) (Figure 1). Those agents are produced by marrow stromal cells and osteoblasts, as is a secreted inhibitor, osteoprotegerin (OPG). The osteoclast progenitors possess cell-surface receptors, such as RANK, for specific growth and differentiation factors. Systemic agents that promote osteoclastogenesis, such as parathyroid hormone, tumor necrosis factor, interleukins, and 1,25-dihydroxyvitamin D, act through those local regulatory mediators.

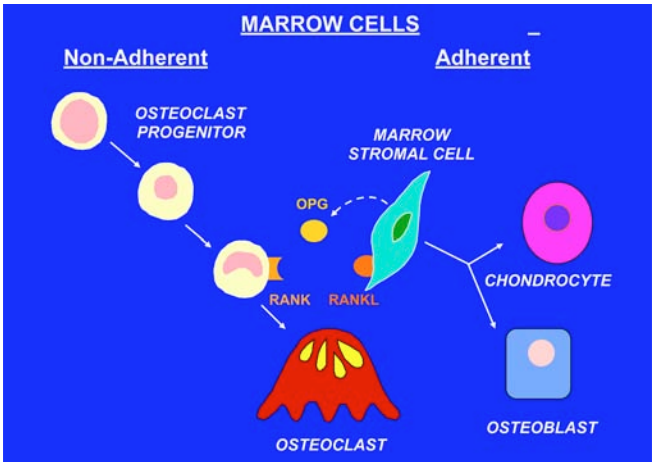

Figure 1. Differentiation of skeletal cells from marrow cells. The non-adherent fraction contains hematopoietic stem cells and the progenitor of osteoclasts. Osteoclast differentiation is regulated by local products such as RANKL, which activates the cell-surface receptor RANK; the secreted protein OPG blocks that interaction. The adherent fraction contains the marrow stromal cell, which can differentiate to osteoblasts, chondrocytes, and other connective tissue cells.

Low-density marrow cells are cultured in phenol red-free α-minimal essential medium with 10% fetal bovine serum and antibiotics. Phenol red, the pH indicator that is added to most media, acts as an estrogen mimetic and needs to be avoided in experiments that can be affected by sex hormones. Osteoclast differentiation is assessed by multinuclearity, tartrate-resistant acid phosphatase activity, calcitonin receptors, or cathepsin K. Cells with those features appear with 10 days *in vitro* [4].

INFLUENCE OF CLINICAL STATUS ON OSTEOCLAST DIFFERENTIATION *IN VITRO*

Many clinical parameters influence the differentiation of osteoclasts, including age, menopausal status, and medications used at the time of surgery. Marrow from older individuals generates more osteoclasts *in vitro* than does marrow from younger individuals; osteoclastogenesis is positively correlated with age ($r=0.98$, $p<0.01$) [4]. Human marrow stromal cells secrete a variety of cytokines that affect development of osteoclasts, such as interleukin (IL)-6 and -11. We tested whether the effect of age on osteoclast differentiation was explained by these mediators [5]. Constitutive secretion of IL-6 and IL-11 was positively correlated with age for marrow from both women and men ($p<0.01$). At the time of those experiments, many women presenting for hip arthroplasty were receiving estrogen replacement therapy (ERT); it was striking that marrow from women receiving ERT showed lower constitutive secretion of IL-6 (7.5%, $p<0.006$) and IL-11 (43%, $p<0.05$) than marrow from age-matched, estrogen-deficient women. In other words, *in vivo* treatment with estrogen rejuvenates the *in vitro* behavior of the marrow cells. The marrow cells reflect the endocrine status of the subject from whom the marrow is obtained.

In the early phase of this research, we obtained marrow from diverse subjects who underwent joint arthroplasty. We had the opportunity to examine cytokine production in marrow from several subjects with rheumatoid arthritis (RA) compared with age-matched women with osteoarthritis (OA). Whereas none of the marrow from OA subjects showed detectable interleukin secretion at 48 hours, many RA samples showed constitutively high secretion of IL-6 and IL-1β. Three samples from RA subjects that did not secrete interleukin were illuminating. Two were from subjects who were receiving corticosteroids at the time of surgery. Thus, marrow production of inflammatory cytokines may have been suppressed by that *in vivo* medication. One sample of marrow was from an RA subject receiving ERT at the time of surgery; that marrow also showed delayed and reduced secretion of interleukins. This is consistent with our hypothesis that ERT inhibits secretion of interleukins, which are osteolytic mediators in bone. In sum, inflammatory status, anti-inflammatory medications, and ERT influence cytokine production by bone marrow. Unless one is investigating inflammation-related mechanisms, marrow from subjects with inflammatory joint disease should be excluded.

Other studies show that there is an age-related decline in marrow production of OPG, a secreted decoy receptor that blocks stimulation of osteoclast differentiation by RANKL [6] (Figure 1). In a small number of samples from post-menopausal women receiving ERT, OPG was expressed at levels like those in marrow from younger women. Age-related loss of this inhibitor may contribute the age-related increases in bone resorption and in fracture risk that are seen in many elders.

Low-density marrow cultures contain the osteoclast progenitors and supportive stromal cells. It is not the nonadherent hematopoietic stem cells that secrete interleukins, either constitutively or with IL-1 treatments [7]. It is the stromal cell fraction that secretes the osteoclastogenic interleukins. We tested whether the age-related secretion of those cytokines could be rejuvenated by *in vitro* treatment of marrow with hormones that are well-known to decline with age, dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), and 17β-estradiol (E2) [7]. Each of those hormones suppresses IL-6 secretion, but the magnitude of suppression depends upon clinical status; *in vitro* inhibition by E2 of IL-6 secretion is abrogated in marrow from women receiving ERT. Human marrow cultures provide a method to examine differences in cytokine secretion while providing information about the impact of clinical features of marrow biology *in vitro*. The conclusion can be drawn that clinical bone resorption could be inhibited by treatment with those hormones, but it would seem wise to identify analogs with less risk for hormone-dependent breast and prostate cancers.

In recent years, few postmenopausal women scheduled for joint arthroplasty are receiving ERT, but more are receiving a bisphosphonate. A pilot study indicates that marrow from women receiving a bisphosphonate showed lower expression of the pro-osteoclastogenic gene RANKL and lower generation of osteoclasts [8]. This is yet another example of marrow displaying different characteristics depending upon medications that are being taken by the individual.

In sum, these studies help to explain mechanisms of age-related bone loss by showing 1) that human marrow generates osteoclasts and factors that stimulate and inhibit their differentiation, 2) that with age there are constitutive increases in osteoclastogenesis and in pro-osteoclastogenic factors and a decrease in an inhibitor of osteoclastogenesis, 3) that marrow from women receiving ERT or bisphosphonate shows suppression of osteoclastogenesis, and 4) that regulation of osteoclast differentiation can be studied with human marrow as long as there are controls for age, estrogen status, and medications that influence bone metabolism.

MARROW-DERIVED PROGENITORS OF OSTEOBLASTS

A subpopulation of marrow stromal cells (MSCs) has the potential to differentiate to osteoblasts and other connective tissue cells (Figure 1). Osteoblast differentiation can be stimulated by addition of osteoblastogenic supplements to the cultures (10 nM dexamethasone, 5 mM β-glycerophosphate, and 170 μM ascorbate-phosphate). Not only are these cells useful for studying the mechanisms of osteoblast differentiation, they also have potential for tissue engineering applications, especially when cultured in 3-dimensional scaffolds. For example, we described how culture conditions can influence the differentiation pathways that human marrow stromal cells follow; they can be con-

trolled to generate either cartilage or bone in collagen scaffolds [9]. Other studies show the influence of growth factors and low oxygen on differentiation [10, 11].

INFLUENCE OF CLINICAL STATUS ON OSTEOBLAST DIFFERENTIATION *IN VITRO*

Discarded human marrow can be used to discover the mechanisms of skeletal aging and the factors involved in the well-described imbalance between bone formation and bone resorption as we age. We found a striking age-dependent decrease in osteoblastogenesis in human MSCs from men [12, 13] and women [13]. Because those recent cohorts did not include women who received ERT, it is not possible to discern the relative contributions of age and estrogen-deficiency.

It is likely that endocrine aging contributes to decreased osteoblast differentiation because addition to the cultures of the hormones that decrease with age (DHEA, DHT, and E2) results in an *in vitro* rejuvenation of osteoblast differentiation [14]. Using human marrow cultures, we have been examining the contributions of such extrinsic factors and well as intrinsic mechanisms of skeletal aging. Extrinsic refers to those systemic age-related changes that have an impact on the skeleton, such as endocrinological aging; intrinsic refers to age-related alterations in bone cells that impair their performance [14]. There are a number of intrinsic cell properties that differ in marrow from young and old subjects [13]. There is an age-related decrease in proliferation of MSCs. This is accompanied by increases in apoptosis and Senescence Associated-β-galactosidase, a marker for *in vivo* age and *in vitro* senescence. These inherent age-related properties of MSCs may contribute to skeletal aging.

We tested whether there is an effect of age on genes known to be involved in regulating proliferation. There are increases in expression of *p53* and its pathway genes, *p21* and *BAX* [13]. In addition, *WNT2* expression in hMSCs is decreased with age [15]. These discoveries lay a foundation for rejuvenation strategies.

Intracellular signaling pathways are used by cells to respond to specific hormonal and cytokine stimulation and they result in amplification that causes changes in gene expression or cell function. For example, low-dose parathyroid hormone treatment is an osteoanabolic therapy and it initiates effects in cells by activating a cell-surface receptor and down-stream signaling pathways. We evaluated signaling response to parathyroid hormone and found a loss of signaling in MSCs from older individuals [16]. Such an intrinsic malfunction may be amenable to novel approaches to enhance signaling in elders.

Age has a profound influence on many features of marrow stromal cells, but other clinical features are likely to do so as well. We had previously discovered vitamin D-deficiency (serum 25-hydroxyvitamin D < 20 ng/mL) in 46% of women with advanced osteoarthritis who presented for hip arthroplasty [17]. We were concerned about vitamin D-deficiency influencing marrow biology. This level 4 study (Table I) required informed consent for measurement of serum 25-hydroxyvitamin D and evaluation of diet and sun exposure; recruitment and testing occurs prior to arthroplasty and experiments with the excess marrow. Preliminary results suggests that *in vitro* stimulation of osteoblast differentiation by activated vitamin D was greatest in cells from vitamin D-deficient elders [18]. This may mean that repletion of vitamin D-deficient subjects may lead to more vigorous bone formation.

CONCLUSION

Excess, discarded human marrow is a valuable resource for testing hypotheses about skeletal cell differentiation and function, with connectivity to the *in vivo* phenotype. Because of the important effects of such clinical parameters as age, menopause, medications, and vitamin D status, it is essential to control for their influences on *in vitro* cell and tissue biology. These investigations establish that human marrow reflects the clinical setting and retains those influences *in vitro*.

References

[1] **Glowacki J, Trepman E, Folkman J.** Cell shape and phenotypic expression in chondrocytes. Proc Soc Exp Biol Med. 1983;172:93-8.
[2] **Eid K, Thornhill TS, Glowacki J.** Chondrocyte gene expression in osteoarthritis: Correlation with disease severity. J Orthop Res. 2006;24:1062-8.
[3] **Anklesaria P, Kase K, Glowacki J, Holland CA, Sakakeeny MA, Greenberger JS.** Engraftment of a clonal bone marrow stromal cell line *in vitro* stimulates hematopoietic recovery from total body irradiation. Proc Natl Acad Sci (USA). 1987;84:7681-5.
[4] **Glowacki J.** Influence of age on human marrow. Calcif Tissue Int. 1995;56S:50-51.
[5] **Cheleuitte D, Mizuno S, Glowacki J.** *In vitro* secretion of cytokines of human bone marrow: effects of age and estrogen status. J Clin Endo Metab. 1998;83:2043-51.
[6] **Makhluf HA, Mueller SM, Mizuno S, Glowacki J.** Age-related decline in osteoprotegerin expression by human bone marrow cells cultured in three-dimensional collagen sponges. Biochem Biophys Res Comm. 2000;268:669-72.
[7] **Gordon C, LeBoff MS, Glowacki J.** Adrenal and gonadal steroids inhibit IL-6 secretion by human marrow cells. Cytokine. 2001;16;178-86.
[8] **Eslami B, Zhou S, LeBoff MS, Glowacki J.** Reduced osteoclastogenesis and RANKL expression in marrow from women taking alendronate. Am Soc Bone Mineral Res, 2008, Montreal, Canada
[9] **Zhou S, Yates KE, Eid K, Glowacki J.** Demineralized bone promotes chondrocyte or osteoblast differentiation of human marrow stromal cells cultured in collagen sponges. Cell Tissue Banking. 2005; 6:33-44.
[10] **Zhou S, Eid K, Glowacki J.** Cooperation between TGF-β and wnt pathways during chondrocyte and adipocyte differentiation of human marrow stromal cells. J Bone Mineral Res. 2004;19:463-70.
[11] **Zhou S, Lechpammer S, Greenberger JS, Glowacki J.** Hypoxia inhibition of adipocytogenesis in human bone marrow stromal cells requires TGF /Smad3 signaling. J Biol Chem. 2005;280:22688-96.
[12] **Mueller SM, Glowacki J.** Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. J Cell Biochem. 2001;82:583-90.
[13] **Zhou S, Greenberger JS, Epperly MW, Goff JP, Adler C, LeBoff MS, Glowacki J.** Age-related intrinsic changes in human marrow stromal cells and their differentiation to osteoblasts. Aging Cell. 2008 Jan 31; [Epub ahead of print]
[14] **Glowacki J, Zhou S.** Extrinsic and intrinsic mechanisms of human skeletal aging. Orthop J Harvard Medical School. 2007;9:80-2.
[15] **Shen L, Zhou S, Glowacki J.** Aging of human bone marrow stromal cells: role of WNT pathways. Am Soc Bone Mineral Res, 2008, Montreal, Canada
[16] **Glowacki J, Zhou S, Amato I, Adler C, Epperly MW, Greenberger JS.** Age-related intrinsic changes in human marrow stromal cells and their differentiation to osteoblasts. Orthopedic Res Soc. 2006, Chicago IL.
[17] **Glowacki J, Hurwitz S, Thornhill TS, Kelly M, LeBoff ML.** Osteoporosis and vitamin D deficiency among postmenopausal osteoarthritic women undergoing total hip arthroplasty. J Bone Joint Surg. 2003; 85A: 2371-2377.
[18] **Glowacki J, Zhou S, LeBoff MS, Mueller SM, Goff J, Greenberger JS.** Effects of vitamin D status on osteoblast differentiation in human marrow stromal cell cultures: Preliminary results. ASBMR Meeting on Contemporary Diagnosis and Treatment of Vitamin D-Related Disorders. 2006, Arlington VA