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Original Article

Hylonome

Measurement of gene expression following cryogenic μ-CT scanning of human iliac crest biopsies

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Abstract

An important consideration in interpreting indices of gene expression in human bone is relating mRNA levels to functional endpoints such as bone architecture. In the present study, a method was developed for quantitative measurement of gene expression and bone morphology in the same specimen. Three-dimensional images of iliac crest bone biopsies from healthy premenopausal women were obtained using a novel high resolution cryogenic μ-CT scanner. RNA was isolated from the biopsies and mRNA levels were measured for genes related to bone metabolism. The gene expression profile and variability of expression within iliac crest biopsies of women was similar to human osteoblastic cell lines and rat long bones. mRNA for alkaline phosphatase, bone matrix proteins, and selected cytokines and cytokine receptors were consistently detected in biopsies. As previously shown in rat bone, there was a tight correlation between mRNA levels for type 1 collagen and osteonectin, a weaker correlation between type 1 collagen and osteocalcin and no correlation between bone matrix proteins and alkaline phosphatase. The relative abundance of the mRNA for the three most prevalent transforming growth factor-β (TGF-β) isoforms in bone (TGF-β_1>> TGF-β_3> TGF-β_2) was the same as the known abundance of the corresponding TGF-β peptides in bone matrix. The results demonstrate the feasibility of analyzing the three-dimensional architecture of a bone biopsy using cryogenic μ-CT imaging and then measuring expression of genes related to bone cell function within the same specimen following RNA extraction and analysis.

Keywords: mRNA, Bone Morphology, Matrix Proteins, Cytokines and Growth Factors

Introduction

Quantitative gene expression analysis using total cellular RNA isolated from skeletal tissues is routinely performed in animals. Extension of these analyses to humans could contribute to our understanding of the molecular mechanisms that underlie regulation of bone metabolism. Such studies are especially important when no validated laboratory animal models are available. Furthermore, gene expression analysis may be valuable as a diagnostic tool and as a method for early determination of the effectiveness of a treatment.

Several difficulties must be overcome before this technology can be effectively transferred to humans. The first important issue is procuring suitable tissue for analysis. Bone obtained as surgical waste material is often available but the patients are generally diseased. Also, surgical waste bone is very heterogeneous, increasing the difficulty in relating gene expression to structural and functional relationships. Gene expression data, without being related to structural, functional, or cellular relationships, would be uninterpretable[1]. Bone biopsies from a standard anatomic site, such as the iliac crest, is more homogeneous than surgical waste bone and specimens can be obtained from healthy as well as diseased individuals. A bone biopsy, however, is highly invasive. Thus, it would be highly desirable to be able to obtain morphological as well as molecular data from a single specimen. To accomplish this goal, it is necessary to use a nondestructive method to obtain morphological data. Standard densitometry lacks sufficient sensitivity and specificity. μ-CT is a nondestructive technique that can provide sufficient resolution. However, conventional μ-CT is performed at ambient temperature which would result in rapid degradation of

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Gene Expression	Iliac Crest Biopsies	MG63 cells	hFOB cells	Rat Tibial Metaphysis
	% SD	% SD	% SD	% SD
Matrix Proteins				
Type 1 Collagen	41	55	34	33
Osteonectin	51	29	23	35
Osteocalcin	26	–	–	26
Alkaline Phosphatase	12	–	–	–
Cytokines				
IL-1ß	26	–	–	29
IL-1Ra	28	–	–	ND
LT-ß	44	–	–	ND
TNF-α	30	–	–	35
TGF-$ß_1$	8	62	42	52
TGF-$ß_2$	24	75	50	25
TGF-$ß_3$	38	100	ND	ND

values are (SD/mean)x100; N=3-4 individuals (bone tissue) or replicates (cell culture).
Not detected (ND); not assayed (–).

Table 1. Variability of mRNA levels obtained from different sources.

mRNA. In this study, we investigated the feasibility of using cryogenic μ-CT scanning to produce 3-dimensional images of bone architecture prior to RNA isolation and gene expression analysis of human iliac crest bone biopsies.

Methods

General

The human and animal studies were performed with the approval of the appropriate animal and human welfare committees. Iliac crest bone biopsies were obtained from 4 premenopausal women in their fourth decade. The proximal tibial metaphysis was obtained from 4 ovary-intact 6-month-old Sprague Dawley rats. The bone specimens were frozen immediately following collection in liquid N_2 and stored frozen at –80°C. MG63 human osteosarcoma cells and human fetal osteoblast (hFOB) cells were cultured as described[2], harvested and used for RNA isolation.

μ-CT Analysis

The cryogenic μ-CT scanner has been described[3]. The method used to analyze bone specimens has also been described[4].

Isolation of RNA

Bone biopsy samples were homogenized in guanidine isothiocyanate using a Spex Freezer mill (Edison, NJ). Total cellular RNA was extracted and isolated using a modified organic solvent method and the RNA yields were determined spectrophotometrically at 260 nm[5].

Northern Blot Hybridization

Total RNA samples (10 μg) were used for Northern blot analysis as described[6]. Labeling of cDNAs, hybridization and quantitation by Phosphorimager (Molecular Dynamics, Sunnyvale, CA) were carried out as described[6]. Labeled cDNAs for type 1 collagen, osteonectin and 18S rRNA were used for probing the blots. The amounts of RNA loaded and transferred were assessed by methylene blue staining of the membranes and hybridization with a cDNA for 18S ribosomal RNA.

RNase Protection Assay for Cytokines

The total RNA (20 μg) isolated was analyzed by RNase Protection Assays using antisense RNA probes as described[6,7]. The antisense RNA was synthesized using the cDNA templates as described (Pharmingen, San Diego, CA). We measured by RNase Protection assay the mRNA concentrations of the following cytokines: Transforming Growth Factor-beta (TGF- β)1, 2, 3; Tumor Necrosis Factor (TNF)-α and -β, Interleukin (IL)-1 α, -1 β, -1Ra, -6, -10, -12 (p35 and p40); Interferon (IFN)-β and γ and Lymphotoxin (LT)-β. Quantitation of protected RNA fragments was per-

formed by PhosphorImager analyses and normalized to 18S ribosomal RNA (Northern blot) and ribosomal structural protein L32 (RNase protection assays).

Statistics

Values for gene expression were expressed as mean ± SD. Variability in gene expression was expressed as % and calculated as % = SD/mean x 100. RNA data for bone matrix proteins and alkaline phosphatase was analyzed by linear regression analysis.

Results

μ-CT images of cryogenic scans of an iliac crest bone biopsy reconstructed at a voxel size of 18 μm on a side are shown in Figure 1. The intact biopsy was scanned at –60°C, a 2 mm diameter core containing cancellous bone removed while frozen and the remaining biopsy re-scanned.

RNA recovered from unscanned and twice cryogenic scanned biopsy was separated by gel electrophoresis and inspected following methylene blue staining. There was no apparent deterioration in the quality of the RNA recovered from cryogenic scanned biopsies. This was confirmed following hybridization with a cDNA to rRNA; cryogenic scanning had no effect on rRNA levels (data not shown).

Figure 2 shows a representative phosphor image of a Northern blot showing mRNA levels for selected bone genes expressed in human iliac crest. mRNA for Type 1 collagen and osteonectin is shown. Alkaline phosphatase and osteocalcin mRNA were also detected in all of the biopsies (Table 1).

Linear regression analysis was used to correlate the relative mRNA levels for selected genes. The mRNA levels for Type 1 collagen and osteonectin were highly correlated (R^2=0.96; $p < .01$). R^2=.80 for Type 1 collagen and osteocalcin but significance was not achieved (p=.07). There was no correlation between mRNA levels for the bone matrix proteins (Type 1 collagen, osteonectin, and osteocalcin) and alkaline phosphatase.

Figure 3 shows phosphor images of RNase protection assays for selected cytokines expressed in human iliac crest. mRNA for IL-1β, IL-1Ra, LT-β, TNF-α, and TGF-β1,2,3 were detected (Figure 3A and 3B). The relative expression (mean ± SE; N=4) of the 3 TGF-β isoforms was TGF-β1 (1.00 ± 0.04), TGF-β2 (0.06 ± 0.01), and TGF-β_3 (0.09 ±0.02).

Table 1 shows a comparison between iliac crest, MG63 osteosarcoma cells, hFOB cells and rat proximal tibial metaphysis. mRNA for TGF-β_3, IL-1Ra, and LT-β were detected in human biopsies, but not in rat bone. TGF-β_3 was not detected in either hFOB cells or rat bone. The variability in mRNA levels of extracts from bone biopsies was similar in magnitude to RNA from rat bone and cultured bone cells.

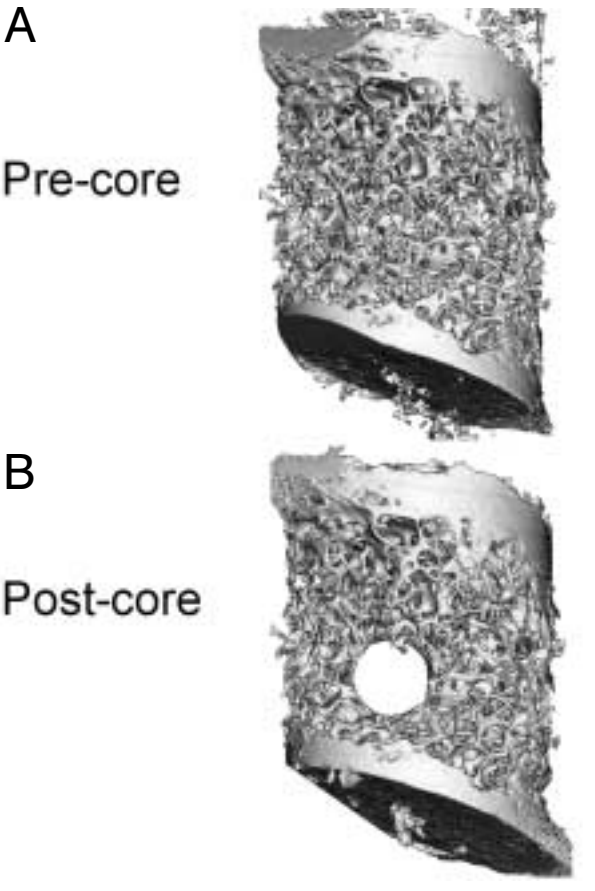

Figure 1. Cryogenic μ-CT of a human bone biopsy reconstructed using 18 μm voxels. A. Pre-core (gradient shading); and B. post-core (gradient shading). The core was 2 mm in diameter and extended through the biopsy.

Discussion

μ-CT, in contrast to conventional light microscopy, is non-destructive. Recent progress in μ-CT imaging has achieved a capability of creating three-dimensional images of bone with sufficient resolution to obtain precise measurement of important indices of bone mass and architecture. The scanner used in the present study can produce images of entire bone biopsies reconstructed from voxels as small as 6 μm on a side[4].

Conventional μ-CT scanning, however, is performed at room temperature. This would insure rapid degradation of mRNA in the tissue, leaving the specimen useless for gene expression analysis. This technical problem was solved in the present study by using a specially designed cryogenic chamber which maintained the temperature of the specimen during the scanning at –60°C[3]. Comparison of scanned and unscanned RNA indicates that the quality of total cellular RNA isolated from iliac crest bone biopsies is not compromised by cryogenic μ-CT scanning (Figures 2 and 3).

Using this novel cryogenic scanning approach, we have

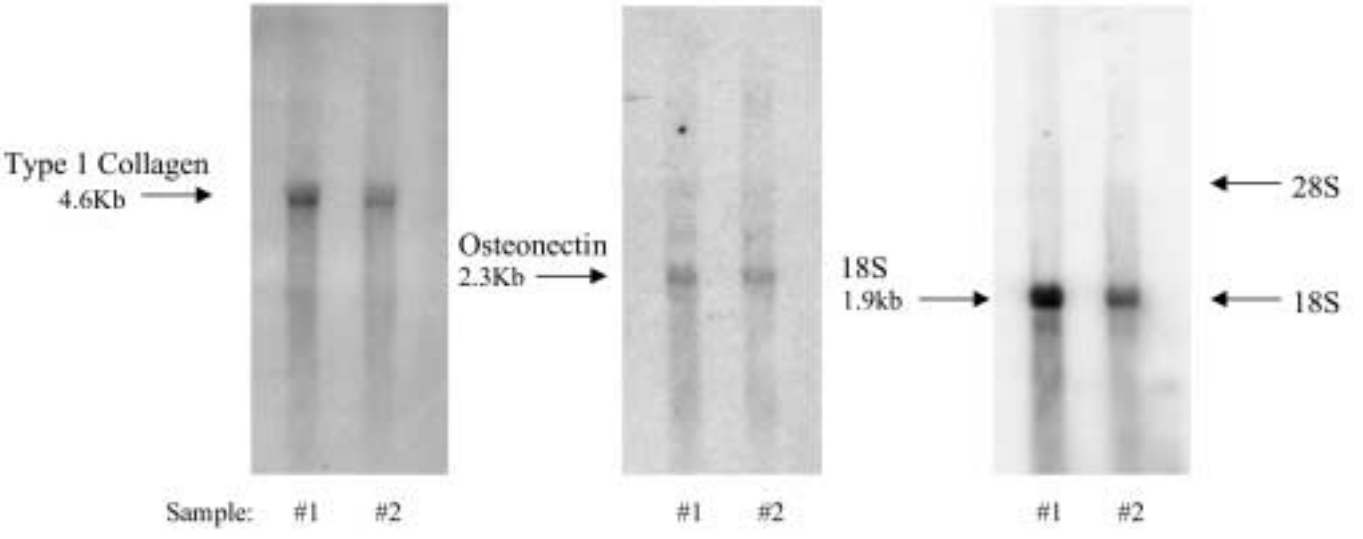

Figure 2. Representative Northern hybridization for type 1 collagen, osteonectin and 18S ribosomal RNA. Samples 1 and 2 are RNA from bone biopsies following cryogenic μ-CT scanning.

demonstrated the feasibility of obtaining bone morphology and gene expression data from the same biopsy. The structural data could include, but would not be limited to, bone mineral content, density, cancellous and cortical bone volume, trabecular number, thickness and separation and connectivity. Finite element modeling and other mathematical models could be applied to investigate the relationships between gene expression, bone architecture and bone mechanical properties[8,9].

Cryogenic sectioning of frozen tissue followed by *in situ* hybridization is another method that is used to identify expression of genes in tissues, including bone. This powerful technique allows the precise localization of gene expression to individual cells but can be practically used to investigate only a small number of genes. Also, the method is generally not quantitative. In contrast, gene expression analysis of total cellular RNA can provide quantitative tissue level measurements. If multiple probe assays are used (Figure 3), expression of dozens of genes are easily measured. Gene arrays could be used to measure thousands of genes. By removing a portion of the biopsy following cryogenic scanning (Figure 1) it should be possible to obtain quantitative tissue level RNA analysis and precise localization of gene expression for a limited number of candidate genes within the same biopsy.

The potential for greater variability of gene expression in humans than in animal models and cell lines was a concern. We, therefore, compared the RNA obtained from the human bone biopsies with RNA from cultured bone cells and rat bone. The variability in mRNA levels for selected genes from human bone was similar in magnitude to the other sources of RNA. Important determinants of bone metabolism, such as aging, hormones (e.g., parathyroid hormone, sex steroids and growth hormone), mechanical usage and life style factors (e.g., alcohol consumption) have been shown in other studies to result in changes in mRNA levels for bone matrix proteins and selected cytokines that greatly exceed individual variation in rats[8,10,11]. Thus, it is likely that individual variability will not preclude the use of RNA analysis as a tool for investigating the molecular mechanism of regulation of bone metabolism in humans.

One of the most powerful applications of conventional bone histomorphometry is the use of fluorochrome labels to measure dynamic analyses of bone formation. Comparable data cannot be obtained by computed tomography. In the rat, mRNA levels for type 1 collagen are generally highly correlated to bone formation as well as to selected other bone matrix protein mRNA levels[12,13]. We observed in human bone a similar tight correlation between type 1 collagen and osteonectin mRNA levels, weak correlation between type 1 collagen and osteocalcin mRNA levels and lack of correlation between bone matrix protein and alkaline phosphatase mRNA levels[12,13]. As previously reported in the rat, the relative expression of TGF-β isoforms (TGF-β1,2,3) mRNA in human iliac crest biopsies is identical to the concentration of the TGF-β peptide isoforms in bone matrix[14,15]. Furthermore, immunohistochemistry has revealed that changes in TGF-β expression largely reflect corresponding changes in osteoblast number (unpublished data). Thus, it may be possible to use mRNA levels for bone matrix proteins and TGF-β as molecular indices of bone formation.

Alternatively, our results suggest that it is possible to obtain tissue level measurements of gene expression from regions of interest within a biopsy by removing a core (Figure 1). It would be possible to analyze fluorochrome labels from the remainder of the tissue following conven-

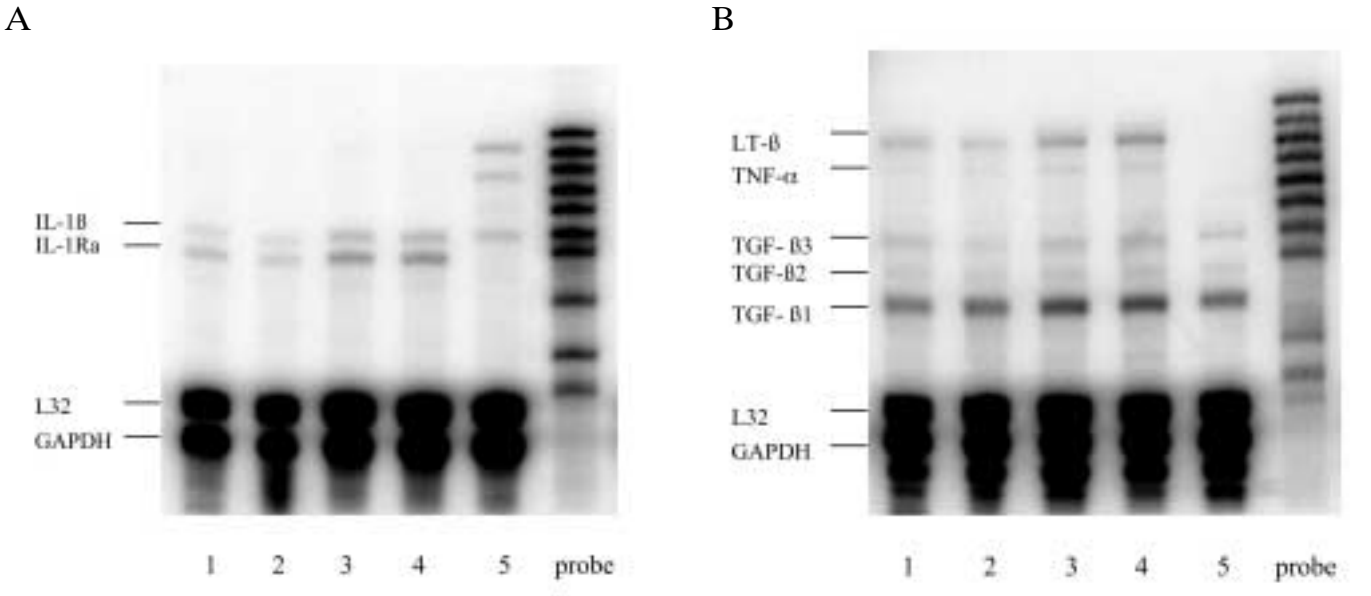

Figure 3. Representative RNase protection assays for cytokines and cytokine receptors. A. IL-1ß, IL-IRa, L32 and GAPDH, and B. LT-β, TNF-α, TGF-β1,2,3, L32, GAPDH. Samples 1 and 2 are RNA from bone biopsies following cryogenic μ-CT scanning. Samples 3 and 4 are RNA from bone biopsies that had not been scanned. Sample 5 is RNA from MG63 cells. Please note that LT-β is expressed in human bone but not in cultured osteosarcoma cells. In contrast, TGF-β isoforms are similarly expressed in bone and in cell culture. The L32 and GAPDH bands appear to be over exposed because of limitations of the printer. The intensity of these bands is well within the linear range of the phosphoimager used to measure their intensity.

tional tissue processing.

Conventional μ-CT is incapable of imaging cells. Future research is required to determine what, if any, cell based information can be obtained from frozen tissue.

In summary, we have shown the feasibility of obtaining morphological and gene expression data from the same human bone biopsy using cryogenic μ-CT. Our results indicate that the capabilities of conventional light microscopy and μ-CT can be greatly extended using this new approach to analyze bone specimens.

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