Maintaining mRNA Integrity during Decalcification of Mineralized Tissues

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Abstract

Biomineralization of the extracellular matrix occurs inappropriately in numerous pathological conditions such as cancer and vascular disease, but during normal mammalian development calcification is restricted to the formation of the skeleton and dentition. The comprehensive study of gene expression in mineralized skeletal tissues has been compromised by the traditional decalcification/fixation methods that result in significant mRNA degradation. In this study we developed a novel RNA/later/EDTA decalcification method that protects the integrity of the mRNA in mature mouse tibial epiphyses. Furthermore, this method preserves the tissue structure to allow histological sectioning and microdissection to determine region-specific gene expression, in addition to immuno- and in situ histology. This method will be widely applicable to the molecular analysis of calcified tissues in various pathological conditions, and will be of particular importance in dissection of the gene expression in mouse bone and joint tissues during development and in important clinical conditions such as arthritis.

Introduction

Degenerative joint disease is a major clinical problem and the molecular study of gene expression networks in joint tissues such as articular cartilage and subchondral bone is of fundamental importance to our understanding of disease mechanisms and to identify potential biomarkers and possible therapeutic targets [1]. The central pathological feature of osteoarthritis is the progressive destruction of articular cartilage and this tissue has been the focus of numerous studies, not least because noncalcified cartilage can be recovered readily by surface dissection of mature human joint tissues recovered during surgery for joint replacements, or post-mortem. However, the use of predominantly noncalcified cartilage because it is readily able to be dissected from the joint surface without decalcification, ignores that fact that osteoarthritis is a disease of the whole joint, including in particular the pathological changes in the underlying calcified cartilage and subchondral bone.

To address the inherent shortcomings in human-based osteoarthritis cartilage studies which rely largely on analysis of end-stage disease, several rodent models have been developed [2] to allow harvesting of joint tissues during the onset and progression of the disease for mRNA expression analysis. The fact that the models develop with a reproducible time course and the age, sex, exercise and genetic background of the animals can be controlled makes them ideal to study the pathological events in osteoarthritis progression. However, the study of degeneration of the calcified tissues of the skeletally mature rodent joint requires first decalcification using either rapid acid-based methods or prolonged exposure to chelating agents, both of which limit downstream analysis to histology and immunohistology after fixation. Since current decalcification methods have been shown to result in significant RNA degradation [3,4], direct mRNA expression analysis has not be possible on calcified cartilage or other calcified joint tissues.

To overcome this limitation we developed a novel decalcification approach that preserves the integrity of the mRNA in mature mouse tibial epiphyses and allows microdissection of cartilage regional zones for mRNA expression profiling. We show that this procedure also conserves the histological morphology which allows in situ hybridization analysis of mRNA and immunohistochemical analysis of protein localization. While this method has been developed for the analysis of cartilage expression in joint degeneration, it will have wide applicability in the transcriptomic analysis of other calcified tissues of the skeleton during normal development and in the pathological calcification that occurs in numerous human diseases such as cancer and vascular disease.

Materials and Methods

Isolation of Tibial Epiphyses

The mouse studies were approved by the Murdoch Childrens Research Institute Animal Ethics Committee (Approval #A672). Male C57BL6 mice were obtained from an inbred SPF colony, where animals were housed in 12 hour light dark cycles and
Mice were sacrificed 10 weeks of age and the tibial epiphyses, which included the articular surface, calcified deep cartilage and subchondral bone were isolated from both hind limbs of all mice, dissected free of the other joint tissues, washed briefly in PBS and either snap frozen or subjected to decalcification within 15 minutes of death.

**Decalcification**

The tibial epiphyses were randomly assigned to one of three decalcification methods. One group of epiphyses were decalcified using conventional ethylene-diamine-tetraacetic acid (EDTA) chelation, where each tibiae was immersed in 5 ml of 20% EDTA/Tris-HCl, pH 7.4, and gently agitated for 72 hrs at 4°C. Following decalcification tibiae were snap frozen and imbedded in OCT and stored at −80°C. Other epiphyses were decalcified

### Table 1. PCR primers.

| Gene   | Forward primer (5’-3’) | Reverse Primer (5’-3’) | Size  |
|--------|------------------------|------------------------|-------|
| Col2a1 | AACGTCCAGTGAACCTTTCCTC | ATTTGCGATGCTGCCAGTTTC | 994 bp|
| Prg4   | TGAAGATGAGATGGAGGTG    | GTCTGGAAAGGGGATGAGCG   | 654 bp|

**Quantitative PCR**

| Gene   | Forward primer (5’-3’) | Reverse Primer (5’-3’) | Size  |
|--------|------------------------|------------------------|-------|
| Col2a1 | AACCTTCCAACCCGCTACA    | GGGAGACCGTTGGGTATCA    | 76 bp |
| Rpl10  | TGAAGACATTTTGCTGAGA    | AGGACCACAGTGGGGATA     | 74 bp |

Figure 1. Analysis of RNA integrity. Microcapillary electrophoresis of total RNA isolated from whole tibial epiphysis (A, C, E) or cryosections of tibiae (B, D, F) decalcified with 0.5 M EDTA (A, B), RNA later/EDTA at pH 9.2 (C, D) or RNA later/EDTA at pH 5.2 (E, F). doi:10.1371/journal.pone.0058154.g001
using specific primer sets for RNA probes. To prepare the probes, RT-PCR was performed using the appropriate RNA polymerase (T7 or SP6; Roche). DIG-labeled [35S]CTP labeled [6] or [35S]CTP (Amersham) labeled [6] or [35S]CTP labeled [7] antisense and sense (control) probes were hybridized to tissue sections overnight at 58°C. After blocking with 1% goat serum in 1% BSA (w/v) in PBS overnight at 4°C, the sections were incubated with mouse anti-collagen II antibody (1:1000; Clone 2B1.5, Thermo Scientific) or affinity purified rabbit anti-human collagen VI polyclonal antibody (1:2000; Fitzgerald Industries) and visualized by immunofluorescence with Alexa-Fluor 488 conjugated secondary antibody (1:200; Molecular Probes). Sections were counterstained with DAPI to visualize nuclei.

**Microdissection, RNA Extraction and Quality Assessment**

Serial 7 μm coronal cryo-sections of EDTA or RNAlater/EDTA decalcified samples mounted on RNase-free SuperFrost slides (Menzel) were fixed in 70% ethanol, washed in RNase-free water, and dehydrated in 70%, 95%, and 100% ethanol for one minute each and air-dried. Slides were then immobilized on an inverted microscope (Leica) and articular cartilage was dissected by using a nitrogen-cooled tissue grinder. The purity and concentration of the RNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and integrity of the RNA samples was determined by capillary electrophoresis with a Bioanalyzer 2100 (Agilent), using a Series II RNA 6000 Pico Kit (Agilent), according to the manufacturer’s specifications.

**Quantitative PCR**

RNA isolated from EDTA or RNAlater/EDTA decalcified whole tibiae (500 ng total RNA) was reverse-transcribed as per the manufacturer’s protocol in a 20 μL reaction using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany). This reaction was incubated at 50°C for 30 minutes, 85°C for 5 minutes. Quantitative PCR (qPCR) reactions containing 2.5 ng cDNA were prepared to 5 μL in 384 well PCR plates (Roche) using the LightCycler® 480 SYBR Green I Master (Roche) as per the manufacturer’s instructions and run on a LC480 II thermocycler (Roche). Col2a1 and Rpl10 primer details are provided in Table 1. Cycling conditions for qPCR reactions were: Pre-incubation, 1 cycle of 95°C (5 minutes, 4.8°C/s ramp rate); Amplification, 45 cycles of 95°C (10 seconds 4.8°C/s ramp rate), 60°C (30 seconds 2.5°C/s ramp rate), 72°C (10 seconds, 4.8°C/s ramp rate); Melting Curve, 1 cycle of 95°C (5 seconds 4.8°C/s ramp rate, 65°C (1 minute 2.5°C/s ramp rate), 97°C (0.11°C/s ramp rate). Analysis of the run file from the cycler was done using the Roche LightCycler® 480 Software release 1.5.0 to calculate Cq values for each sample.
Results and Discussion

We found that standard EDTA decalcification procedures severely impacted on the quality of the RNA extracted from both whole tibiae (Fig. 1A) or from microdissected tibial cartilage (Fig. 1B), as determined by microcapillary electrophoresis. In both cases there was little or no 18S or 28S RNA reflected in the low RNA Integrity Numbers (RIN) of 2.3 and 1.0, respectively. These

Figure 3. Cartilage morphology after EDTA or RNA/EDTA decalcification. Tibial epiphyses were decalcified for 72 hrs at 4°C with 0.5 M EDTA (A) or RNA/10% EDTA at pH 5.2 and cryosections were stained with toluidine blue/fast green. The medial tibial plateau is shown. Cartilage morphology and aggrecan staining is preserved in the RNA/10% EDTA, pH 5.2 decalcified samples. Scale bar = 100 μm.
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Figure 4. In situ hybridization for Prg4 and Col2a1. Cryosections from tibia decalcified with EDTA (A,B) or RNA/EDTA at pH5.2 (C–F) were hybridized with DIG-labeled Prg4 antisense (A,C) or sense (B,D) RNA probes, and against 35S-labeled Col2a1 antisense (E) or sense (F) RNA probes for Col2a1. Arrows show representative regions of target gene mRNA expression. Scale bars = 100 μm (A–D), 10 μm (E, F).
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data were consistent with recent studies demonstrating the deleterious effects of current decalcification methods on RNA integrity [5,4]. In an attempt to overcome this serious problem with the need to decalcify mineralized tissues prior to any downstream mRNA profiling studies, we combined the RNA stabilizing agent RNA-later (Ambion) with EDTA in the decalcification protocol. Using decalcification times of 72 hrs (at 4°C; equivalent to standard 20% EDTA procedures), we first decalcified the tibiae in RNA-later containing 10% EDTA at pH9.2 (Fig. 1C, D) which was the adjusted pH required to allow initial EDTA dissolution. These results showed very little protection of the RNA from degradation, with RINs of 2.7 (whole tibia) and 2.3 for the microdissected samples. In an attempt to further improve RNA quality we reduced the pH of the RNA-later/EDTA to pH5.2 (Fig. 1E, F) which corresponded to the usual working pH of RNA-later. With this decalcification method RNA was completely protected from degradation with RINs of 9.2. While this method provides an important new approach to obtaining high quality mRNA for expression analysis, it was important to determine if the RNA-later/EDTA decalcification was compatible with other downstream analyses such as qPCR, histology, in situ mRNA expression analysis and immunohistochemical detection of protein localization. For qPCR, equal amounts of RNA isolated from EDTA or RNA-later/EDTA decalcified whole tibiae were reverse-transcribed and analyzed by qPCR for the expression of Col2a1 and the house-keeping gene Rpl10 (Fig. 2). For both genes EDTA and RNA-later/EDTA, pH 9.2 decalcification performed similarly with threshold cycles of amplification (C\textsubscript{t}) of ~29.3 cycles for Col2a1 (Fig. 2A) and ~27.4 cycles for Rpl10 (Fig. 2B). In contrast, when RNA was extracted after RNA-later/EDTA, pH 5.2 decalcification, the detection of both gene transcripts was far more robust with C\textsubscript{t} of ~22.0 cycles for Col2a1 (Fig. 2A) and ~22.1 cycles for Rpl10 (Fig. 2B), representing an increased abundance of quantifiable, the signal in the RNA-later/EDTA samples was stronger than that seen in the samples decalcified by EDTA alone (Fig. 2A), consistent with the protection of RNA from degradation in the RNA-later/EDTA samples (Fig. 1).

In addition to mRNA integrity in the decalcified tissue sections, immunohistochemical staining for cartilage proteins was possible. We demonstrated the cartilage localization of collagen II (Fig. 5B) and collagen VI (Fig. 5D) which is expressed throughout the articular cartilage. Both aggrecan and collagen VI (Fig. 5D) was preserved compared to the conventional EDTA-decalcified samples (Fig. 5A, C) and it is important to note that the pericellular collagen VI extracellular matrix characteristic of chondrocytes was also intact after RNA-later/EDTA treatment (Fig. 5D).

In this study we have developed a simple method for decalcification of mineralized tissues that protects RNA integrity for downstream expression analysis by quantitative PCR, microarray expression profiling or in situ hybridization, while preserving tissue morphology for histology and immunohistochemistry. In addition to these advantages, this method does not affect the protein composition of the tissue (data not shown), allowing parallel proteomic studies to be conducted, providing the opportunity for a systems biology level analysis of the biology of calcified tissues. In these studies we have applied this technique to mouse tibial epiphyseal cartilage, which has immediate application to the study of joint degeneration and arthritis mechanisms. However, there are other important developmental contexts, and pathological situations such as vascular calcification and cancer, where this approach should be of value.

**Author Contributions**

Conceived and designed the experiments: DB JFB CBL. Performed the experiments: DB LR. Analyzed the data: DB LR JFB CBL. Wrote the paper: JFB DB LR CBL.
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