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Delineating bone's interstitial fluid pathway in vivo

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Abstract

Although interstitial fluid flow has been suggested to play a role in bone adaptation and metabolism, the constituents and ultrastructure of this interstitial fluid pathway are not well understood. Bone's lacunar–canalicular porosity is generally believed to be a continuous interstitial fluid pathway through which osteocytes sense external mechanical loading as well as obtain nutrients and dispose of wastes. Recent electron microscopy studies have suggested that a fiber matrix surrounds the osteocytic cell processes and fills this pericellular fluid space. However, studies injecting tracer molecules into the bone vasculature have provided conflicting results about the pore size or the fiber spacing of the interstitial fluid pathway. In addition, whether the smaller collagen–apatite porosity in adult bone is also a continuous fluid pathway is still unclear. To delineate bone's interstitial fluid pathway, four tracers of various size were injected into rats: reactive red (approximately 1 nm), microperoxidase (MP, approximately 2 nm), horseradish peroxidase (HRP, approximately 6 nm), and ferritin (approximately 10 nm). Five minutes after injection, the tibiae were harvested and processed using histological protocols optimized to minimize processing time to reduce possible redistribution of tracer molecules. The number of blood vessels and osteocytic lacunae labeled with the tracers per unit bone area was then measured for mid-diaphysal cross-sections of the tibia. While none of the tracers was detected within the mineralized bone matrix (the collagen–apatite porosity) using light microscopy, all the tracers except ferritin were found to pass through the canaliculi and appear in the osteocytic lacunae. These results indicate that while small tracers (< 6 nm) readily pass through the lacunar–canalicular porosity in the absence of mechanical loading, there appears to be an upper limit or cutoff size between 6 and 10 nm for molecular movement from bone capillaries to osteocytic lacunae in rat long bone. This range of pore size contains the most likely fiber spacing (approximately 7 nm) that has been proposed for the lacunar–canalicular annular space based on the presence of a proteoglycan fiber matrix surrounding the osteocyte.

Keywords

Bone pore size; Bone permeability; Bone interstitial fluid; Tracer movement; Mass transport

Introduction

Interstitial fluid flow in bone tissue has been suggested to play a role in bone adaptation and metabolism. Mechanical loading has been proposed to induce interstitial fluid flow in the

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porous bone matrix that results in shearing stresses on the osteocytic cell process membrane [3,5,9,24,28] or circumferential deformation of the cell process cytoskeleton [29,31], which may enable the bone cells to detect the external mechanical signals, thus beginning the process of adapting bone's mass and structure to its environment. Moreover, the interstitial fluid flow induced by mechanical loading has also been proposed to enhance mass transport in bone to ensure the metabolic function of bone cells that is crucial for bone growth and maintenance [10–15,19,25]. However, the constituents and ultrastructure of bone's interstitial fluid pathway are not well understood due to the technical difficulties of handling calcified tissues and bone's complex microstructure.

There are three levels of porosity containing bone fluid within cortical bone and the trabeculae of cancellous bone. The largest pores are found in the vascular porosity (approximately 3- to 35- μ m radius [4]), which consists of the osteonal and Volkmann's canals that surround the blood vessels. A much smaller lacunar–canalicular porosity (lacunae: approximately 5- μ m radius; canaliculi: approximately 200-nm radius) houses the osteocytic cell bodies and their protrusive processes and is generally believed to be a continuous fluid pathway through which the osteocytes obtain nutrients from the blood supply and dispose of wastes [4,10–12,14,15,19,25]. Within the lacunar–canalicular channels, a gel-like fiber matrix with a fiber spacing of approximately 7 nm, similar to the surface glycocalyx on endothelial cells, has been proposed to fill the pericellular fluid space [5,26,28,31]. Although electron microscopy studies have suggested the existence of such a fiber matrix [1,21,22,27,30], the ultrastructure of the lacunar–canalicular porosity remains elusive. The smallest porosity, the collagen–apatite porosity (approximately 10-nm radius), has been postulated to form between apatite crystallites during the process of collagen mineralization. Because bone fluid in this porosity is thought to be bound to the mineral [28], whether the collagen–apatite porosity is part of the continuous interstitial fluid pathway is not clear.

Tracer molecules of various sizes have been injected into bone to explore tracer movement through the bone interstitial fluid spaces, and no agreement has been achieved on the size of the largest tracer molecule that can pass through lacunar–canalicular or collagen–apatite pores in mature mineralized bone. For example, ferritin (approximately 10 nm in diameter) injected into mature dogs [18] and goats [17,20] has demonstrated halo-like labeling surrounding the vascular canals, shown as discrete lines with a spacing similar to that observed for canaliculi [18] or multiple rings within the matrix with a clear margin from the osteonal canal surface [17,20]. These results suggest that ferritin may be small enough to pass through the canalicular pores and possibly through the collagen–apatite pores in the mineralized matrix. Smaller tracers such as horseradish peroxidase (HRP, approximately 6 nm in diameter) [7,8,23], microperoxidase (MP, approximately 2 nm in diameter) [2,23], and procion red (<1 nm in diameter) [10–14] have been found to pass through the lacunar–canalicular porosity and appear in the osteocytic lacunae of mature bone, although there are conflicting reports on the detailed tracer distribution patterns. Moreover, discrepancy exists as to whether these tracers penetrate the collagen–apatite pores in the mineralized matrix. Some studies have found that microperoxidase [2,23] and procion red [14] are excluded from the collagen–apatite pores, while others report or imply that not only microperoxidase [14] and procion red [13] but also the much larger tracer ferritin [17,18,20] appear in the collagen–apatite pores within the mineralized matrix.

To address the conflicting results from previous tracer studies and to further delineate bone's interstitial fluid pathway, we performed this study using four tracers of various size. The goals of this study were (1) to begin to systematically delineate the smallest pore size of the interstitial fluid pathway in vivo; (2) to determine whether the collagen–apatite porosity in

the mineralized matrix of adult bone is a continuous fluid pathway; and (3) to quantify tracer movement and labeling patterns in the absence of mechanical loading.

Methods

To explore the ultrastructure of bone's interstitial fluid pathway, we injected the following exogenous tracers into the rat blood system: reactive red, a dye usually used as an affinity chromatography medium; microperoxidase (MP) and horseradish peroxidase (HRP), enzyme proteins derived from equine heart cytochrome and horseradish, respectively; and ferritin, an iron storage protein. We chose to use these tracers because most of them have been safely used previously, and the tracer molecular weights span three orders of magnitude, from approximately 1000 Da (reactive red) up to approximately 400000 Da (ferritin) (Table 1), which is a good representation of solute molecules (e.g., nutrients, hormones, cytokines, and other signaling molecules) in bone. The tracer sizes are in the range of approximately 1 nm (reactive red) to 10 nm (ferritin), a range in which the most likely fiber spacing of the pericellular fiber matrix (7 nm) is thought to fall [5,28].

Animal preparation

Male Sprague–Dawley rats (body weight 335 ± 50 g, 8–11 weeks old, $n = 15$) were divided into five groups (three animals per group) receiving reactive red, MP, HRP, ferritin, and saline (controls), respectively. Permission for this in vivo study was granted by the Institutional Animal Care and Use Committee at the Hospital for Special Surgery. To optimize the consistency of tracer labeling and minimize diffusion artifacts, preliminary work was performed before the present study to test different injection locations, tracer dosages, circulation times (the time interval between tracer injection and the stopping of blood circulation), as well as the subsequent histological procedures. The experimental protocol was established as follows:

Rats were first anaesthetized via an intraperitoneal injection of a mixture of ketamine (80 mg/kg body weight) and xylazine (5 mg/kg body weight). The left jugular vein was then exposed surgically and a 25 G5/8 needle attached to a syringe was inserted into the vein to inject a bolus of tracer solution. The jugular vein was chosen because it is relatively easy to access and a reproducible injection was achieved at the site.

Tracer solutions were prepared from powder except for ferritin, which was supplied as a liquid. All the tracers were purchased from Sigma (St. Louis, MO). The reactive red solution (concentration: 0.8%) was prepared by dissolving 1.4 g of reactive red powder (purity: 60%) in 100 ml phosphate-buffered saline (PBS) and then filtering with Whatman paper (grade 2). The MP and HRP solutions were prepared by dissolving 25 mg MP or HRP in 1 ml PBS. Ferritin solution (Type I, from equine spleen, concentration: 85 mg/ml) was concentrated to 270 mg/ml using Amicon Ultra-4 centrifugal filter devices (100000 molecular weight cutoff, Millipore, Billerica, MA). Reactive red, MP, HRP, and saline solutions were administered at 1 ml per 100 g of body weight while ferritin was administered at 0.57 ml per 100 g of body weight, giving dosages of 8, 25, 25, and 154 mg per 100 g of body weight for reactive red, MP, HRP, and ferritin, respectively (Table 1). The injected fluid was slowly flushed into the jugular vein over 2 min and allowed to circulate for 5 min post-injection. The rat was then sacrificed by carbon dioxide inhalation, which took approximately 5 min, and both tibiae were harvested and immersed into the fixatives immediately (100% alcohol for reactive red- and ferritin-injected samples and 0.5% glutaraldehyde, 2% paraformaldehyde in 0.05 M sodium cacodylate buffer for MP-, HRP-, and saline-injected samples). Anatomical positions (anterior and posterior lateral corners) were marked on the bone surfaces using colored markers to maintain orientation for later imaging and data analysis.

Histological processing of bone samples

To preserve the tracer distribution in its *in vivo* condition, the histological processing time was minimized to reduce the possible redistribution of tracer molecules during processing. The histological processing for each tracer was indicated in the following sections (Fig. 1).

Reactive red—The mid-diaphyses of the tibiae from rats injected with reactive red were initially cut into 0.3-mm-thick cross-sections using a diamond blade saw (Buehler, Lake Bluff, IL) and then fixed in 100% alcohol for 4 h. These calcified sections were ground down to approximately 0.2 mm using Carbimet paper discs (800–1200 grit; Buehler), and then were mounted on glass slides and coverslipped with microscopy oil. Nail polish was used to seal the edges of the coverslip to prevent oil leakage.

Microperoxidase and horseradish peroxidase—The mid-diaphyses of the tibiae from rats injected with MP and HRP were initially cut into 1.5- to 2.0-mm-thick small blocks using the diamond blade saw and then fixed in 0.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M sodium cacodylate buffer while being agitated in a shaker for 48 h. The small blocks were then decalcified in 10% nitric acid while being agitated in a shaker for 48 h. Thin cross-sections (5–10 μ m) were then cut from the blocks using a cryostat (Model OTF 5030, Bright Instrument Company, England) and air-dried for 12 h.

Both MP and HRP were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB), a peroxidase substrate suitable for use in immunoblotting and immunohistological staining procedures. This substrate produces an insoluble end product that is brown to allow visualization of the tracers. Fresh DAB solution was prepared by dissolving 10 mg of DAB tablet (Sigma) in 15 ml of PBS, which was then filtered; 12 μ l of hydrogen (H_2O_2) was added before use. Drops of DAB solution were applied to the thin sections for 1 h at room temperature. After staining, the slides were rinsed in distilled water, air-dried, and coverslipped with mounting media.

Ferritin—The mid-diaphyses of the tibiae from rats injected with ferritin were initially cut into 1.5- to 2.0-mm-thick small blocks using the diamond blade saw and then fixed in 100% alcohol for 4 h. Since immersion into any acid interferes with staining of ferritin, the calcified blocks were first stained en bloc for 1 h in Perl's reagent, a freshly made 1:1 mixture of 4% potassium ferrocyanide and 4% hydro-chloric acid (Polysciences, Warrington, PA). Ferritin is split by hydrochloric acid, allowing potassium ferrocyanide to combine with the ferric ion to form the end product Prussian Blue that enables visualization of the ferritin. The stained blocks were then decalcified in 10% nitric acid while being agitated in a shaker for 24 h. Frozen cryostat sections (5–10 μ m) were cut from the decalcified blocks. The cross-sections were then counterstained using 0.1% acid fuchsin for 5 s to achieve a pink background. After staining, the slides were rinsed in distilled water briefly and coverslipped with mounting media.

Controls injected with saline—The mid-diaphyses of the tibiae from rats injected with saline (controls) were initially cut and cryosectioned identically as the MP- and HRP-injected samples. The saline-injected group served both as the negative control for the MP- and HRP-injected groups and as the baseline control for counting the labeled osteocytic lacunae and blood vessels for all the tracer-injected groups. The thin sections used for negative controls were stained with DAB following the same procedure as the MP and HRP-injected sections. The thin sections used for baseline controls were stained with 0.1% toluidine blue O (molecular weight 305–383, Aldrich Co., Milwaukee, WI) for 5 s.

Microscopic imaging and data analysis

We examined the tracer distribution for the three animals per group using three cross-sections of the right tibia from each animal. Due to the large area of the tibial cross-section, measurements were performed at three sampling regions (anterior corner, posterior lateral corner, and posterior medial corner) in each cross-section (Fig. 2). The mean values of the measurements from these three regions in the three cross-sections were calculated for each animal and used in the subsequent statistical analyses. To achieve consistency of sampling of the three regions, efforts were made to cut, collect, and image the sections in the same orientation for all animals.

The fluorescent labeling of reactive red was examined using selective green light excitation, while the sections injected with MP, HRP, and ferritin as well as the controls stained with DAB or toluidine blue were examined using routine light microscopy (Nikon Microphot-FXA). Digital images were taken using a 10× objective (field area: approximately 0.18 mm²) and the Bioquant Nova image analysis system (R&M Biometrics, Nashville, TN) with a camera and an image intensifier (Optronics, Goleta, CA). Detailed tracer labeling was also examined and imaged using a 40× objective in some reactive red- and ferritin-injected sections.

Three parameters, the bone area (B.Ar), the number of the labeled blood vessels (N.Bv), and the number of labeled osteocytic lacunae (N.Ot), were measured from the 10× images of the thin cryosections injected with MP, HRP, ferritin, and the toluidine blue-stained control sections. Two ratios were derived from these direct measurements: density of the labeled blood vessels (i.e., the number of blood vessels labeled per unit area [#mm²], $\text{Den.Bv} = \text{N.Bv} / \text{B.Ar}$); and the density of labeled osteocytic lacunae (i.e., the number of osteocytic lacunae labeled per unit area [#mm²], $\text{Den.Ot} = \text{N.Ot} / \text{B.Ar}$). For the control group where all the osteocytic lacunae and blood vessels were stained with toluidine blue, the two calculated ratios served as references (i.e., 100% labeled) for the experimental tracer groups. The number of the labeled blood vessels (N.Bv) was not reported for the reactive red-injected sections, which, being cut fresh without decalcification, were relatively thick (approximately 200 μm) and contained a three-dimensional vascular network, rendering it difficult to quantify the number of labeled vessels. However, the number of labeled osteocytes could still be counted for the reactive red group using the epifluorescent microscopy because the osteocytes on the sample surface were bright with a well-defined boundary.

A one-way ANOVA (analysis of variance) was conducted on the densities of the labeled blood vessels and osteocytic lacunae for each animal to find out whether the difference between the group means was significant among the four injected tracers, and then Bonferroni tests were performed using StatView (SAS Institute Inc., Cary, NC) to reveal which pairs of groups had significant differences. For all the statistical tests, a significance level of $P < 0.05$ was used.

Results

Five minutes post-injection, the four tracers showed different labeling patterns depending on the tracer size (Fig. 3). The labeling of reactive red was extensive with dye found in most blood vessels and osteocytic lacunae (Fig. 3a). At higher magnification, the reactive red was found to fill the lacunae and the fine canalicular channels as well, but it did not appear to penetrate the smaller collagen-apatite porosity in the mineralized matrix (Fig. 3b). The labeling of MP and HRP was similar, with tracer found in blood vessels and the osteocytic lacunae in the cortex, but not in the mineralized matrix (Figs. 3c, d). The negative controls injected with saline did not show any DAB staining (data not shown), confirming that the DAB staining in the MP- and HRP-injected sections was not an artifact. Ferritin, the largest

tracer used in this study, was found inside the blood vessels with no osteocytic lacunae labeled (Fig. 3e). At higher magnification, ferritin appeared to be confined to the vascular canals without penetrating the lacunar–canalicular or collagen–apatite porosities (Fig. 3f).

The ANOVA analyses showed no significant difference of the densities of labeled blood vessels (Den.Bv) and labeled osteocytic lacunae (Den.Ot) among the examined groups, except that no osteocyte lacunae were labeled in the ferritin group (Fig. 4). The density of the labeled blood vessels (Den.Bv) was roughly the same for MP, HRP, ferritin, and controls stained with toluidine blue (Ctrl) (Table 2, Fig. 4a). Compared to the controls, the density of osteocytic lacunae (Den.Ot) labeled with reactive red (R- Red), MP, HRP, and ferritin decreased as the tracer size increased from approximately 1 to 10 nm (Table 2, Fig. 4b). Since no osteocytic lacunae were labeled with ferritin (Den.Ot = 0 for all three animals), the ferritin group was excluded from the labeled osteocyte ANOVA analysis because of the zero variance of the measurements (Fig. 4b). No pairwise differences were found to be significant ($P < 0.05$) among the examined group means of the labeled osteocytic lacunae density (Den.Ot), although the difference between the control and HRP groups was approaching significance ($P = 0.054$) (Fig. 4b).

Discussion

Although electron microscopy studies have suggested the existence of a pericellular structure in the canaliculi [1,21,22,27,30], there are no quantitative data on the fine structure of the pericellular matrix. The current study provides indirect evidence of the fiber spacing of the pericellular matrix in the lacunar–canalicular pores, and suggests that this pore size falls in the range of 6–10 nm in rat long bone, because reactive red (approximately 1 nm), microperoxidase (approximately 2 nm), and horse-radish peroxidase (approximately 6 nm) could pass through the lacunar–canalicular pores and reach osteocytic lacunae away from the blood vessels, while the 10-nm ferritin was excluded from the osteocyte population and was confined to the vascular canals. This range of pore size (6–10 nm) contains the most likely fiber spacing (approximately 7 nm) that has been proposed for the lacunar–canalicular annular space based on the presence of a proteoglycan fiber matrix that lines the surface of vascular endothelial cells and serves as a filter to prevent leakage of serum albumin [5,28].

Our results do not show penetration of the four tracers into the collagen–apatite pores within 5 min after injection, which suggests that either the collagen–apatite porosity may not be a continuous fluid pathway or its pore size may be smaller than 1 nm (the size of reactive red, the smallest tracer used in this study). We further increased the circulation time (up to 1 h) for reactive red, but the tracer still did not appear to penetrate the collagen–apatite porosity. However, due to the limited resolution of detecting tracer distribution used in this study, further investigations are needed to elucidate the permeability of the collagen–apatite porosity using other microscopy techniques and smaller tracers.

The following four paragraphs summarize tracer labeling from previous studies along with the findings from this study for the four tracers used, highlighting any contradictory findings (further details are listed in Table 3).

Reactive red

Our results indicate that reactive red (approximately 1 nm) is excluded from the collagen–apatite pores of the mineralized matrix following 5 min of perfusion (Figs. 3a, b), which is consistent with a study by Knothe Tate et al. [14] where similar-sized procion red (molecular weight 300–400 Da, <1 nm in diameter) was not found in the collagen–apatite porosity of adult rat bone 30 min post-injection in fresh-prepared undecalcified ground sections. However, a more recent study by Knothe Tate et al. [13] reports that procion red

penetrates into the collagen–apatite porosity of the mineralized matrix within 9 s post-injection in mechanically loaded rat ulnae.

Microperoxidase

Tanaka and Sakano [23] and Ayasaka et al. [2] demonstrated that microperoxidase (MP, approximately 2 nm) passed through unmineralized alveolar bone in the developing rat, but was excluded from the collagen–apatite porosity of the mineralized matrix 2 h post-injection, while Knothe Tate et al. [14] demonstrated that this tracer appeared in the collagen–apatite porosity of the rat tibia along two bands close to bone surfaces and was absent from the mid-cortex 2 h after injection. In our study, in 5 min following injection, we found that MP labeled both the blood vessels and the osteocytic lacunae across the whole rat tibia including the mid-cortex, and that MP did not appear to penetrate into the collagen–apatite porosity 5 min post-injection (Fig. 3c).

Horseradish peroxidase

Doty and Schofield [8] found quite uniform labeling of horseradish peroxidase (HRP, approximately 6 nm) in the blood vessels and the osteocytic lacunae in the rat femur within 30 min of injection. Similarly, Dillaman et al. [7] demonstrated HRP labeling in blood vessels and osteocytic lacunae in the rat femur 5 and 15 min post-injection. Knothe Tate et al. [14] reported that HRP labeling of blood vessels and osteocytic lacunae was present in the inner half of the cortex of the rat tibia where the bone was well-organized 2 h post-injection. Our study shows HRP labeling of the blood vessels and osteocytic lacunae across the whole bone 5 min post-injection (Fig. 3d).

Ferritin

Previous studies report ferritin labeling as halos surrounding blood vessels in 2-day-old chick bone [6] and adult dog bone [18], or multiple halos with a clear margin (15–18 μm) from the osteonal canal surface in adult goat bone [17,20] after the tracer was circulated for 10–60 min. These results suggest that ferritin (approximately 10 nm) might pass through not only the lacunar–canalicular porosity but also the collagen–apatite porosity in both young and adult bone. Young bone may be more permeable than adult bone [16], and therefore, it is possible for ferritin to penetrate into the collagen–apatite porosity in the 2-day-old chick bone. However, unlike these studies, our study shows that in adult rat bone, ferritin labels blood vessels but does not appear outside of the vascular porosity (Figs. 3e, f).

These different tracer labeling results suggest that the outcome of tracer distribution depends on many factors, including driving forces (such as mechanical loading and circulatory hydraulic pressure), the molecular size of the tracer, the tracer dosage, how long the tracer is allowed to circulate in the vascular system, as well as the species and age of the experimental animals. All these important factors should be considered when interpreting the labeling results. In the present study, we focused on tracer perfusion without any mechanical loading applied to the adult rat tibiae. The tracers we used, reactive red, MP, HRP, and ferritin, differ by two orders of magnitude in molecular weight, and their diameters are estimated to be from approximately 1 to 10 nm if assuming spherical molecular shapes. The tracer dosages for reactive red, MP, and HRP used in this study were chosen to be similar to those used previously for procion red, MP, and HRP, respectively (Table 3). Compared to previous studies [17,18,20] where ferritin was injected via the tibial nutrient artery of adult tibiae, we increased the ferritin dosage by threefold to compensate for the possibility of less tracer being delivered to the bone via the jugular vein (Table 3). Although the total number of molecules injected into the animals was not equal for the four tracers, the tracer distribution patterns probably would not differ significantly from the case of injecting the same number of tracer molecules because each tracer was visualized using

different histological processing methods to produce optimal staining. As to the circulation time, we allowed the tracer to circulate 5 min post-injection for two reasons: (1) the time was found to be long enough for all the tracers to enter bone vasculature throughout the tibiae; and (2) longer circulation times (up to 1 h) showed the same labeling patterns as those in the case of 5 min of circulation.

In addition to the factors just described, we also found that the histological processing procedure has a strong influence on the final labeling results, and we believe that the different histological processes used in different studies may be the cause of the conflicting tracer experiment results summarized above. When the blood circulation is stopped and the histological process begins, the injected tracer may continue to move in the interstitial fluid space unless the tracer molecules are well fixed spatially. Although chemical fixatives are able to preserve biological tissues and to render the cell constituents (proteins, lipids, and carbohydrates) insoluble and resistant to subsequent processes, whether the injected tracers are stabilized in situ with these fixatives is not clear. A significant potential artifact for tracer studies, therefore, is the redistribution of the soluble tracer particles due to diffusion during the long period of histological processing. For example, the differing results from procion red studies could possibly be because some of the samples were embedded in methyl methacrylate (MMA) following a long processing period. While the tracer distribution can be examined in the fresh-prepared undecalcified ground sections within hours after the experiments, it usually takes many days (up to weeks) to complete MMA embedding, which involves immersing samples in dehydrating agents, clearing agents, and then plastic resins, and could result in redistribution of the injected tracer. In this study, the experimental protocol was optimized to reduce the processing time to avoid redistribution of tracer by initially sectioning into smaller pieces, using rapid decalcification methods to shorten decalcification time, and using cryosectioning instead of plastic embedding. However, the processing times for our four tracers still varied greatly because they had to be processed and visualized using different methods (Fig. 1). It would be of great advantage to use tracers that can be processed and visualized using the same protocol. Further use of fluorescent tracers is such an option that would even allow two different-sized tracers to be visualized at the same time.

Despite the small number of animals per group, this study allows us to draw the conclusion that the osteocyte pericellular pore size falls between 6 and 10 nm based on the labeling results from the four tracers used. The most significant finding of our study is that ferritin (approximately 10 nm) is confined to the vascular porosity and does not pass through the lacunar–canalicular porosity. Because the ferritin labeling reported here contradicts previously published studies, we have conducted several subsequent ferritin experiments. We have increased the circulation time up to 1 h, where we found the same pattern of ferritin confined to the vascular pores with no staining of osteocytic lacunae in the tibiae. We have also varied the histological processing in an attempt to create halos found by other investigators. Using histological processing protocols similar to previous investigators (Table 3), we have been able to obtain ferritin “halos” appearing to enter the bone matrix beyond the vascular porosity (Fig. 5). To determine the cause of the halos, we are looking into possible structural changes of the tissue induced by the histological processing. For example, while ferritin-injected bone is being stained in the acidic Perl's reagent, the size of bone pores may be enlarged as the acidic reagent removes calcium from the mineralized matrix, and thus the injected unbound ferritin may be able to penetrate the more porous bone. We are currently investigating the effect of staining time in Perl's reagent on the ferritin staining distribution, and our preliminary results suggest that prolonged staining time may account for the diffusive boundaries of the ferritin “halos” as reported in the adult dog [18]. Clearly, more work needs to be performed to further verify that molecules the size of

ferritin are excluded from the lacunar–canalicular porosity and to confirm the source of the ferritin halos found by other investigators.

In summary, the present study provides quantitative data on tracer movement in the absence of mechanical loading. Our results show that 5 min after tracer injection, the percentage of the osteocytic lacunae labeled with reactive red (approximately 1 nm), microperoxidase (approximately 2 nm), horseradish peroxidase (approximately 6 nm), and ferritin (approximately 10 nm) decreases from 88%, 67%, 57%, to 0% (Table 2), suggesting (but not yet proving) the decreased transport of larger molecules in bone in the absence of mechanical loading. Therefore, convective transport from mechanical loading may be needed to ensure timely transport of the relatively larger molecules in the lacunar–canalicular porosity. However, due to the sieving effect of the lacunar–canalicular porosity, there probably exists an upper limit of the molecular size (cutoff size) for the tracers that can pass through the canalicular pores even with the help of convection in mechanically loaded bone. The present perfusion study suggests that this cutoff size in rat long bone falls between the size of horseradish peroxidase (40000 Da, approximately 6 nm) and ferritin (440000 Da, approximately 10 nm). To refine the pore size of the lacunar–canalicular porosity and the cutoff molecular size for mass transport, tracers with a molecular size between 6 and 10 nm (e.g., albumin, approximately 7 nm) will be used for further investigations, which should help us to better understand bone metabolism and molecular signaling among bone cells in vivo.

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Fig. 1.

Histological processing protocols for the rat tibiae injected with control (saline), and microperoxidase (MP), horseradish peroxidase (HRP), ferritin, and reactive red tracers. Visualization of the four tracers was achieved by the colored end products of either DAB staining (for MP and HRP) or Prussian Blue (for ferritin), or by fluorescence (for reactive red). The control group served as a negative control of DAB staining for the MP and HRP groups and as a baseline measurement control (stained with toluidine blue) for all the tracer groups. EM-Fix: 0.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M sodium cacodylate buffer. Perl's reagent: a freshly made 1:1 mixture of 4% potassium ferrocyanide and 4% hydrochloric acid. DAB: 3,3'-diaminobenzidine tetrahydrochloride.

Fig. 2.

A cross-section of the mid-diaphysis of the rat right tibia. Measurements of bone area (B.Ar) and the numbers of labeled osteocytic lacunae (N.Ot) and blood vessels (N.Bv) were performed on three sampling regions: anterior (A), posterior lateral (L), and posterior medial (M) regions in the cortex between the endosteal surface (Es) and the periosteal surface (Ps). The mean values of the measurements from these three regions in three cross-sections were reported for each animal and used in the subsequent statistical analyses (magnification: 35 $\times$; scale bar: 0.5 mm).

Fig. 3.

Tracer labeling at the posterior medial region of the rat tibia. (a) Reactive red appeared in most blood vessels (v) and osteocytic lacunae (Ot) (magnification: 350 $\times$, scale bar: 40 μ m). (b) Reactive red appeared in canaliculi (Ca) but did not appear to be present in the mineralized matrix (magnification: 1400 $\times$, scale bar: 10 μ m). (c) MP labeled blood vessels and osteocytic lacunae (shown in brown) (magnification: 350 $\times$, scale bar: 40 μ m). (d) HRP showed similar labeling as MP (magnification: 350 $\times$, scale bar: 40 μ m). (e) Ferritin was found in blood vessels (shown in blue) without labeling the surrounding osteocytic lacunae (magnification: 350 $\times$, scale bar: 40 μ m). (f) Ferritin labeling was confined to vascular pores and did not appear to penetrate the collagen-apatite pores within the mineralized matrix (magnification: 1400 $\times$, scale bar: 10 μ m).

Fig. 4.

(a) The density of labeled blood vessels (Den.Bv, #mm²) showed no significant differences among control (Ctrl), MP, HRP, and ferritin (Fe) groups. (b) The density of the osteocytic lacunae (Den.Ot, #mm²) labeled with reactive red (R-Red), MP, and HRP decreased as the tracer size increased from approximately 1 to 6 nm. All the osteocytic lacunae were stained with toluidine blue in the control group (Ctrl), while none of the osteocytic lacunae was labeled with ferritin (approximately 10 nm diameter). Due to the zero variance of the measurements, the ferritin group was not included in the statistical analysis and is not shown in this plot. Despite the decreasing trend, no significant differences of group means were found among the groups examined here, although the difference between HRP and control was approaching significance ($P = 0.054$).

Fig. 5. Unlike those confined to the vascular pores shown in Figs. 3e and 3f, ferritin “halos” (indicated with arrows) were found to appear inside the bone matrix after processing rat samples in Perl's reagent for 3 h, followed by decalcification in sodium citrate/formic acid for 5 days and paraffin embedding with hematoxylin counterstain.

Table 1

Four tracers injected into the rat

	Reactive red	Microperoxidase	Horseradish peroxidase	Ferritin
Molecular weight (Da)	1470	1862	40 000	440 000
Approximate molecular diameter (nm)	1	2	6	10
Dosage (mg/100 g body weight)	8	25	25	154

Table 2Tracer labeling 5 min post-injection in rat tibia without mechanical loading^a

Measurements	Control	Reactive red	Microperoxidase	Horseradish peroxidase	Ferritin
Density of labeled blood vessels (Den.Bv, #/mm ²)	116 ± 26	N/A	90 ± 11	101 ± 13	98 ± 6
Percentage (%)	100	N/A	78	87	84
Density of labeled osteocytic lacunae (Den.Ot, #/mm ²)	1248 ± 35	1101 ± 152	838 ± 222	712 ± 272	0
Percentage (%)	100	88	67	57	0

N/A indicates no measurements available. The densities of the labeled blood vessels (Den.Bv) and the osteocytic lacunae (Den.Ot) for each animal were the averaged measurements from the anterior, posterior lateral, and posterior medial regions in three cross-sections (see Fig. 2). The percentage was calculated by dividing the mean value of each tracer group with that of the control group, which was stained with toluidine blue and served as a reference of 100%.

^aData are reported as mean ± standard error of mean (*n* = 3 per group).

Table 3

Summary of bone tracer studies

Tracer	Study	Animal/bone	Dosage/injection site	Circulation time ^a	Fixation/decalcification/staining/ embedding/section size/preparation time (if given)	Tracer labeling patterns
<i>Procion red</i>						
Imperial	[14]	rat tibia, metacarp (young and mature)	0.8%; 0.01 ml/g BW lateral tail vein	10, 30 min	40% ethanol; no decalcification; MMA-embedded or freshly ground ^b to 50 μ m	appeared in VP, LCP, not in MM
Imperial	[12]	rat tibia	0.8%; 0.01 ml/g BW lateral tail vein	loading time: 7 s to 3 min ^c	processed as Ref. [14] except that all samples were MMA-embedded	appeared in VP, LCP; enhancement of molecular transport in loaded limb (does not say within MM)
Imperial	[13]	rat ulna	0.01 ml/g BW lateral tail vein	loading time: 9 s	40% ethanol; no decalcification; MMA-embedded	appeared in VP, LCP, and MM; enhanced transport in loaded limb
<i>Reactive red</i>						
Sigma	present study	rat tibia	8 mg/ml; 0.01 ml/g BW; jugular vein	5 min	100% alcohol for 4 h; no decalcification; ground to 200 μ m; ~ hours	appeared in VP, LCP, not in MM
<i>MP</i>						
Sigma	[23]	rat alveolar bone (5-day-old)	0.25 mg/g BW jugular vein	2 h	glutaraldehyde/paraformaldehyde ^d for 3 h; EDTA/sucrose for 2-3 days; DAB for 90 min; ethanol series, embedded in Spurr resin; 1 μ m	appeared in VP, LCP, not in MM
Sigma	[2]	rat alveolar bone	50 mg/(140-165 g animal); jugular vein	2 h	processed as Ref. [23] except 3-4 weeks of decalcification	appeared in VP, LCP, not in MM
MP-11, Sigma	[14]	rat tibia, metacarp (lateral tail vein)	25 mg/ml; 0.01 ml/g BW; lateral tail vein	30 min, 2 h	glutaraldehyde/paraformaldehyde ^d for 3 h; EDTA/sucrose for 10 days (metacarp) or 2 weeks (tibiae); DAB staining (2 h); ethanol series and xylol; 50 μ m	two bands of labeling possibly in MM near bone surfaces
MP-11, Sigma	[12]	rat tibia	25 mg/ml; 0.01 ml/g BW; lateral tail vein	2 h plus loading time ^c	processed according to protocols from Ref. [14] except sections 6 μ m	appeared in VP, LCP; enhancement of transport in loaded limb
MP-11, Sigma	present study	rat tibia	25 mg/ml; 0.01 ml/g BW; jugular vein	5 min	glutaraldehyde/paraformaldehyde ^e for 48 h; nitric acid for 48 h; DAB staining for 1 h; 5-10 μ m; ~ 5 days	appeared in VP, LCP, not in MM
<i>HRP</i>						
	[8]	rat femur	25-50 mg/(200 g animal), vein not specified	15, 30 min	6% glutaraldehyde for 3 h; EDTA for 48 h; DAB staining	appeared in VP, LCP, not in MM

Tracer	Study	Animal/bone	Dosage/injection site	Circulation time ^a	Fixation/decalcification/staining/ embedding/section size/preparation time (if given)	Tracer labeling patterns
Type II, Sigma	[23]	rat alveolar bone (5-day-old)	0.25 mg/g BW jugular vein	2h	glutaraldehyde/paraformaldehyde ^d for 3 h; EDTA/sucrose for 2-3 days; DAB for 90 min; ethanol series, embedded in Spurr resin; 1 µm	appeared in VP, LCP, not in MM
Sigma	[7]	rat femur	0.25 mg/g BW; left femoral vein	5, 15, 30, 60, 240 min, 1 day	glutaraldehyde/paraformaldehyde ^e fixation; no decalcification; DAB staining; JB-4 embedding; 10 µm	appeared in VP, LCP, and endosteal MM
Sigma	[14]	rat tibia, metacarp	25 mg/ml; 0.01 ml/g BW; lateral tail vein	2h	glutaraldehyde/paraformaldehyde ^d for 3 h; EDTA/sucrose for 10 days (metacarp) or 2 weeks (tibiae); DAB staining (2 h); ethanol series and xylol; 50 µm	appeared in VP, LCP, concentrated in inner half of cortex
Sigma	present study	rat tibia	25 mg/ml; 0.01 ml/g BW; jugular vein	5 min	glutaraldehyde/paraformaldehyde ^e for 48 h; nitric acid for 48 h; DAB staining for 1 h; 5-10 Am; ~5 days	appeared in VP, LCP, not in MM
Ferritin						
horse spleen	[6]	chick femur (2-day-old)	0.2 ml solution; 0.5 mg ferritin/g BW jugular vein	5, 240 min	Karnovsky's fixative ^f for 24 h; Perl's reagent for 3 h; sodium citrate/formic acid for 24 h; ethanol series, embedded in Paraplast Plus; 10 µm; at least 3 days	halo-shaped labeling within MM
cadmium-free horse spleen, Sigma	[18]	dog tibia	0.5 mg/g of tibia weight; tibial nutrient artery	0, 1, 2, 5, 7.5, 10, 15, 20, 25, 30, 60 min	Karnovsky's fixative ^f for 30 h; Perl's reagent for 5-8 h; sodium citrate/formic acid for 2-4 weeks; ethanol series, embedded (embedding media not specified); 10 µm	fuzzy halo-like labeling (possibly within MM)
cadmium-free, Sigma	[20]	goat tibia	0.5 mg/g of tibia weight; tibial nutrient artery	0 min	Karnovsky's fixative ^f for 30 h; Perl's reagent for 8 h; no decalcification; ethanol series and acetone; MMA-embedded; 30-50 µm; at least 4 days	halos observed (possibly within MM)
cadmium-free, Sigma	[17]	goat tibia	0.5 mg/g of tibia weight; tibial nutrient artery	2 - 3 min under loading	processed according to protocols from Ref. [20] except sections 40 µm; at least 4 days	halos observed (possibly in MM); enhanced transport in loaded bone
cadmium-free horse spleen, Sigma	present study	rat tibia	1.54 mg/g BW jugular vein	5 min	100% alcohol for 4 h; Perl's reagent for 1 h; 10% nitric acid for 24 h; cryosectioning; 5-10 µm; ~1 day	appeared in VP, not in LCP and MM

Key: BW = body weight; VP = vascular pores; LCP = lacunar-canalicular pores housing osteocytes and cell processes; MM = the extracellular mineralized matrix containing the collagen-apatite porosity but excluding the vascular and lacunar-canalicular pores; MMA = methyl methacrylate; EDTA = ethylenediaminetetraacetic acid; DAB = 3,3'-diaminobenzidine tetrahydrochloride.

^aCirculation time is defined as the time period between the end of tracer injection and the beginning of animal sacrifice.

^bThis study used a fresh grinding technique for the 180-day-old (mature) rat tibia and metacarpus as well as the 60-day-old (immature) rat tibia. MMA embedding was used for the immature rat metacarpus.

^c Mechanical loading was applied at three frequencies (0.2, 2.0, 5.0 Hz) for 35 cycles, which corresponds to 3 min to 7 s of loading time.

^d Fixative: 2.5% glutaraldehyde, 2% paraformaldehyde, and 4% sucrose in a 0.1 M sodium cacodylate buffer.

^e Fixative: 0.5% glutaraldehyde and 2% paraformaldehyde in a 0.05 M sodium cacodylate buffer.

^f Karnovsky's fixative: 3% glutaraldehyde and 3% paraformaldehyde in a 0.2 M sodium phosphate buffer.