Short Communication

Application of Flash and Cumulative Labeling with Bromodeoxyuridine for Analysis of Cell Kinetics of Bone Cells and Chondrocytes in Young Growing Rats

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Abstract: We examined cell kinetics of bone cells and chondrocytes in the femur of young growing rats using flash and cumulative labeling with bromodeoxyuridine (BrdU). As flash labeling, rats received an intravenous injection of BrdU. As cumulative labeling, rats were implanted with osmotic pumps filled with BrdU into the peritoneal cavity. The BrdU-labeled nuclei were immunohistochemically identified in decalcified paraffin-embedded specimens using anti-BrdU antibody. In the articular cartilage, BrdU-positive chondrocytes were observed in the middle of the cartilage by flash labeling, and the positive cells were constantly increased with the cumulative labeling days. In the growth plate, BrdU-positive chondrocytes were observed in the proliferating zone by flash labeling, and almost all chondrocytes were positive for BrdU by cumulative labeling. In the bone tissues, by flash labeling, the undifferentiated mesenchymal cells which seem to be a source of osteoblast were positively stained for BrdU. By cumulative labeling, BrdU-positive osteoblasts, osteocytes, and osteoclasts were observed and these cells were mainly localized in the metaphysis. The results suggest that the technique using the combination of flash and cumulative labeling with BrdU is useful for analysis of cell kinetics of bone cells and chondrocytes, and will be applicable to the analysis of bone and cartilage changes induced by various compounds and observed in animal models of skeletal diseases. (J Toxicol Pathol 1998; 11: 65~68)

Key words: Cell kinetics, Bromodeoxyuridine, Rat, Chondrocyte, Osteoblast, Osteocyte, Osteoclast

Introduction

Rats are widely used to test the effects of various compounds and diseases on the structure and composition of bone and cartilage. The bone tissue contains various types of cells, that is, osteoblasts, osteocytes, and osteoclasts, and the cartilage tissue contains chondrocytes. Cell kinetics of bone cells and chondrocytes in rats have traditionally been investigated by detecting the uptake of tritiated thymidine (³H-thymidine) into DNA using autoradiography¹⁻⁵. These studies based on following the fate of cells labeled by ³H-thymidine *in vivo* have provided the basis of current knowledge about cell kinetics of bone cells and chondrocytes.

Recently, a new method employing 5-bromo-2'-deoxyuridine (BrdU) has become available for the analysis of cell kinetics⁶. BrdU is a thymidine analogue which is incorporated into DNA-synthesizing nuclei in the S-phase of the cell cycle, and can be identified immunohistochemically using an anti-BrdU monoclonal antibody. Various methods of BrdU-labeling, such as flash, pulse, and cumulative labeling are used according to the purpose of the study. By flash labeling, the DNA-synthesizing cells in the S-phase of the cell cycle at the time of BrdU-injection are observed. On the other hand, by pulse labeling, the fate of labeled cells is traced

at intervals (several days or weeks) after BrdU-injection. By cumulative labeling, all the proliferated cells which replicate their DNA during the continuous injections of BrdU are labeled regardless of their proliferative or non-proliferative state at necropsy. Several attempts are being made to analyze cell kinetics of bone cells and chondrocytes using flash and pulse labeling⁷⁻⁹.

The aim of this study is to clarify the normal cell kinetics of bone cells and chondrocytes using flash and cumulative labeling with BrdU in the femur of young growing rats as a basis for the future studies of bone and cartilage changes induced by various compounds and observed in animal models of skeletal diseases. These experiments on animals were approved by the Laboratory Animal Use and Care Committee for the Faculty of Agriculture, the University of Tokyo.

Materials and Methods

Male Sprague-Dawley rats were purchased from Japan SLC Inc. (Shizuoka) and subjected to the experiments at the age of 6 weeks. The animals were housed in cages in an animal room maintained at a temperature of 23±2°C and a humidity of 55±10%, with 14 to 16 air changes per hour and a 14-hour light and 10-hour dark cycle, and they were fed pelleted chow (CE-2; Clea Japan Inc., Tokyo) and tap water *ad libitum*.

As flash labeling, rats received an intravenous injection of BrdU [BrdU and 5-fluoro-2'-deoxyuridine (10:1 ratio) aqueous solution, Amersham, Bucks, UK] in the tail vein at

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the dose level of 30 mg/kg and were sacrificed 2 hours later.

As cumulative labeling, rats under ether anesthesia were implanted with mini-osmotic pumps [2 (Model 2001) or 4 (Model 2002) pumps per rat, Alza Co., Palo Alto, CA] filled with 12% BrdU (Sigma Chemical Co., St. Louis, MO) in distilled water containing 50% dimethyl sulfoxide into the peritoneal cavity, and injected with 240 μ g of BrdU per hour for 4, 7, and 14 days before sacrifice.

Animals were sacrificed by exsanguination under ether anesthesia. At necropsy, the femurs (diaphysis to distal epiphysis) were removed from each animal, and fixed in 10% neutral-buffered formalin for 24 hr. The bones were decalcified for 14 days in 20% EDTA, pH 7.5, and embedded for routine paraffin sectioning. Paraffin-embedded tissues were cut into sections.

Sections were deparaffinized in xylene, rehydrated in ethanol and incubated in 0.3% H₂O₂-containing methanol for 30 min to inactivate endogenous peroxidase activity. After washing in distilled water and 0.05 M tris buffered saline, pH 7.6 (TBS), the sections were digested in 0.1% trypsin in 0.05 M tris buffer, pH 7.6 (TB), containing 0.1% CaCl₂ for 20 min at 37°C. Immunohistochemical staining was performed according to the labeled streptavidin-biotin

method, as follow. After incubation in skim milk for 30 min to reduce non-specific staining, sections were incubated successively in the mouse anti-BrdU monoclonal antibody/nuclease mixture (Amersham) for 120 min at 37°C, the biotinylated anti-mouse IgG (Dako, Carpinteria, CA) for 45 min at room temperature and the streptavidin conjugated to horseradish peroxidase (Dako) for 45 min at room temperature, respectively. These sections were washed in TBS for 15 min after each staining step. The sections were then incubated in a medium containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride, 0.01 M imidazole and 0.005% H₂O₂ in TB to visualize immunoreaction. The sections were finally counterstained lightly with hematoxylin and eosin.

Results and Discussion

In the articular cartilage, BrdU-positive chondrocytes were scarcely observed in the middle of the cartilage by flash labeling (Fig. 1a). By cumulative labeling, the BrdU-positive chondrocytes were constantly increased. The positive cells were mainly distributed in the middle of the articular cartilage, and the areas of positive cells were constantly expanded with the labeling days (Fig. 1b).

Fig. 1. Articular cartilage sections of rats with flash (a) and cumulative (b) labeling with BrdU. (a): BrdU-positive chondrocytes are scarcely observed in the middle of the articular cartilage. (b): A large number of chondrocytes show BrdU-positive nuclei at 7 days. Immunostaining, $\times 175$.

Fig. 2. Growth plate sections of rats with flash (a) and cumulative (b) labeling with BrdU. (a): BrdU-positive chondrocytes are observed in the proliferating zone. (b): BrdU-positive cells are observed from the proliferating to the hypertrophic zones at 4 days. Immunostaining, $\times 140$.

In the growth plate, the BrdU-positive chondrocytes were observed in the proliferating zone by flash labeling (Fig. 2a). By cumulative labeling, almost all chondrocytes were stained positively for BrdU, and the positive cells were observed from the proliferating to the hypertrophic zones at and after 4 days (Fig. 2b). BrdU-negative chondrocytes were observed in the uppermost region of the resting zone at any day of cumulative labeling.

In the bone tissues, typical osteoblasts, osteocytes, and osteoclasts were negative for BrdU by flash labeling. Some of the undifferentiated mesenchymal cells showing fibroblast-like spindle-shaped cytoplasm and spindle-to-ovoid shaped nucleus were positively stained for BrdU. These cells were localized in the inner layer of the periosteum and near the endosteal surface of the spongy trabeculae and the compact bones, and were most abundant in the metaphyseal spongiosa subjacent to the growth plate (Fig. 3a).

By cumulative labeling, in addition to the aforementioned mesenchymal cells, osteoblasts, osteocytes, and osteoclasts were positive for BrdU. BrdU-positive bone cells were mainly localized in the metaphyseal spongiosa subjacent to the growth plate, and were constantly increased and expanded to the diaphyseal side of spongiosa with the

period of labeling days (Fig. 3b). BrdU-positive osteoblasts were observed on the surface of the trabeculae and the compact bones, and BrdU-positive osteocytes were observed inside the bone matrix (Fig. 4). The osteoclasts with BrdU-positive nuclei were observed on the surface of trabeculae (Fig. 5).

It is known that the cartilage tissue contains proliferating chondrocytes. By flash labeling, BrdU-positive chondrocytes were observed in the middle of the articular cartilage and in the proliferating zone of the growth plate. The distribution of BrdU-positive chondrocytes by flash labeling is in agreement with that of previous reports using ^3H -thymidine¹⁻³ and BrdU⁸. By cumulative labeling, the BrdU-positive chondrocytes were constantly increased in the articular cartilage with the period of labeling days. In the growth plate, almost all chondrocytes except for several ones

Fig. 3. Metaphysis sections of rats with flash (a) and cumulative (b) labeling with BrdU. (a): BrdU-positive undifferentiated mesenchymal cells are observed near the endosteal surface of the spongy trabeculae subjacent to the growth plate. (b): A large number of bone cells show BrdU-positive nuclei at 7 days. Immunostaining, $\times 80$.

Fig. 4. Metaphysis section of a rat with cumulative labeling with BrdU for 7 days. BrdU-positive osteoblasts are observed on the surface of the trabeculae, and BrdU-positive osteocytes are observed inside the trabeculae. Immunostaining, $\times 220$.

Fig. 5. Metaphysis section of a rat with cumulative labeling with BrdU for 7 days. Osteoclast (arrow) including BrdU-positive nuclei is observed and this cell has both BrdU-labeled and unlabeled nuclei. Immunostaining, $\times 670$.

in resting zone were positive for BrdU. These results implied that, by cumulative labeling, the differentiated chondrocytes in the non-proliferative state are positive for BrdU and the fate of labeled chondrocytes is traced.

In the bone cells, osteoblast, osteocyte, and osteoclast are believed to be in the non-proliferating state. Osteoblasts are believed to be derived from undifferentiated mesenchymal cells, which further differentiate into osteocytes¹⁰. Osteoclasts are believed to be recruited from hematopoietic stem cells, and osteoclast progenitors proliferate and differentiate into mononuclear preosteoclasts and fuse with each other to form multinucleated osteoclasts¹¹.

By flash labeling, BrdU-positive undifferentiated mesenchymal cells which seem to be a source of osteoblast were observed, and these cells were mainly localized in the metaphysis. The osteoblasts, osteocytes, and osteoclasts were negative for BrdU. These results are in agreement with those of previous reports using ³H-thymidine^{1,2,4,5}. On the other hand, by cumulative labeling, osteoblasts and osteocytes were positively stained for BrdU. Moreover, the BrdU-positive nuclei inside a osteoclast were also detected. The fact that the differentiated bone cells in the non-proliferative state were positive for BrdU may indicate that these cells are recruited from progenitor cells during the labeling period, and the fate of labeled bone cells can be traced by cumulative labeling.

In addition, we examined the satisfactory dose of BrdU for cumulative labeling in the bone and cartilage tissues of young growing rats. The nuclei of cartilage tissues of rats injected with 120 and 240 μ g of BrdU per hour were both stained satisfactorily with anti-BrdU antibody. In the bone cells of rats injected with 120 μ g per hour, the reaction products were insufficient to identify BrdU-positive nuclei. On the other hand, BrdU-labeled nuclei of bone cells of rats injected with 240 μ g per hour were readily identified. Therefore, the dose level of 240 μ g of BrdU per hour seems to be required to identify BrdU-positive nuclei in bone cells by cumulative labeling.

In conclusion, the technique using the combination of flash and cumulative labeling with BrdU is useful for analysis of cell kinetics of bone cells and chondrocytes in young growing rats, and will be applicable to the analysis of bone

and cartilage changes induced by various compounds and observed in animal models of skeletal diseases.

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