SHORT COMMUNICATION

Demonstration of mononuclear tartrate-resistant acid phosphatase-positive cells in mouse embryonic mandibles using the simultaneous azo dye technique and a confocal laser scanning microscope

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Introduction

The histochemical detection of hydrolytic enzymes, e.g., acid and alkaline phosphatases (ACPase and ALPase), using naphthol AS phosphate as a substrate and azo dye as a coupler, was simplified by Burstone^{1,2)}. Although the fluorescence in reaction products has been described³⁾, diffuse localization in sections has affected the detectability of fluorescent products. Recently, the application of the fluorescent simultaneous coupling azo dye technique to tissues and cells has been reported⁴⁻⁶⁾. The histochemical staining for ALPase activity under fluorescence microscopy, was extremely sensitive and highly specific, in comparison with that under light microscopy⁵⁾. More recently, with the development of confocal laser scanning microscopes (LSM), the distinct localization of tartrate-resistant ACPase (TRAPase) products has been observed within the cytoplasm of osteoclastic cells in culture and in microslipped bone⁶⁾. Thus, the fluorescent assay with the simultaneous azo dye technique is extremely sensitive. In the present study, we examined whether mononuclear TRAPase-positive cells could be detected by application of the simultaneous coupling azo dye technique and use of a confocal LSM, in paraffin-embedded mouse embryonic mandibles. The combina-

tions of laser emission wavelength and long pass filter setting are also discussed.

Materials and Methods

Pregnant ddY mice were killed by cervical dislocation under anesthesia. Embryonic day 11, 13 and 15 heads were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 3 h at 4°C and then refixed in the same fixative overnight. Use of glutaraldehyde was avoided because of induction of autofluorescence⁵⁾. The fixed specimens were dehydrated in a graded series of ethanol following thorough rinsing with the buffer, then cleared with xylene and embedded in paraffin. The paraffin-embedded tissues were stored at -20°C until use. Sections were cut at 5 µm in thickness on a microtome. For the detection of TRAPase activity in the deparaffinized sections, the following incubation medium was used : 50 ml/ 0.1 M sodium acetate buffer (pH 5.2), 0.25 ml/ N, N'-dimethyl formamide, 5 mg naphthol AS-MX phosphate (Sigma, St. Louis, MO) as substrate, 30 mg fast red violet LB salt or fast blue RR salt (Sigma, St. Louis, MO) as coupler, and 50 mM L(+)-tartaric acid (Wako, Osaka). The incubation medium was prepared and filtrated immediately before use, and the deparaffinized sections were incubated for 15 min at 37°C in a moisture chamber, to avoid non-specific

Fig. 1 Sections from mouse embryonic mandible at day 13. Localization of TRAPase around the molar tooth, with staining apparent in erythrocytes. Osteoclast shows marked staining (arrowhead). Fast red violet LB as coupler. Laser emission wavelength : 568 nm, long pass filter : 590 nm. $\times 144$

fluorescence. After incubation, the sections were mounted in aqueous mounting medium (AQUATEX, Merck, Germany). The incubated sections were observed both with a light microscope and a confocal LSM (TCS 4D, Leica, Cambridge) equipped with a differential interference device. Differential interfering and fluorescent images were stored in a workstation computer, superposed by installed PhotoshopTM software (Adobe Systems Incorporated, CA) and then printed on superfine glossy paper by a color printer (Epson, Tokyo). Fluorescence of TRAPase activity, presented as black, indicated the presence of enzyme.

Results and Discussion

When deparaffinized sections were stained in incubation medium containing fast red violet LB, only weak staining was visible in mononuclear cells under a light microscope (not shown). However, osteoclasts showed marked staining.

When sections stained as above, were observed under a confocal LSM at similar settings (Ar-Kr laser emission wavelength 568 nm, long pass filter 590 nm)

Fig. 2 Sections from mouse embryonic head at day 15. Fast blue RR fluorescence, an azo dye, is visible erythrocytes within blood vessels (arrowhead). Fast blue RR as coupler. Laser emission wavelength : 568 nm, long pass filter : 590 nm. $\times 144$

to those used previously^{5,6}), they demonstrated bright fluorescence in many mononuclear cells surrounding the molar tooth of embryonic day 13 mice (Fig. 1). Almost all positive cells, with the exception of osteoclasts, appeared to be erythrocytes and nucleated erythroblasts. ACPase activity in erythrocytes is considered to be inhibited by 0.5% formaldehyde⁷). In a previous study, an isoenzyme of ACPase confined to the cytoplasm, was shown to be inhibited by Cu^{2+} , HCHO and fast blue RR, but insensitive to fluoride and L (+)-tartrate, while another isoenzyme localized in organelles, indicated sensitivity⁸). HCHO decreased but did not inhibit completely tartrate-insensitive ACPase activity. Therefore, although inhibition of ACPase activity with formaldehyde is apparent at the light microscopic level, it is in fact incomplete with some activity detected as bright fluorescence under a confocal LSM. To test whether another coupling agent, fast blue RR, inhibits ACPase activity in erythrocytes and erythroblasts, sections of the embryonic day 15 head were incubated in staining medium that substituted fast blue RR for fast red violet LB, and then observed under a confocal LSM at the same wavelength and filter settings as above. Erythrocytes

within blood vessels were stained (Fig. 2). This suggests that the combination of laser emission wavelength of 568 nm and long pass filter of 590 nm is not useful for visualizing mononuclear TRAPase-positive cells, even with fast red RR as coupler. In a previous

Fig. 3 The same section and region of Fig. 2. Note no fluorescence in any erythrocytes (arrowhead). Fast blue RR as coupler. Laser emission wavelength : 660 nm, long pass filter : 665 nm. $\times 144$

study, ALPase staining products with naphthol AS-MX phosphate and fast blue RR, were visible as a blue staining precipitate under light microscopy, but no fluorescence was detected under epifluorescence illumination with a rhodamine filter set⁵⁾. The difference between the previous and the present studies suggests that confocal LSM is more sensitive than conventional fluorescence devices for detection of azo dye precipitates.

Various combinations of laser emission wavelength and long pass filter settings were examined. No fluorescence was detected in erythrocytes in the same section as those examined in Fig. 2, at a laser emission wavelength of 660 nm and long pass filter of 665 nm (Fig. 3). However, various intensities of fluorescence of TRAPase activity were visible in the mandible (Fig. 4). Interestingly, mononuclear TRAPase positive cells were localized around the incisor, indicating that they may be involved in the morphogenesis of the tooth⁹⁾. Surprisingly, in embryonic day 11 mandibles, the mononuclear cells were diffuse, and almost all were present in capillaries (Fig. 5). However, the cell type has yet to be established. The appearance of mononuclear TRAPase positive cells in the early stage (day 11) of mandibular morphogenesis, may be

Fig. 4 Region of incisor within mandible in the same section of Fig. 2 and 3. Specific fluorescence of TRAPase localizes around the incisor (arrowhead).

Fast blue RR as coupler. Laser emission wavelength : 660 nm, long pass filter : 665 nm. M : Meckel's cartilage, EO : enamel organ of incisor. $\times 144$

Fig. 5 A rostral region of mouse embryonic day 11 mandible. Almost all of the mononuclear TRAPase positive cells are present in blood capillaries (arrowhead), indicating weak activity of TRAPase.

Fast blue RR as coupler. Laser emission wavelength : 660 nm, long pass filter : 665 nm. $\times 144$

associated with the mouse fetal hemopoietic system, in which yolk sac-derived primitive erythroblasts enter circulation before day 10¹⁰), and in which miscellaneous blood cells, e. g., macrophages, are occasionally present in vitelline vessels¹¹). However, we could not distinguish mononuclear TRAPase positive cells from red blood cells with fast red violet LB staining under a confocal LSM. The maximum peak of fluorescence wavelength in fast red violet LB both in azo dye solution and staining products, has been reported to be 650~670 nm⁶). Further examination is required to determine whether autofluorescence of erythrocytes is detectable in deparaffinized, non-stained sections. Fluorescence staining was demonstrated in cells at 568 nm (laser emission wavelength) and 590 nm (long pass filter), but not at 600 nm and 665 nm (not shown).

In conclusion, both the application of fast blue RR as coupler, and the confocal LSM settings described in the present study, are useful for clear visualization of mononuclear TRAPase positive cells, probably precursors of osteoclasts, during mouse mandibular morphogenesis.

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References

- 1) Burstone, M. S. : Histochemical demonstration of acid phosphatases with naphthol AS-phosphates. *J. Natl. Cancer Inst.* **21** : 523~539, 1958.
- 2) Burstone, M. S. : The relationship between fixation and techniques for the histochemical localization of hydrolytic enzymes. *J. Histochem. Cytochem.* **6** : 322~339, 1958.
- 3) Burstone, M. S. : Histochemical comparison of naphthol AS-phosphates for the demonstration of phosphatase. *J. Natl. Cancer Inst.* **20** : 601~615, 1958.
- 4) Dolbeare, F., Vanderlaan, M. and Phares, W. : Alkaline phosphatase and an acid arylamidase as marker enzymes for normal and transformed WI-38 cell. *J. Histochem. Cytochem.* **28** : 419~426, 1980.
- 5) Ziomek, C. A., Lepire, M. L. and Torres, I. : A highly fluorescent simultaneous azo dye technique for demonstration of nonspecific alkaline phosphatase activity. *J. Histochem. Cytochem.* **38** : 437~442, 1990.
- 6) Tsuchiya, T., Matsumoto, Y. and Kurihara, S. : The fluorescent simultaneous azo dye technique for demonstration of tartrate-resistant acid phosphatase (TRAPase) activity in osteoclast-like multinucleate cells. *J. Bone Miner. Metab.* **13** : 71~76, 1995.
- 7) Pearse, A. G. E. : Acid phosphatases. In : *Histochemistry, theoretical and applied*, Vol. 1, Third edition, (edited by Pearse, A. G. E.) pp. 545~575, Churchill Livingstone, Edinburgh, London, New York, 1968.
- 8) Chen, C.-H. and Chen, S. C. : Evidence of acid phosphatase in the cytoplasm as a distinct entity. *Arch. Biochem. Biophys.* **262** : 427~438, 1988.
- 9) Nagata, M., Ohashi, Y. and Ozawa, H. : A histochemical study of the development of premaxilla and maxilla during secondary palate formation in the mouse embryo. *Arch. Histol. Cytol.* **54** : 267

~278, 1991.

- 10) Kovach, J. S., Marks, P. A., Russell, E. S. and Epler, H. : Erythroid cell development in fetal mice : Ultrastructural characteristics and hemoglobin synthesis. *J. Mol. Biol.* **25** : 131~142, 1967.
- 11) Haar, J. L. and Ackerman, G. A. : A Phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse. *Anat. Rec.* **170** : 199~224, 1971.