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# The Distribution of Dividing Endothelial Cells of Chick Embryonic Aortae Studied by Vital Labeling with Bromodeoxyuridine

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**ABSTRACT**—The proliferation of aortic endothelial cells of 10, 14, and 20 day-old embryonic and 3 day post hatch chicks was studied by vital labeling with bromodeoxyuridine (BrdU) and immunofluorescent visualization using anti-BrdU. We modified the DNA denaturation step using 0.2% Triton X-100 and incubation with 0.1 M hydrogen chloride for 15 min at 30 to 35°C. These modifications were effective to stain the chromosomes with anti-BrdU in the whole mounted tissues. The BrdU-positive endothelial cells were present in the whole aortic areas without showing any mitotic hot spots. The average labeling index of the endothelial cells was high in the young stages; 3.7% at 10 day-old embryos, and then it reduced to 1.4% at 3 day-old chicks.

## INTRODUCTION

The frequency and distribution of dividing endothelial cells in the embryonic blood vessels have been collected few attentions, even though the cell proliferation may play an important role in the development of blood vessels (D'Amore and Thompson, 1987). The chick embryonic blood vessels provide us a convenient model to study the cell proliferation in which the vital labeling of replicating DNA may be easier than that of mammalian embryos. The identification of DNA-synthesizing cells by labeling with BrdU and visualization with immunocytochemistry is now of a popular way to examine the cell proliferation (Gratzner, 1982; Vogel *et al*., 1986; De Fazio *et al*., 1987; Sasaki *et al*., 1987). This is a time saving and convenient tool avoid problems remained in the method using radioactive materials. For the study of endothelium in wide areas of blood vessel, we applied the immunocytochemistry to tissue blocks without thin sectioning (Jinguji and Fujiwara, 1994). Thus, we attempted to combine the immunocytochemistry and whole mounting to detect the BrdU positive cells in aortae. In our preliminary experiments, the methods used by other studies (for examples, Beisker *et al*., 1987; Soriano *et al*., 1991) caused the destruction of the embryonic arterial endothelia or loss of stainability for anti-BrdU. In the present study, we modified the method to reduce the damages of endothelium during DNA denaturation step, and applied it to examine the distribution of dividing cells in the chick embryonic aortae.

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## MATERIALS AND METHODS

### Biological materials

The aorta was obtained from chick embryos of 10, 14, and 20 day of incubation, and 3 day-old young chicks.

### Antibodies and fluorescent reagents

5-bromodeoxyuridine (BrdU), nuclease, and the mouse monoclonal antibody to BrdU were purchased from Sigma Chemical Co. (St Louis, MO, USA). Rhodamine conjugated rabbit anti-mouse IgG was from Cappel Laboratories (Malvern, PA, USA). Propidium iodide was from Wako Chemicals (Osaka, Japan).

### BrdU labeling

The labeling solution was 10 mM BrdU in distilled water. For embryos, a window sized 2 cm × 2 cm was made on the egg shell and the chorioallantoic membrane was exposed. A single shot (0.1 ml) of BrdU solution was given through a small vena (approximately 1 mm in outer diameter) of the chorioallantoic membrane using a 33 gauge needle (Hamilton Co., Nevada, USA). The window of the shell was sealed by a sterile cellophane sheet. The eggs were incubated further in humidified air at 38°C. The young chicks received 0.2 ml of the labeling solution intraperitoneally injected by a syringe.

### Immunofluorescence microscopy

At 1.5 hr after injection of the labeling reagent, chicks were fixed by vascular perfusion with 3.5% paraformaldehyde in phosphate buffered saline (PBS) for 5 min. The aortae were excised out and cut into small pieces, and then were put in the fresh fixative for over a half day in the cold place. The wall of aortae was cut longitudinally to expose the endothelium before cytochemical treatments.

The tissue pieces were treated as follows: 1) washing in PBS for 30 min with three exchanges; 2) treatment with 0.2% Triton X-100 in PBS for 3 min; 3) washing in PBS for 30 min with several exchanges; 4) treatment with 0.1 M hydrogen chloride (HCl) for 15 min at 30 to 35°C; 5) rinsing in distilled water; 6) incubation with 50 mM Tris buffer (PH 8.0) for 3 min to neutralize the HCl solution remained in the tissues (Tris buffer may also work in quenching the aldehyde residues in the tissues); 7) washing in PBS for 30 min with several exchanges;

8) incubation with 5% low fat milk-PBS for 1 hr to block the nonspecific antibody binding. 9) After being washed in PBS, the tissue pieces were treated with a mixture of mouse monoclonal anti-BrdU IgG diluted 50 times with PBS, and 10 unit of nuclease for overnight in a refrigerator. Tissue pieces were washed in PBS for 2 hr with several exchanges, and then were labeled by rhodamine conjugated secondary antibody, diluted 100 times with PBS, for 2 hr. Tissue pieces were then washed in PBS for 1 hr with several exchanges. Some pieces of the tissues prepared as described above were stained further with 1.2 μg/ml propidium iodide in PBS for 5 min. Propidium iodide was the reagent generally used for the staining of nucleic acid that revealed the cell localization in the tissues.

All of the fluorescence labeled tissues were placed on slide glasses and mounted using 60% glycerol in PBS. The samples were examined under an Olympus MT-2 microscope with a X20 (D Plan Apo, N: 7.70) and X40 (D Plan Apo, 40 UV, N: 1.00) lenses. An ACAS570 Interactive Laser Cytometer (Meridian Ins., USA) mounted on an Olympus MT-2 microscope with a X40 lens was used to obtain confocal fluorescence images.

**Calculation of BrdU labeling index**

The total number of endothelial cells in tissue blocks was estimated as follows: 1) distribution density of cells in a unit area of the endothelium (0.05 $mm^2$) was measured from a fluorescence micrograph magnified to X315; 2) approximate size of the endothelial surface of the tissue block (that was square in micrographs) was measured from the series of fluorescence micrographs of the same magnification; 3) from these two results, the total number of endothelial cells in the tissue block was estimated (Table 1). All of the BrdU positive cells in the tissue block was counted from the same series of fluorescence micrographs. Cell counting was done on the micrographs obtained from 2 or 3 chicks per each stages (Table 1).

## RESULTS AND DISCUSSION

**Immunofluorescence staining**

The confocal laser scanning microscopy showed that our modified method was available to maintain the structures of embryonic aorta (Figs. 1, 3a, 3b). The results of propidium iodide staining also showed the normal pattern of cell distribution in the tissues treated with HCl (Fig. 2). In our protocol, the concentration and incubation time with HCl solution were critical. The solution of HCl denser than 0.2 M and incubation for over 30 min at temperature over 40°C frequently denatured the endothelium and caused a loss of stainability for anti-BrdU. The membrane solubilization using Triton X-100 may reduce the time needed for the DNA denaturation. The enzymatic digestion of chromosomes may not be strong, because the tissues were treated with the solution containing nuclease at room temperature (20–25°C) for 30 min before transfer them into refrigerator. Although the enzymatic digestion was thought as a less essential in the immunocytochemical procedures (Gonchoroff *et al.*, 1985), the relatively mild treatment with nuclease was effective in our methods. In the present study, the proliferating smooth muscle cells in the vessel wall also were visualized (Fig. 3b). To increase the fluorescence signals from smooth muscle cells of the deep regions, we recommend the long incubation with HCl solution for 30 min or little more. As shown here, our methods might be applicable to the mono-layered tissues such as cells lining the luminal surface of tube-like organs. The blood vessels preserved in the cold fixative for at least 6 months maintained a good stainability for anti-BrdU.

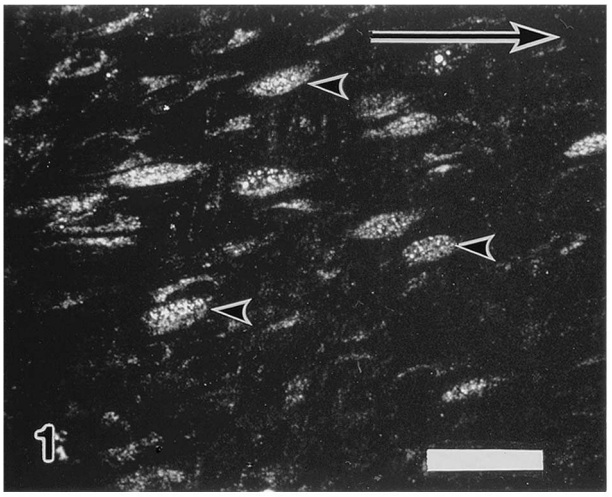


**Fig. 1.** Confocal micrograph of the aortic endothelium of 10 day-old chick embryo at 1.5 hr after injection of BrdU solution. BrdU positive nuclei were demonstrated by anti-BrdU and rhodamine conjugated secondary antibody. The distribution of endothelial cells was shown by faint background stain. Arrow indicates direction of blood flow. Bar: 20 μm.

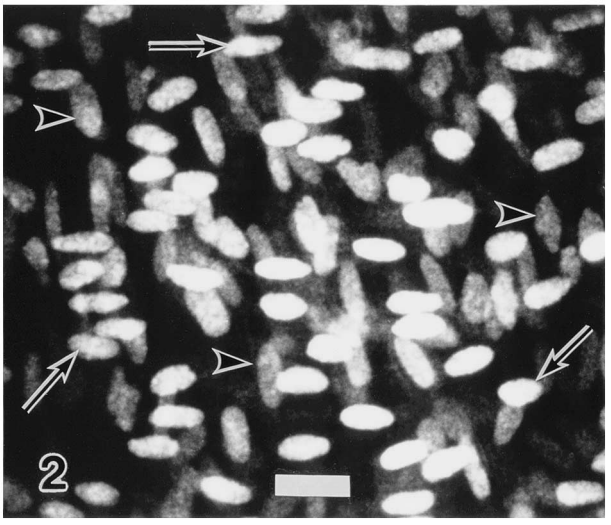


**Fig. 2.** Confocal image of aortic wall of 14 day-old chick embryo stained with propidium iodide. The nucleus of endothelial cells (arrows) maintained their normal distribution pattern. The nucleus of smooth muscle cells (arrowheads) present below the endothelium also were shown in the same field. Propidium iodide staining was done on the sample used for immunofluorescence microscopy. Bar: 20 μm.

**Distribution of BrdU-positive cells**

The intensity of fluorescence signals varied in BrdU labeled nuclei. This may depend on the variation of time length passed after BrdU uptake by replicating DNA. Therefore, the nucleus which had only few dot-like signals also was counted

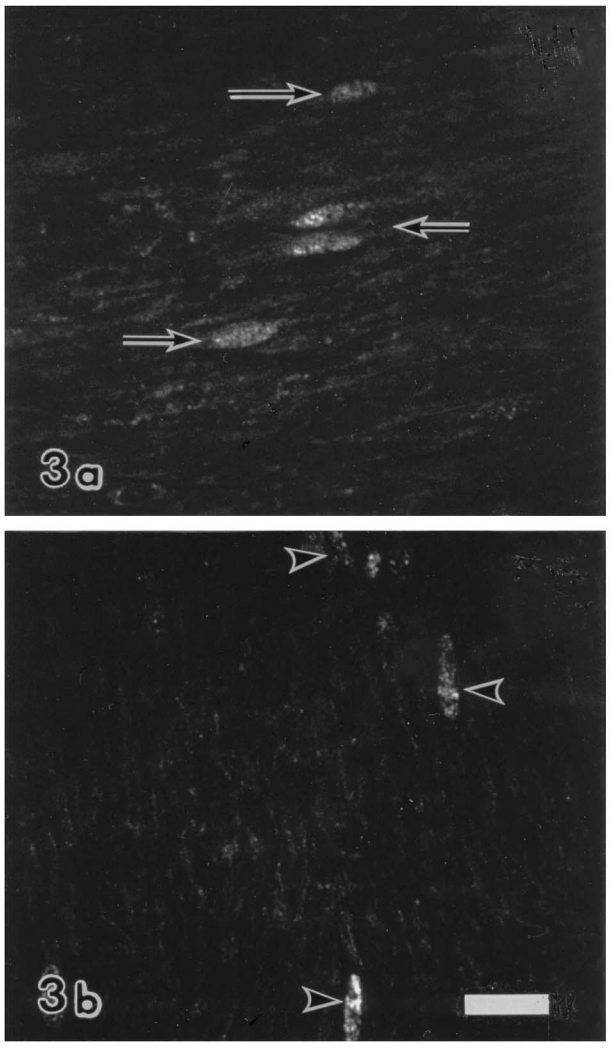


**Fig. 3.** Two semiserial confocal images of BrdU-positive endothelial cells (**a**) and smooth muscle cells (**b**) in the same field of 14 day-old embryonic chick aorta. The major axis of endothelial cell nucleus (**a**, arrows) was parallel to the direction of blood flow, whereas that of smooth muscle cells (**b**, arrowheads) was perpendicular to blood flow. Bar: 20 μm.

**Table 1.** BrdU labeling index of aortic endothelial cells in embryonic and young chicks

| stage (day) | BrdU-positive cells | total endothelial cells | ratio of mitotic cells (%) |
|---|---|---|---|
| 10E | 260 | 7310 | 3.6 |
| | 405 | 10500 | 3.9 |
| 14E | 238 | 9630 | 2.5 |
| | 344 | 14510 | 2.4 |
| 20E | 101 | 5760 | 1.8 |
| | 107 | 9730 | 1.1 |
| | 114 | 11470 | 1.0 |
| 3P | 88 | 7360 | 1.2 |
| | 179 | 10360 | 1.7 |

E: embryo. P: posthatch.

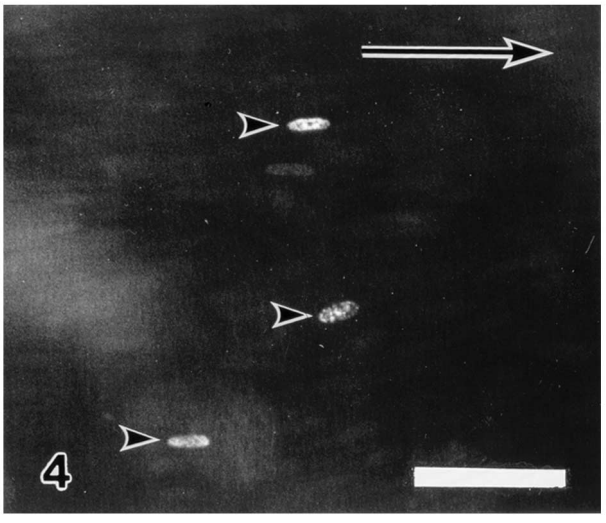


**Fig. 4.** Fluorescence micrograph of 20 day-old embryonic chick aorta labeled with BrdU. This was obtained with conventional fluorescence microscopy. BrdU-positive endothelial cells (arrowheads) were distinct from other cells and showed no clustering in the endothelium. Arrow: blood flow direction. Bar: 50 μm.

as the BrdU-positive one. The BrdU-positive endothelial cells were present random in all of the aortic regions (Figs. 1, 3a, 4, 5). This suggests there was no mitotic hot spot in the embryonic aortae. Similar results were obtained with immunofluorescence staining using anti-myosin on the aortae after mitotic arrest induced by colchicine (data not shown). Schwartz and Benditt (1976) found the clustering of replicating cells in rat aortae. However, other reports including their next works suggested the absence of such mitotic hot spots in rat aortae (Schwartz and Benditt, 1977; Hasson *et al*., 1985; Linn *et al*., 1989). The BrdU labeling index of the endothelial cells was calculated using the areas randomly selected from the tissue blocks (Table 1). The high labeling index was found in the young stage; in 10 day-old embryos (Fig. 1), the average labeling index was 3.7%. In 14 day-old embryos (Fig. 3a), it was 2.4%, and then at the stages of around hatching the index reached 1.2% (20 day-old embryos, Fig. 4) or 1.4% (3 day-old chicks, Fig. 5). Studies of other investigators using adult rats showed that less than 0.55% endothelial cells replicated at 24 hr after injection of tritiated thymidine (Schwartz and Benditt, 1976 and 1977; Hasson *et al*., 1985; Linn *et al*., 1989). Schwartz and Benditt (1976) also reported on young rats that the cell replication rate was high (13%) at just after birth and it fell down largely to less than 0.3% at about 6 month later. Although the experimental schedules and the methods of these previous works were different from the present study, our results showed a similar tendency in the changing of cell replication rate. In the chick aortae, endothelial cells replicated with higher rate during young embryonic stages than that of post hatched chicks. Such rapid cell replication may contribute to the large growth of blood vessels during embryonic development.

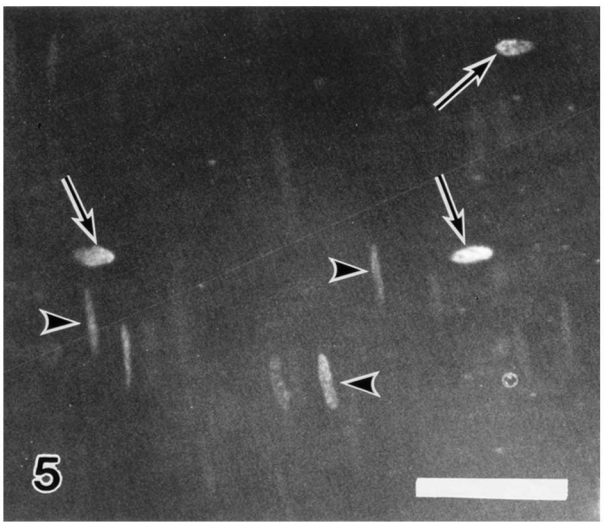


**Fig. 5.** Fluorescence micrograph of BrdU labeled aorta of 3 day-old chick. The BrdU-positive nuclei of endothelial cells (arrows) and smooth muscle cells (arrowheads) showed no clustering in the aortic wall. Bar: 50 μm.

## ACKNOWLEDGEMENT

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