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AUTORADIOGRAPHIC STUDY USING ^{3}H-THYMIDINE ON RAT INCISAL PULP CELLS *in vitro*

BY

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ABSTRACT

An autoradiographic study using ^{3}H-thymidine (^{3}H-TdR) was carried out to investigate which cells migrate from the pulp explant of the rat incisor *in vitro*. The cells close to the arterioles (perivascular cells) were especially labeled after ^{3}H-TdR incubation following the dissection of the pulp explant. In tracing these ^{3}H-TdR-labeled perivascular cells, about 15 per cent of the outgrowth cells were the perivascular cells after 2 days of culture and these perivascular cells had a high mitotic activity *in vitro*. On the other hand, it was ascertained that the odontoblasts in the pulp explant underwent necrotic changes and that the odontoblasts had no ability to migrate from the pulp explant.

INTRODUCTION

In the dental pulp, there are many kinds of cells such as odontoblasts, fibroblasts, structural cells of blood vessels, undifferentiated mesenchymal cells and so on (Avery [1]). Previous studies have shown that many cells migrated out of the pulp explant in the culture (Pinkerton and Boyle [2]), and the morphologic studies of such outgrowth cells from the dental pulp explant have been carried out (Szabo [3]; Niizima and Cattoni [4]; Zussman and Ioachim [5]; Vidic *et al.* [6]; Magloire and Joffre [7]; Prime and Reade [8]). Also, the culture of the odontoblasts using the dental pulp explant has been reported (Zussman and Ioachim [5]).

However, it has not been established whether the odontoblasts have the ability to migrate out of the pulp explant or not and what kind of cells in the dental pulp migrate. These problems were investigated in the present study using ^{3}H-thymidine (^{3}H-TdR) autoradiography on the pulp explant of the rat incisor in the tissue culture.

MATERIALS AND METHODS

Fifty-one male Wistar rats aged seven weeks with a mean body weight 180 g were used.

I. Preparation of pulp explants

The animals were killed by decapitation under ether anesthesia and the upper incisors were isolated and dipped in a dilute iodine tincture for a moment. The apical part of the incisor was bared; the surrounding dentine and enamel were removed with fine forceps; and the dental pulp was carefully pulled out. The dental pulp was rinsed three times with ice cold Hanks' balanced salt solution (BSS) containing 200 IU per ml of penicillin G and 200 μg per ml of streptomycin. The pulp of the same area in every tooth was dissected by cutting transverse-

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ly with a sharp blade under a microscope (Fig. 1). All of the above procedures were performed within one hour.

II. ^{3}H-TdR labeling in pulp explant

The purpose of this experiment was to investigate what kinds of pulp cells were labeled with ^{3}H-TdR as the course of culture time progressed. Ten groups of three rats each were used. The pulp explants were placed in plastic Petri dishes (35×10 mm, Lux Scientific Corportation). Two ml of the culture medium was added; the depth of the medium was about 2 mm. The culture medium was F12 (Nissui) supplemented with 10 per cent (v/v) fetal bovine serum (Flow Laboratories), 100 IU per ml of penicillin G and 100 μg per ml of streptomycin, controlled at pH 7.2 with $NaHCO_3$ under an atmosphere of 99 per cent air, 1 per cent CO_2 and maintained at 37°C. The medium was changed every two days and the outgrowth cells were observed using a phase contrast microscope. After each was cultured respectively for 0, 12, 24 and 36 hours and 2, 3, 4, 5, 6 and 7 days, the medium was removed and the explants were incubated in the ^{3}H-TdR medium containing 10 μCi per ml of ^{3}H-TdR (s.a. 25 Ci per m mole, New England Nuclear) for 90 minutes. The explants of 0 hour of culture were incubated in the ^{3}H-TdR medium immediately after the preparation of the pulp explants. The explants were washed in BSS after the incubation in the ^{3}H-TdR medium and fixed in the phosphate-buffered (pH 7.2) glutaraldehyde-formalin solution. After dehydration, the explant was embedded in glycol methacrylate resin (GMA, Nissin). One side of the explant was sectioned longitudinally and the other side of the explant was sectioned transversely into 1-μm thickness. These sections were then used for the autoradiographic study.

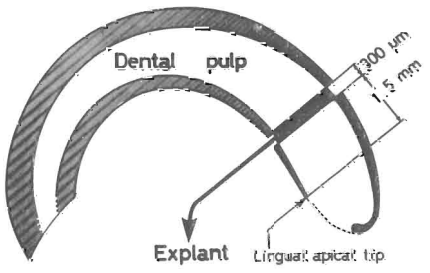

Fig. 1. The pulp explant for culture was dissected from the region of the rat upper incisor pulp, 1.5 mm from the lingual apical tip and 300 μm in thickness.

III. Tracing the ^{3}H-TdR-labeled cells

The purpose of this experiment was to trace the ^{3}H-TdR-labeled cells after the preparation of the pulp explants. Seven groups of three rats each were used, and the pulp explants were prepared according to method I. The pulp explants were incubated in the ^{3}H-TdR medium containing .1 μCi per ml for 90 minutes, washed three times with BSS at 37°C and transferred to the culture medium without the ^{3}H-TdR and each cultured respectively for 12, 24 and 36 hours and 2, 3, 4 and 5 days. In this experiment, the pulp explants were cultured on a slide glass which was placed in a flat Petri dish (85×17 mm, Ikemoto) and 14 ml of the culture medium was added; the depth of the medium was about 2 mm. After culturing, the pulp explants were fixed, embedded and sectioned according to the procedure described above. The sections were then used for autoradiography. The outgrowth cells on the slide glasses were washed with BSS, fixed gradually with acetic acid-ethanol solution (v/v 1:3), treated with 2 per cent (v/v) solution of perchloric acid and then used for autoradiography (Yamada and Takeda [9]).

IV. Autoradiography

Autoradiography was performed by the usualy dipping procedure (Adams [10]). The sections or the outgrowth

cells on the slide glasses were dipped in the autoradiographic emulsion (Sakura NR-M2, Konishiroku Co.), exposed at 4°C for 10–28 days, then developed and fixed. The sections were stained with azure-eosin (Bennet *et al.* [11]) for histological oservation and the outgrowth cells were stained with Giemsa. The cells were considered as "labeled" when their nuclei had five or more grains. In the sections of the pulp explants, the labeled cells were counted in each of the five sections which were not serial but taken 20 μm apart. The dental pulp cells in the sections were divided into four groups: 1) odontoblasts, 2) endothelial cells, 3) perivascular cells arranged circumferentially around the arterioles and 4) fibroblasts.

Results

The schematic illustration of the findings obtained from the experiments is shown in Fig. 2.

I. Autoradiographic observations in pulp explant

When the pulp explant was incubated in the ^{3}H-TdR medium just after the dissection, a large number of the labeled perivascular cells was noted. The labeled endothelial cells or the labeled fibroblasts were observed only rarely (Figs. 3, 4). Eighty-five per cent of the ^{3}H-TdR-labeled cells were perivascular cells. The odontoblasts remained partially attached to the pulp explant. However, their processes were torn off. The ^{3}H-TdR-labeled odontoblasts was not observed (Fig. 4).

All odontoblasts were noted having a remarkable karyolysis and pyknosis after 12 hours of culture. The nuclei of most of the fibroblasts showed similar but less pronounced changes (Fig. 5).

At 24 hours of culture, the perivascular cells and some of the fibroblasts which were free from necrotic change began to migrate out of the pulp explant and these migrating cells had characteristic pointed cytoplasmic extensions and were of slender form (Fig. 6). The endothelial cells maintained a normal structure.

After 36 hours of culture, no cells were labeled with ^{3}H-TdR after the incubation in the ^{3}H-TdR medium (Fig. 4). On the other hand, the migrating cells increased in the pulp explant.

After 3 days, the necrosis of the pulp explant progressed even more and the migrating cells in the pulp explant decreased. After 5 days, a large part of the pulp explant underwent necrosis and a

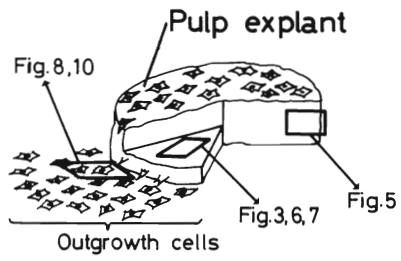

Fig. 2.

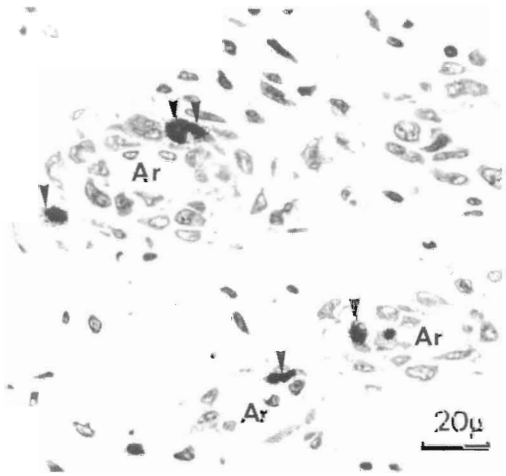

Fig. 3. Transverse section of pulp explant incubated in ^{3}H-TdR medium for 90 minutes after dissection. The perivascular cells close to the arterioles (Ar) were especially labeled (arrows). Azure and eosin stain.

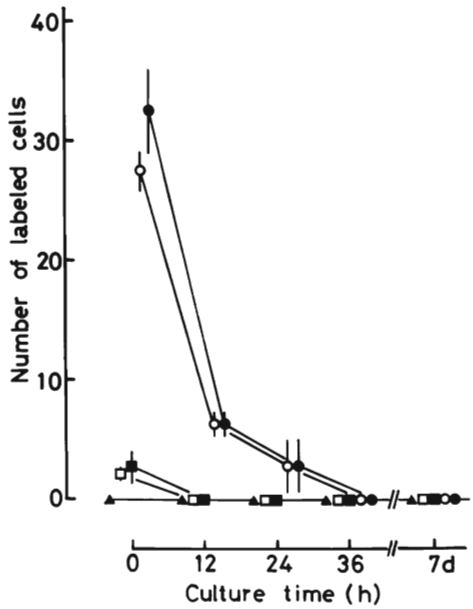

Fig. 4. Number of ^{3}H-TdR-labeled cells in the transverse section. Odontoblasts (▲), fibroblasts (□), endothelial cells (■), perivascular cells (○), total labeled cells (●). Each point and bar represents the mean±SD (n=3).

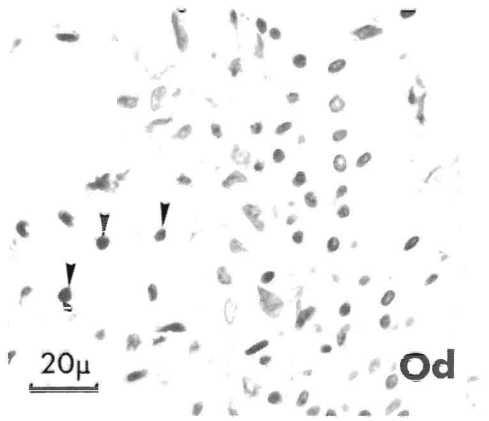

Fig. 5. The histologic change in plup explant after 12 hours of culture. The processes of odontoblasts (Od) were torn away and their nuclei underwent a marked pyknosis. The fibroblasts at the subodontoblastic layer also showed initial signs of necrosis (arrows). Azure and eosin stain.

large amount of the eosinophilic materials was found in the pulp explant. However, the endothelial cells still remained

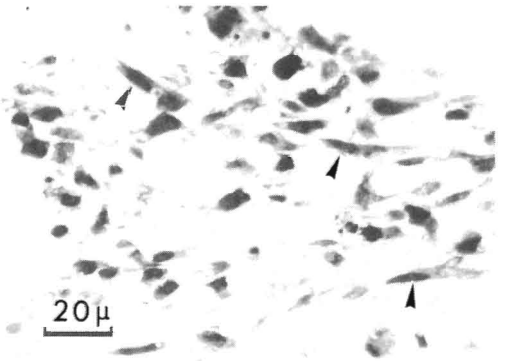

Fig. 6. The migrating cells after 24 hours of culture. Note their characteristic cytoplasmic extensions (arrows). Azure and eosin stain.

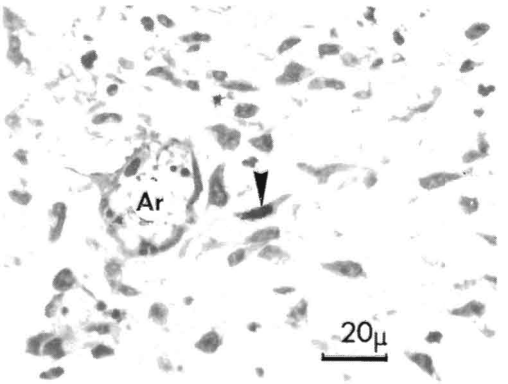

Fig. 7. Migrating cells in the pulp explant after 36 hours of culture. They had characteristic cytoplasmic processes. The ^{3}H-TdR-labeled migrating perivascular cells (arrow) around the arterioles (Ar) were observed. Azure and eosin stain.

along the blood vessel walls. After 7 days, the necrotic changes were noted in all parts of the pulp explant.

II. Tracing the ^{3}H-TdR-labeled cells

After 12 hours of culture, mitotic figures of ^{3}H-TdR-labeled perivascular cells were observed around the arterioles. At 36 hours, ^{3}H-TdR-labeled cells were found among the migrating cells in the pulp explant (Fig. 7).

On the 2nd day of culture, the outgrowth cells were found around almost every pulp explant under the phase contrast microscope. These outgrowth cells

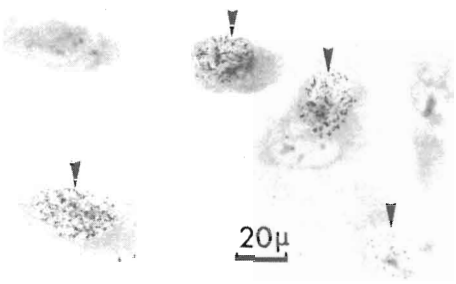

Fig. 8. The outgrowth cells from the pulp explant after 2 days of culture. Some of their nuclei were ^{3}H-TdR-labeled (arrows). These labeled cells might have been located at the perivascular region, then proliferated once in the pulp explant and migrated outward. Giemsa stain.

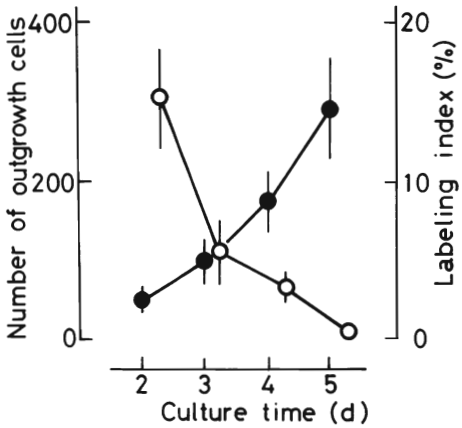

Fig. 9. Number of outgrowth cells (●) and ^{3}H-TdR-labeling index (○). Each point and bar represents the mean±SD (n=6). After the preparation of the pulp explants, the explants were incubated in the ^{3}H-TdR medium and cultured for different periods of time.

had cytoplasmic processes and existed apart from the explant. Fifteen per cent of the outgrowth cells were labeled with ^{3}H-TdR at 2 days (Figs. 8, 9).

After 3 days of culture, mitotic figures of the ^{3}H-TdR-labeled cells were observed among the outgrowth cells. The labeling index, i.e., the ratio of the ^{3}H-TdR-labeled cells to all the outgrowth cells, decreased in contrast with the increase of the outgrowth cells (Fig. 9).

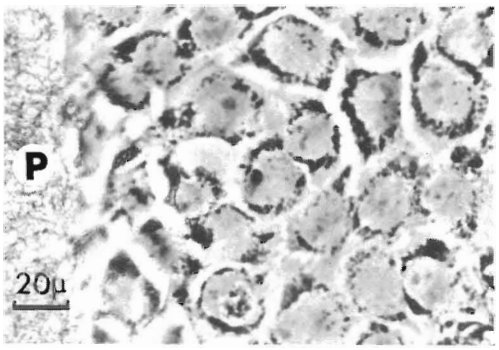

Fig. 10. The outgrowth cells from the pulp explant (P) after 7 days of culture. By phase contrast microscope.

While the cell density increased, the outgrowth cells became rounded with shorter cytoplasmic processes, and the cells adjacent to the explant showed a polygonal form, having many characteristic cytoplasmic granules around the large round nuclei. These cells, which were morphologically similar to the epithelial cells, became more and more predominant as the time of culture progressed. (Fig. 10).

Discussion

The present experiment showed that the necrotic changes of the pulp explant were found at a rather early period. However, others (Szabo [3]; Vidic *et al.* [6]) have demonstrated that the pulp explants remained alive for a long time *in vitro*. The difference between these results might be due to the discrepancy of the culture condition, mainly the lower oxygen concentration in the present experiment. The culture condition for this experiment was the same as is usual for the explant culture (Paul [12]). The difference in the results is thought to be not so important for investigating the origin of the outgrowth cells; the morphological finding of the outgrowth cells in the present study seemed to be identical in structure with the previous report

using the pulp explant of the rat incisor (Zussman and Ioachim [5]).

The possibility of the migration of the odontoblasts has been reported by a similar method used in the present study (Niizima and Cattoni [4]; Zussman and Ioachim [5]), however, the odontoblasts are considered to be static, postmitotic cells (Robins [13]; Chiba *et al.* [14]), and highly differentiated cells which produce a dentine matrix (Weinstock and Leblond [15]). As shown by the histological observations of the present study, the cytoplasmic processes of the odontoblasts were severely injured by the experimental procedure (Fig. 5), and they underwent necrosis. These findings suggest that the odontoblasts colud not migrate out of the pulp explant.

In the *in vitro* experiment, it was ascertained that 85 per cent of the total labeled cells were perivascular cells immediately after the preparation of the pulp explant (Figs. 3, 4).

With regard to the mitotic activity of the rat incisor pulp *in vivo*, most mitoses occurred at the apical region and generally fewer mitoses were found in the incisal and the central part of the pulp (Robins [13]; Chiba [16]). However, from 500 μm to 5000 μm from the apical foramen, mitoses still occurred frequently in all the arterioles, the highest density being observed in the large arterioles in the central part of the pulp and most of them occurring at perivascular cells, especially the "smooth muscle cells" (Moe *et al.* [17]).

In the present study, the ^{3}H-TdR-labeled perivascular cells were found not only in the large arterioles but also in the more peripheral fine arterioles. The smooth muscle cells as indicated by Moe *et al.* [17] were too difficult to be identified in the present study. Therefore, it seems to be reasonable that the ^{3}H-TdR-labeled cells observed in the present study is called "perivascular cells". The results of tracing the ^{3}H-TdR-labeled cells showed that the perivascular cells were involved certainly in the outgrowth cells.

Considering the early necrotic change in the pulp explant, the increase of the outgrowth cells might be caused by the migration of the pulp cells up to 2 or 3 days of culture. After that time the increase of the outgrowth cells might be caused mostly by the proliferation of the outgrowth cells. The total number of cells in the pulp explant in this experiment was estimated to be $1-2\times10^4$ cells. It seems to be possible to produce a larger cell population by a rather small cell population, maybe less than one hundred cells in the pulp explant. It was ascertained by tracing the ^{3}H-TdR-labeled cells that 15 per cent of the outgrowth cells were labeled with ^{3}H-TdR and that the labeling index decreased in contrast with the increase of the outgrowth cells (Fig. 9). It was considered that the decrease of ^{3}H-TdR labeling index with time was mostly due to the proliferation of the labeled cells. Thus, the perivascular cells have a high mitotic potentiality and proliferate actively on the culture dish surface, exhibiting morphologically a uniform feature (Fig. 10).

It has been reported that the undifferentiated mesenchymal cells which have a great capacity for further differentiation exist usually outside the vessel wall in the dental pulp and that they may be the precursor cells of the new odontoblasts (Seltzer and Bender [18]; Sveen and Hawes [19]; Senzaki [20]; Fitzgerald [21]). These undifferentiated mesenchymal cells seemed to be identical with the perivascular cells observed in the present study.

On the other hand, the differentiation

of the human pulpal outgrowth cells into the odontoblast-like cells *in vitro* has been reported (Magloire and Joffre [7]; Magloire *et al.* [22]). Although the differentiation of the outgrowth cells into the odontoblasts was not observed in this study, the histochemical stain reaction of the collagen fiber by Azan-Mallory staining was observed around the outgrowth cells (Kasugai [23]). However, further investigations are necessary to elucidate the function of the outgrowth cells obtained by the present study.

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