

Original

Investigation of Structure of Dentin Formative Disturbances Caused by Antineoplastic Agents

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Abstract: In this research, we aimed to clarify change of old odontoblasts as well as mineralization level of formative disturbances line upon administration of antineoplastics. Male Wistar rats weighing 100 g were given injection of 2 mg/kg of Vinblastine (VBL). 1, 3, 5, 7, and 14 days after the administration, perfusion fixation was performed and upper incisors were removed. Then specimens with and without demineralization were embedded in epoxy resin and were observed by light microscopy, contact microradiography and transmission electron microscopy. At 1 day after the administration, necrosis of many old odontoblasts, abnormality of direction of collagen secretion and plenty of collagen fibers deposition were seen. Detection of this alteration at even 7 days after the administration suggested that disorder of secretion function associated with microtubule abnormality continues for a long period. The collagen secreted after VBL administration did not contribute increase of thickness and density of the predentin collagen formed before the drug injection and was deposited extremely dense along distal end of the cells. Thus the original predentin remained with low collagen density and a wide interfibrillar space. Regarding with mineralization process of formative disturbances line, density of collagen fibers was extremely low with thick needle shaped crystals in hypermineralization layer, and the density was oppositely very high with small crystals in hypomineralization layer. Although irregular dentin formed at medial side of formative disturbances line increases with time, mineralization degree is very low. The reason for this phenomenon was considered not only due to embedment of vessels and cellular particles but also observation of an unmineralized region around thick dentinal tubule, thus resulting in a decrease of general mineralization level.

Key words: Dentin, Formative disturbance, Vinblastine, Rat incisor

Introduction

Disturbance of matrix formation in the developing tooth during administration of chemotherapy for childhood cancer has already been known¹⁻³⁾. Especially formative disturbances line of the dentin is clearly seen and abnormality of mineralization has been reported. However, fine structure of this abnormality has been remained unknown. There are several reports for effect of antineoplastic agents on dentin formation. In one of these reports, vinblastin has been mentioned to deteriorate dentin formation through abnormal secretion of collagen from young odontoblasts due to change of their function during early phase of dentin formation⁴⁾ and through change of direction of collagen secretion⁵⁻⁷⁾.

However, there has been no research for alteration of old odontoblasts⁴⁾ and expression of associated abnormality of dentin formation. In this research, we aimed to clarify change of old odontoblasts and fine structure of dentin formation as well as mineralization state associated with this alteration upon administration of antineoplastic agent.

Materials and Methods

In this experiment, young male Wistar rats weighing 100 g were given subcutaneous injection of 0.2 mg of vinblastine (VBL) (Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in 1.0 ml of a saline solution. Normal saline was administered to control animals. At 1, 3, 5, 7 and 14 days after administration, the animals were anesthetized with ketamine-hydrochloride (Ketalar, Park-Daves and Sankyo Ltd., Tokyo, Japan) and fixed by perfusion

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for 20 minutes with a mixture of 2.5 % glutaraldehyde and 2.0 % paraformaldehyde. The upper incisors were removed from the animals and divided into two groups.

In the first group, the incisors were demineralized in a 10 % EDTA solution for 4 weeks at 4 °C. The incisors were postfixed for 90 minutes with a 1 % osmium tetroxide solution, dehydrated in graded ethanol, and embedded in epoxy resin (Epok 812, Oken Shoji Co., Tokyo, Japan). The sections 2µm in thickness were cut with a microtome and were stained with toluidine blue.

The incisors of the second group were not demineralized and embedded in polyester resin (Rigolac, Oken Shoji Co., Tokyo, Japan). Ground sections (about 60µm thick) were prepared, and contact microradiograms (CMR) were obtained with a soft X-ray unit (CMR-3, Softex Co., Tokyo). Ultrathin sections were cut with an ultramicrotome (LKB Type IV, LKB, Bromma, Sweden) equipped with a glass or a diamond knife and were observed in a transmission electron microscope (H7000, Hitachi Co., Tokyo, Japan) after either double staining with uranyl acetate (U) and lead citrate (Pb). Undemineralized sections were observed in a transmission electron microscope without staining. In some cases, however, undemineralized sections were stained with uranyl acetate that had been dissolved in absolute ethanol (alcoholic U) to minimize its demineralizing effects.

About 250 ~ 350 µm central part of incisor dentin was observed and observation area was moved into direction of the free edge when growth of incisor continues. All animal studies were performed in conformity to the guidelines for animal experiments of Tokyo Dental College.

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Results

1. Control group

Light microscopic findings: Multilayer arrangement of high columnar shape odontoblasts is observed. Nuclei of odontoblasts deviate to mesial side and Golgi field in center of the cell body is clearly seen. Many capillaries enter into area between odontoblasts near vicinity of distal side of the cells. Width of the predentin is 20~30 µm and it is almost unstained. Calcospherite at the dentin mineralization front and dentinal tubules in the dentin are clearly observed (Fig. 1).

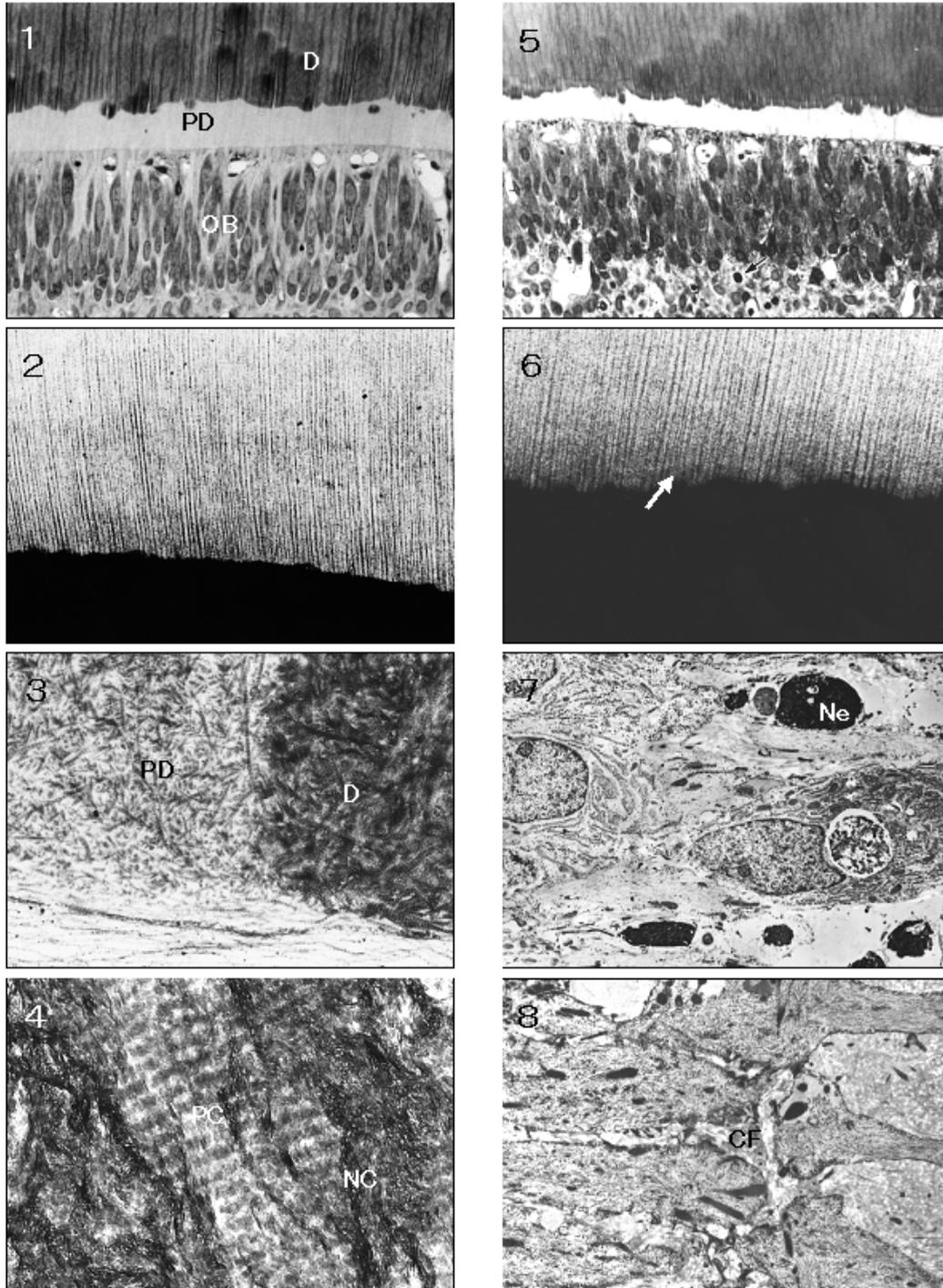
CMR findings: Almost homogeneous mineralization of the dentin is shown. Dentinal tubules are clearly observed as a black vertical line (Fig. 2).

Electron microscopic findings: Plenty of cellular organelles such as rough endoplasmic reticulum, Golgi apparatus and mitochondria are seen. A clear area at distal end of the cells without organelles is extending. Abundant amount of amorphous substance without collagen fibers exists in predentin that is approaching to distal end of the odontoblasts. However, amount and density of collagen fibers increase in direction of mineralization front (Fig. 3). Interfibrillar space of the dentin becomes dense and is covered by amorphous substance (Fig. 3). When undemineralized section is observed, plate-like crystals are seen along the collagen fibrous period and thick needle-like crystals are deposited between fibers (Fig. 4).

2. One day after VBL administration

Light microscopic findings: Length of odontoblasts becomes shorter and Golgi field disappears. Spheroid shape cell necrotic particles darkly stained with toluidine blue in the cell inside and intercellular area are observed especially between pulpal cells near mesial side of the odontoblasts (Fig. 5). Width of predentin is narrow (10~20 µm) as compared to the control group. A lightly stained layer (4 µm width) with toluidine blue appears at the dentin mineralization front (Fig. 5).

CMR findings: Although a low mineralization area is noticed at



Figs. 1-4. Controls

Fig. 1. It shows old odontoblasts (OB) and dentin (D). PD: Predentin. x 230

Fig. 2. Homogenous mineralization of dentin and clear appearance of dentinal tubule are observed by CMR image. x 460

Fig.3. Wide interfibrillar space in predentin (PD) collagen and dense interfibrillar space in dentin (D) covered by high electron density material are observed. x 8 000

Fig.4. In undemineralized sections, plate-like crystals (PC) are deposited along circular structure of collagen fibers and thick needle-like crystals (NC) at interfibrillar space are seen. x 56 000

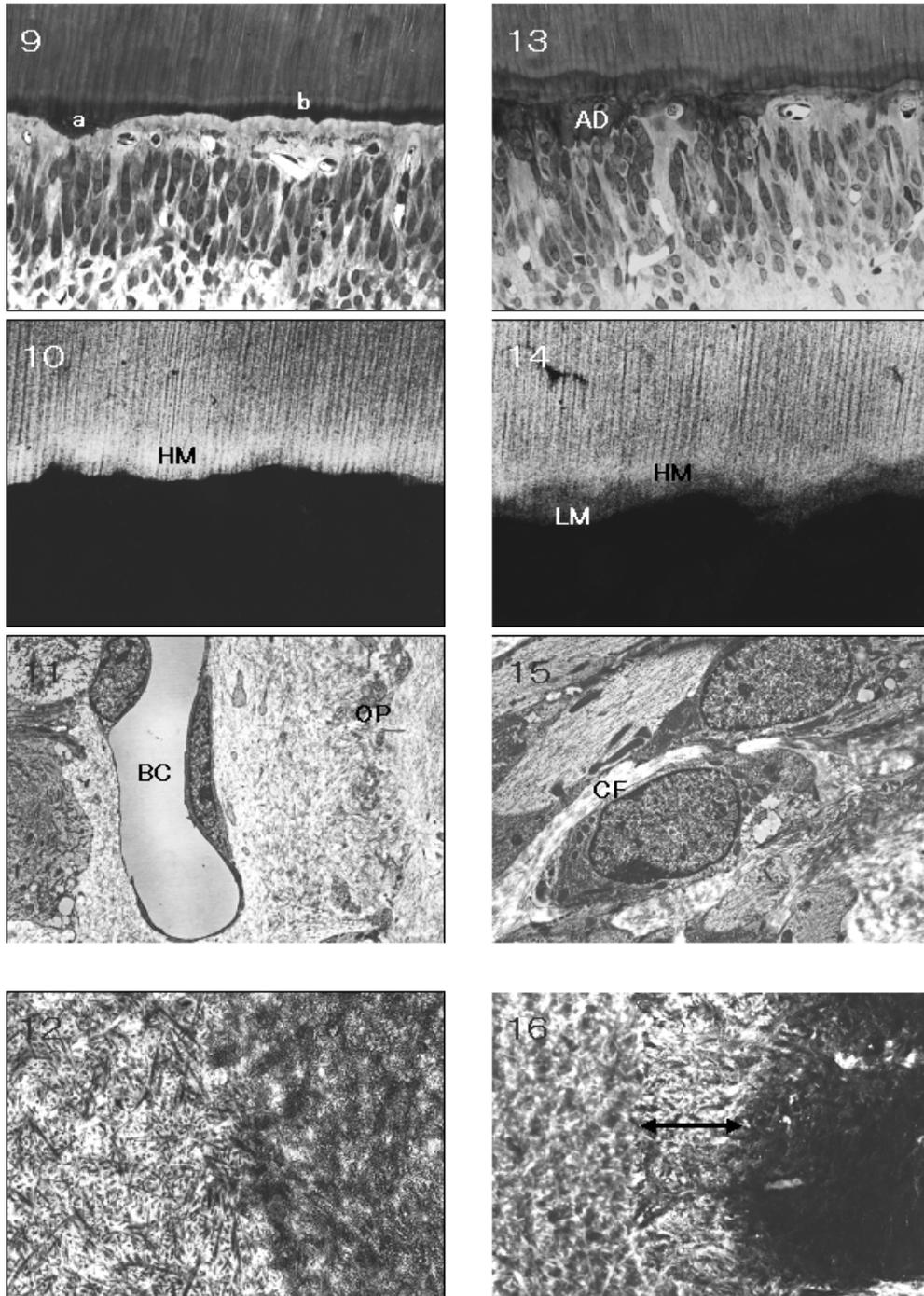
Figs. 5-8. Example of 1 day after VBL administration.

Fig. 5. Odontoblasts layer and darkly stained material (arrow) by toluidine blue in the pulp appear. Width of predentin decreases. x 230

Fig. 6. A layer with hypomineralization level at mineralization front (arrow) is narrowly formed. x 460

Fig. 7. Necrotic material (Ne) in odontoblasts inside and intercellular space is observed. x 2 800

Fig. 8. Collagen fibers (CF) are seen at intercellular space of cell distal end. x 4 200



Figs. 9-12

Example of 3 days after VBL administration

Fig. 9. Lightly (a) and darkly (b) stained layers by toluidine blue at mineralization front are seen. x 230

Fig. 10. Hypermineralization layer (HM) at mineralization front is seen in CMR findings. x 460

Fig. 11. Embedment of odontoblasts processes (OP) and vessel (BC) in predentin are observed. x 2 800

Fig. 12. There is no big difference between structure of mineralization front neighborhood and corresponding controls. x 16 700

Figs.13-16

Example of 5 days after VBL administration

Fig. 13. Plenty of collagen fibers at intercellular space and irregular dentin (AR) at mineralization front part are formed. x 230

Fig. 14. Hypomineralization layer (LM) at medial side of hypermineralization layer (HM) are seen in CMR image. x 460

Fig. 15. A lot of collagen fibers (CF) are deposited at intercellular space. x 4 200

Fig. 16. Region with low crystal density (arrow) at mineralization front in undemineralized section are observed. x 8 000

the dentin mineralization front, it is extremely unclear and small. But homogenous mineralization of dentin is observed at lateral side of this area (Fig. 6).

Electron microscopic findings: Plenty of necrotic material in the odontoblasts as well as at intercellular space is seen. Nuclei move to central area and disorder arrangement of organelles is observed (Fig. 7). The cell morphology is slightly irregular and deposition of collagen fibers is seen at intercellular space (Fig. 7). A lot of collagen fibers are also deposited in vicinity of intercellular space of the odontoblasts (Fig. 8). In the predentin, thickness and density of collagen fibers increase towards mineralization front and mineralization occurs by crystal deposition at the dentin. The crystal is thin and small with a needle-like structure and is almost same with the control group.

3. Three days after VBL administration

Light microscopic findings: Although length of odontoblasts is uneven and short as compared with the control group, Golgi field at cell body is recovered and is clearly seen. Predentin is narrow and irregular with a width of 10~20 μm at distal end of the odontoblasts, and cellular particles as well as embedment of vessels are noticed. Dentin mineralization front is darkly stained with toluidine blue. A layer lightly stained with toluidine blue is seen at lateral side of this line (Fig. 9).

CMR findings: A hypermineralization layer is seen in the dentin mineralization front, and the dentinal tubule of this area is unclear (Fig. 10).

Electron microscopic findings: Shape of odontoblasts becomes further irregular, and plenty amount of collagen fibers forms especially at intercellular space, resulting in applanation of the cells. This collagen bundle of the intercellular space and collagen fibers of the predentin are united. Amorphous substance and thin collagen fibers observed in the control group are not seen in this area. Cellular particles possibly belong to odontoblast processes and capillaries are abundantly embedded in the predentin (Fig. 11). At the mineralization front, density of collagen fibers is slightly low as compared to the control group and it is covered by a material with high electron density (Fig. 12). When undemineralized section is observed, crystal deposition occurs not only at the mineralization front but also at intercellular space from collagen fibers. Individual crystal is thin and small with a needle-like shape, and shows almost same morphology with the control group.

4. Five days after VBL administration

Light microscopic findings: Although individual odontoblasts appear to be almost normal, cellular arrangement is broken and formation of irregular predentin at distal end as well as at intercellular space is seen. The part of the irregular dentin stained dark irregularly with toluidine blue and embedment of vessel as well as cellular particles are observed. This irregular dentin

continues with the mineralization front which showed the dark staining in the previous group. The layer lightly stained with toluidine blue is seen at lateral side of this area (Fig. 13).

CMR findings: The mineralization front is irregular with a shape of wave, and shows slightly lower level of mineralization as compared to original dentin (Fig. 14). The hypermineralization layer is seen in the lateral side of the hypomineralization layer.

Electron microscopic findings: Although most odontoblasts show almost normal morphology, a lot of collagen fibers form at the distal end of the cells or intercellular space, and embedment of the cells into the collagen bundles is also seen in some part. In this part, the collagen fibers are thick and dense (Fig. 15). And predentin is united with intercellular collagen fibers and cannot be differentiated. In irregular dentin area, density of collagen fibers is considerably different in various parts. A relatively high density collagen bundle is seen near odontoblasts, but is lower than this in lateral sides. When undemineralized section is observed, crystal at the mineralization front is thin and small with needle shape, and its density is low as compared to the control group. At the lateral side of this area, dense crystal deposition as compared with the control group is observed (Fig. 16).

5. 7 days after VBL administration

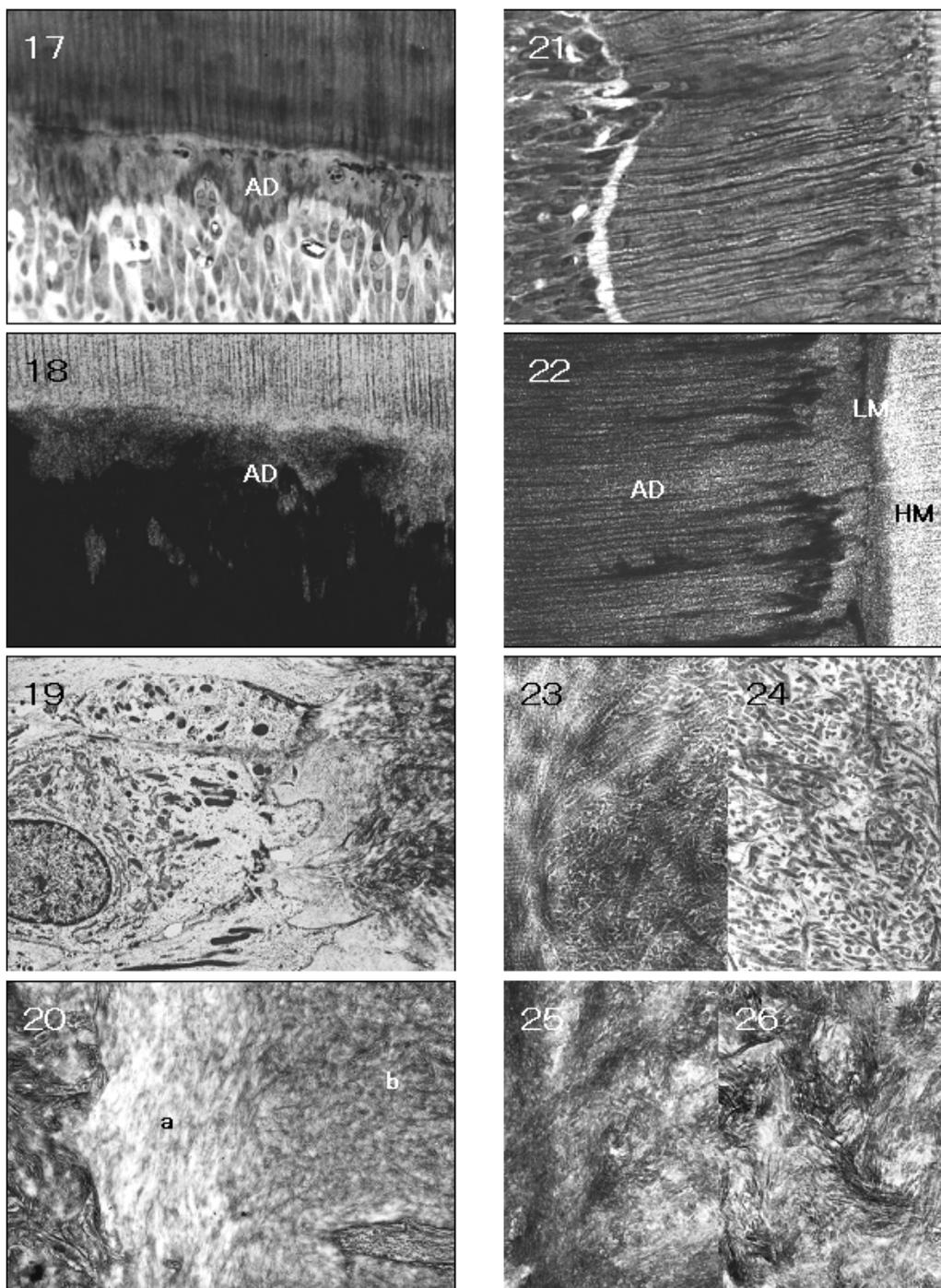
Light microscopic findings: Odontoblasts are almost normal and capillary invasion at intercellular space are seen. However predentin of irregular dentin is almost not formed, and is not more than a layer with an approximate width of 5 μm . Structure of mineralization front as well as staining is extremely irregular, and dentinal tubules arrays are unclear in irregular dentin which is formed after 3 days administration (Fig. 17).

CMR findings: A small amount of island or stick like mineralization part is seen near odontoblasts in irregular dentin. At the mineralization front, although a peninsula like mineralization area continued with the hypermineralization layer is formed, mineralization density is very low as compared with the normal dentin (Fig. 18).

Electron microscopic findings: Abnormal odontoblasts are seen in some part, and width of predentin is extremely narrow with little amount of amorphous substance at the distal end of the odontoblasts (Fig. 19). At lateral vicinity of irregular dentin, many remnants of cellular processes are observed, and collagen fibers are highly thick and dense at this area. However, at lateral side from this part, collagen density is low and mesh like arrangement of the fibers with space formation between collagen fibers is noticed (Fig. 20).

6. 14 days after VBL administration

Light microscopic findings: Although odontoblasts almost seem to be normal, width of predentin of irregular dentin is narrow and it is not detected in some parts. Although mineralization front



Figs. 17-20

Example of 7 days after VBL administration

Fig. 17. Width of irregular dentin (AD) increases. x 230

Fig. 18. Irregular dentin (AD) shows hypomineralization level in CMR image. x 460

Fig. 19. Morphological abnormality of odontoblasts is seen and width of predentin is also narrow. x 2 300

Fig. 20. Areas with high (a) and low collagen fiber density in lateral side of irregular predentin are seen. x 5 000

Figs. 21-26

Example of 14 days after VBL administration

Fig. 21. Thick irregular dentin is formed. x 230

Fig. 22. High (HM) and low (LM) calcification layers and irregular dentin (AD) with hypomineralization level at the medial side are observed in CMR image. x460

Fig. 23. Enlargement image of collagen fiber with high density is shown. x 15 300

Fig. 24. Enlargement image of collagen fiber with low density is shown. x 15 300

Fig. 25. Undemineralized section of collagen fiber with high density is shown. x 80 000

Fig. 26. Undemineralized section of collagen fiber with low density is shown. x 80 000

shows unevenness, arrangement of dentinal tubule and dentin structure is relatively normal. Structure of irregular dentin becomes abnormal through lateral direction, and around abnormal formation line, disappearance of dentinal tubule and embedment of vessels are observed. The layers darkly and lightly stained with toluidine blue are unclear (Fig. 21).

CMR findings: Prominent radiolucency of irregular dentin by x-ray image is observed due to low density of mineralization, embedment of vessel and cells as well as existence of unmineralized matrix. Formative disturbances line is formed by hypermineralization layer and hypomineralization layer near irregular dentin (Fig. 22).

Electron microscopic findings: Although old odontoblasts are almost same with those in the control group, and predentin width is narrow. Though dentin approaching odontoblasts shows relatively normal structure, density of collagen fibers are extremely high at formative disturbances line, and both of close attachment of collagen fibers (Fig. 23) as well as extending of interfibrillar space (Fig. 24) due to their low density are observed. When undemineralized section is observed, crystal amount is low in the area with dense collagen fibers (Fig. 25), and is high in the area with low density of collagen fibers (Fig. 26). When crystal of this area was enlarged, small plate-like or needle-like crystals were numerous seen in the area with high fiber density. On the other hand, thick, long needle-like crystals were observed in the area with low fiber density.

Discussion

1. Change of old odontoblasts

Although several studies have been reported for morphological alteration of odontoblasts after administration of antineoplastic agents, almost all is at light microscopic level⁸⁻¹³⁾, and researches for observation of ultrastructural change upon VBL administration are limited to few works⁵⁻⁷⁾. VBL has been known to be a microtubule inhibitor. It disturbs directivity of secretory granule, or break microtubule structure in ameloblasts¹⁴⁾. VBL has also been reported to inhibit collagen secretion in osteoblasts¹⁵⁻¹⁶⁾. In researches for young odontoblasts so far, it has been reported that microtubule inhibition resulted in abnormality for secretory direction of collagen matrix⁵⁻⁷⁾.

In the current research for old odontoblasts, it has also been shown that plenty of collagen fibers have been deposited at intercellular space due to abnormality of collagen secretory direction and necrosis of many cellular organelles even one day after administration of the drug. Since this kind of alteration has been seen at 7 days after administration of VBL, it has been suggested that disorder of secretory function associated with microtubule change occurred for long term. And morphological alterations are thought to occur due to necrotic decrease of organelles as well as effect of collagen deposition at intercellular space. Morphological change of most cells recovered at 14 days

after administration of the agent. However, width of the predentin is narrow and dentin mineralization level is low as compared to the control, recovery of cellular function was considered not to be enough.

2. Formative disturbances line

In current observation, we classified the dentin formed until 3 days after VBL administration as formative disturbances line and the dentin embedded with vessels and cellular particles after 3 days of VBL administration as irregular dentin. In this period, arrangement of collagen fibers was mainly observed.

Formation process of the formative disturbances line is divided into secretion of organic matrix and its mineralization stages. Since predentin width is extremely narrow one day after the administration, collagen secretion was considered to be prominently inhibited, which then resulted in decrease of secretion of organic matrix. And because a lightly stained layer by toluidine blue treatment appeared, it has been suggested that secretion of non-collagenous proteins has also been influenced. However, in current research identification of non-collagenous proteins was out of our purpose, whether any abnormality has been occurred remained unknown.

At 3 days after the administration, an irregular predentin has been formed over the original predentin. This formation has been thought to occur due to alteration of collagen secretion amount as well as direction because of defective function of microtubules regenerated in odontoblasts. It has been known that formation of collagen fibers by odontoblasts occurs at predentin. An important and interesting point is that collagen secreted after VBL administration does not contribute thickness and density of already formed predentin before the drug administration, and deposits along distal side of the cells. Thus density of collagen fibers of original predentin is low and it remains with structure of wide interfibrillar space. This newly deposited collagen fibrils density is very high and a matrix almost with no interfibrillar space has been formed. Formation of such a matrix with high density suggests that abnormality of non-collagenous proteins is likely to occur. In previous researches regarding with young odontoblasts, although unusual appearance of collagen aggregates has been reported⁵⁻⁷⁾, unusual collagen aggregates were not observed in old odontoblasts part. And appearance of unusual collagen aggregates is suggested to be related with over-secretion of non-collagenous proteins¹⁷⁾. Since non-collagenous proteins almost don't appear after the administration, and although collagen synthesis in old odontoblasts recovers early, recovery of non-collagenous proteins delays and progress of only collagen synthesis occurs, resulting in dense fibril formation.

Regarding with mineralization process, a clear hypermineralization layer appears at mineralization front part at 3 days after the administration, and a hypomineralization layer appears at medial side of this area after 5 days. When these layers

were compared with the corresponding demineralized sections, density of collagen fibers was extremely low in hypermineralization layer and thick, needle shape crystal deposition was shown. On the other hand, in hypomineralization layer, density of collagen fibers was high and small crystal deposition was observed. This result suggested that although enough growth of dentin crystal occurred in the area with low collagen density, space for crystal growth cannot be secured in the area with high collagen density. Therefore mineralization of formative disturbances line was suggested to be oppositely proportional with collagen amount of the matrix. And because crystal size observed in hypermineralization layer was almost same with crystal size of normal dentin, hypermineralization layer was thought to be formed through increase of crystal density.

Formation of irregular dentin begins at medial side of the formative disturbances line after 5 days administration of the VBL. Formation of this irregular dentin progresses with time and its structure becomes close to original dentin. Although this phenomenon happens through either recovery of cellular function or formation by newly differentiated odontoblasts, mineralization was shown to be very low by CMR observation. As a cause for this situation, not only embedment of vessels and cellular particles in irregular dentin but also thickness of dentinal tubule with circumferential unmineralized region was observed. This is supposed to be reason for decrease of general mineralization level.

Conclusion

In this research, change of old odontoblasts and alteration of microstructure as well as mineralization level of formative disturbances line were observed after administration of antineoplastic agents. At 1 day after the administration, necrosis of many cells was observed, abnormality of direction of collagen secretion occurred and this resulted in deposition of many collagen fibers at intercellular space. And also secretion inhibition of non-collagenous proteins was observed. Collagen secreted after VBL administration densely deposited along distal end of the cells. However, the predentin part before the administration remained with low collagen fiber density and wide interfibrillar space. While collagen fiber density was extremely low at hypermineralization layer of formative disturbances line and crystals deposited there were thick and needle shaped, density of collagen fibers was high at hypomineralization layer of formative disturbances line and crystals deposited there were small. Formation of irregular dentin with low mineralization degree medial to the formative disturbances line was observed, and thick dentinal tubule with embedment of vessels and cellular particles in this region as well as circumferential unmineralized area were shown.

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