Compositional and morphological imaging of CO_2 laser irradiated human teeth by low vacuum SEM, confocal laser scanning microscopy and atomic force microscopy

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Enamel and dentin of human teeth irradiated by CO_2 laser were investigated by confocal laser scanning microscopy (CLSM), low vacuum scanning electron microscopy (WET-SEM) and atomic force microscopy (AFM). Optical tomographic imaging by CLSM, compositional imaging based on atomic number effect of reflected electrons by WET-SEM, high resolution observation of surface morphology by AFM were done for both the irradiated and non-irradiated area of the same specimen throughout. The crystals of about 50 μm length and the bright spots were observed by CLSM at the bottom of the cavity induced by laser irradiation. They turned out from the observation by WET-SEM as the acicular crystals with the cross section of an irregularly hexagonal shape situated parallel and perpendicular, respectively, to the inner surface of the cavity. The thickness of the thermally deteriorated zone of the cavity was about 25 μm. The crystals unidirectionally grown up to the size of several hundreds nm were also observed by AFM, while the apatite crystallites of 50–150 nm were recognized all over in non-irradiated area. All the results suggest that after instantaneous melting at the surface of teeth by CO_2 laser shot the crystals of calcium phosphate were recrystalized and grown to a large size. The compositional imaging in addition to morphological observation was useful to obtain the information of the change in materials induced by laser irradiation.

1. Introduction

Laser has been used for various purposes in dentistry [1]. So-called soft lasers with athermal low energy are used to help healing, reduce inflammation and pain, and relieve hypersensitivity [2]. Hard lasers are used for surgical applications such as vaporization, roughening tooth surface and cavity formation. They are effective in clinics when used in an appropriate manner and operation can be done quickly without pain. In other times they result in little effect. The mechanism of laser irradiation effect is often unknown.

In the investigation of such phenomena with biological specimens, however, there is always problem of individual difference. The results and conclusions obtained by different observation methods lead sometimes to ambiguity whether they are of intrinsic nature or they originate from individual difference. The use of the same specimen would be much advantageous to obtain the clear conclusions.

The recently developed new microscopes including the confocal laser scanning microscope (CLSM) [3, 4], low vacuum scanning electron microscope (WET-SEM) [5, 6], atomic force microscope (AFM) [7–9] are all the scanning type of microscopes which are suitable for the control and data process by computer. They have the functions different from the conventional microscopes such as non-contact analysis of surface roughness, light tomography of internal structure, direct observation of the wet or raw specimen without pre-treatment and quantitative analysis of data.

In this study three different imaging methods: CLSM, WET-SEM, AFM were applied intensively on the same spot of the same specimen throughout to investigate the effect of CO_2 laser irradiation on enamel and dentin. The morphological and compositional informations derived by these methods were used complementarily and comparatively for analysis.

Low vacuum SEM is called in various ways, for example environmental SEM, natural SEM and wet SEM. In this paper the word WET-SEM was used for brevity.

2. Experimental procedure

Vertical cross section in buccolingual direction and horizontal one perpendicular to tooth axis were prepared

from human lower molars conserved in air after extraction by mechanical cut and polishing with diamond paste. Opelaser-3(Yoshida Seisakusyo) was used for CO_2 laser irradiation experiment. One pulse irradiation was done onto enamel and dentin with the power 0.5–6.1 W and the pulse duration time 0.05–1 sec.

Observation was carried out using CLSM (Bio-Rad MRC500J/600 and Lasertec 1LM21), WET-SEM (Topcon ABT-32WT) and AFM (Topometrix TMX-2000 Explorer) for both the irradiated and non-irradiated area. No pre-treatment for observation was done on specimens and the same spot of the same specimen was used commonly for the observation by three different microscopes.

3. Results

Fig. 1 is the vertical cross section of human lower molar tooth before CO_2 laser irradiation. They are the optically sectioned tomographic images using autofluorescence light by CLSM. Fig. 1(a) is the image in focus just on surface and (b) is the sectioned image at 20 μm below surface. In Fig. 1(a) the fissure and dentinoenamel junction are clearly seen. In the lower part nothing is seen because the tooth is concave to the vertical direction where there was caries. Because of the scars made by mechanical polish the intrinsic structure of enamel and dentin is not well recognized. In the sectioned image underneath the surface (Fig. 1(b)), the scars are no more observed and the growth line of enamel in a ring manner and the dentinal tubules extending from dental pulp towards dentinoenamel junction are observed. The fissure and dentinoenamel junction show the bright contrast. The concave part in the lower which was not seen in Fig. 1(a) is now observed at 20 μm below the surface.

The vertical cross section of molar imaged by reflection electrons in WET-SEM is shown in Fig. 2 after the lower half was cut off. The image shows the compositional contrast where enamel is brighter than dentin. The cavities A–D were formed by one pulse irradiation of laser. For A and B the laser power 6.1 W was applied for the duration of 0.5 s. Likely the laser power 2.1 W for 0.5 s and 1.1 W for 0.2 s were applied respectively for C and D. The contrast of the laser irradiated areas in dentin (B, C, D) where the cavity was formed is similar to that of enamel. The cracks induced by thermal shock are also clearly seen in the cavity. The laser irradiated area in enamel (A) has indifferent contrast from non-irradiated enamel. The scars in enamel and amelodentinal junction observed by optical microscope in Fig. 1 are invisible by SEM.

Fig. 3 is the cross section image of the cavity of Fig. 2B observed by CLSM using the fluorescent light. The cavity is recognized with the inner surface composed of the zone of the thickness about 25 μm which looks gray with the brighter contrast. The cavity has the semicircular pan bottom shape. The diameter and depth of the concave cavity are estimated as 550 μm and 200 μm, respectively, as indicated by white and black arrows. The extended diameter influenced by laser irradiation is about 1 mm. In the surrounding area some distance apart from the cavity the autofluorescence light was strongly emitted, which look white in the Fig. 3.

Fig. 4 is the reflection electron image of the surrounding area of the cavity induced in dentin by laser irradiation. The dentinal tubules are observed as

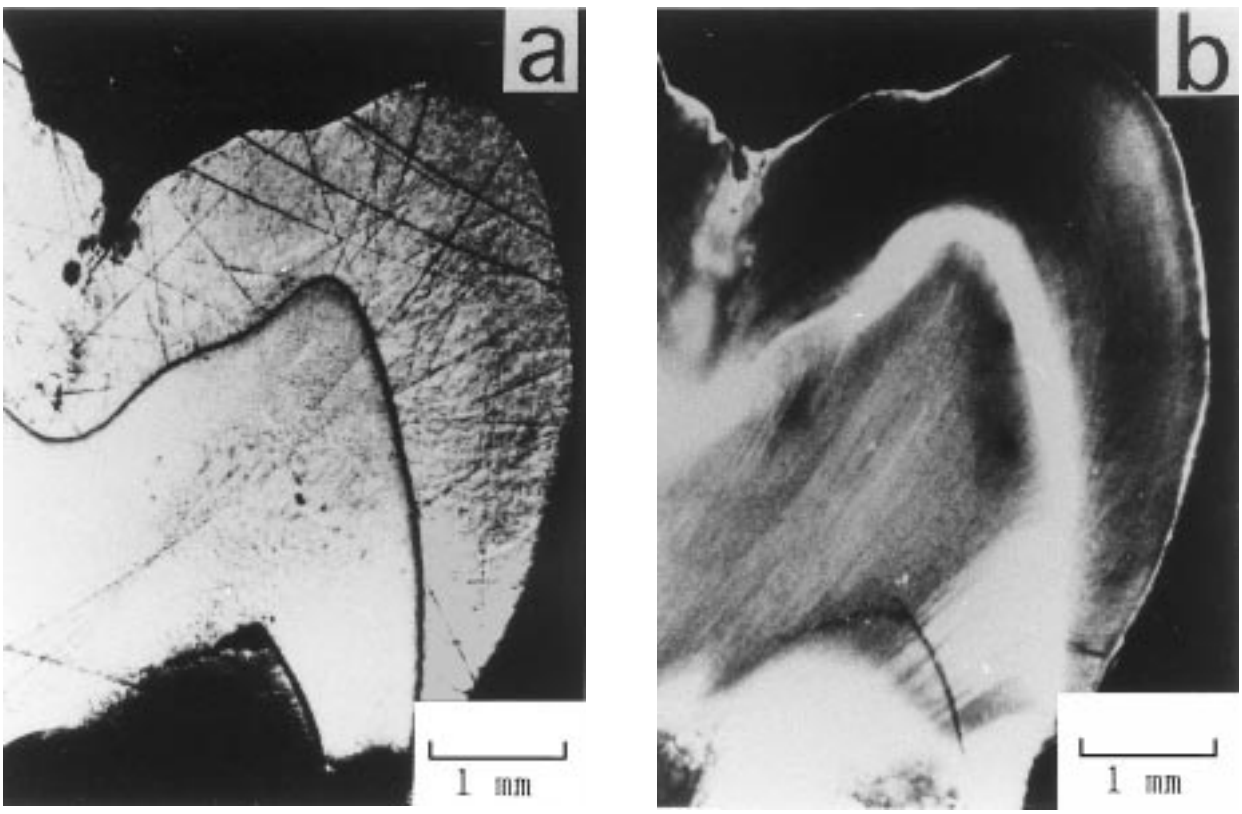

Figure 1 Optically sliced images of the vertical cross section of human molar formed by CLSM using an autofluorescence light. (a) in focus just on surface, (b) sectioned image at 20 μm below surface.

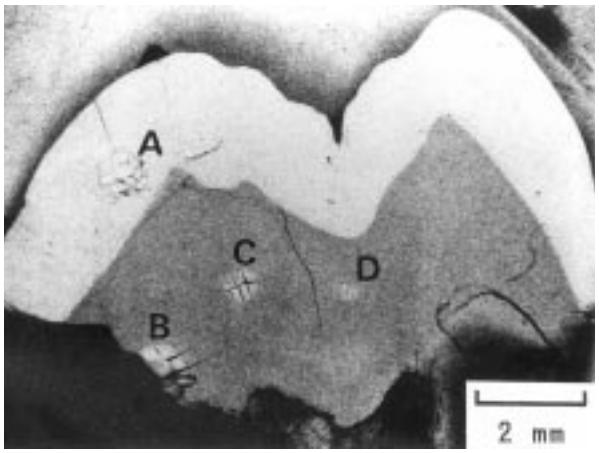

Figure 2 Reflection electron image of the vertical cross section of human molar by WET-SEM. Note the contrast of the laser irradiated area B,C,D in dentin.

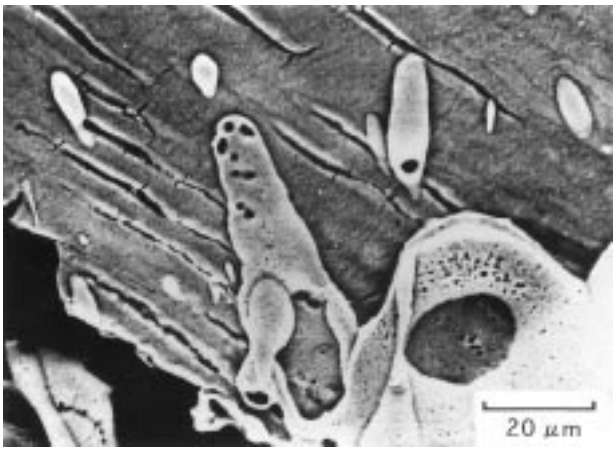

Figure 4 The reflection electron image of the splashed drops in the surrounding area of the cavity in dentin observed by WET-SEM.

more or less straight grooves aligned parallel each other. All of them have approximately the same length, about 50 μm, which means that the direction of dentinal tubules are slightly inclined to the surface. Both sides of dentinal tubules look white compared to gray in the intertubular dentin. The drops were observed with the form splashed from molten material and condensed on the surface of dentin. The direction of the splashed form suggests they flew out isotropically from the center towards the circumference. Some drops have pores inside. The wettability of these drops with the dentin surface is not so good since the contact angle of peripheral parts of drops looks large with the tendency to form the spherical shape. The contrast of these drops is all white compared to dentin surface.

Fig. 5 shows the thickness measurement of the drops splashed around cavity in dentin. Fig. 5(a–d) are the optically sliced images taken by CLSM using an autofluorescence light. They are the images at surface (a) and 4 μm (b), 8 μm (c), 16 μm (d) below surface, respectively. As the sectioned images are observed in series from surface to the inside, the drops on the surface disappeared at a certain depth (Fig. (5d)) underneath the surface. From the disappearing depth the thickness of the drops was estimated as about 10 μm.

Fig. 6 is the SEM image of the cross section of the inner surface of the cavity in dentin, which is identical with Fig. 2B and also Fig. 3. In lower right of figure one can see the normal morphology of unirradiated dentin with the openings of dentinal tubules. The cross section of inner surface in the center of the figure is composed of the zone of the thickness about 15 μm with white contrast where the shape of dentinal tubules are thermally deteriorated or disappeared. This is changed to the zone with the a little gray contrast of about 10 μm width including the deteriorated dentinal tubules and many fine pores inside. It then continues to the normal dentin area. In the upper-left area of the figure many dots of the size a few microns are observed together with pores.

Fig. 7 is the bottom surface of cavity degenerated by CO_2 laser irradiation in dentin observed by CLSM. The cavity is the same as Fig. 2B and Fig. 3. The image is formed using reflection light. The acicular crystals with about 50 μm in length were observed. There are also numerous bright spots. Identification of these spots is practically difficult with the resolution of CLSM using a light.

Fig. 8 is the SEM image of the bottom in the cavity of Fig. 7. The enlarged image by SEM with higher resolution shows clearly the part of acicular crystals in lower right. The width was about 3 μm. The spots observed by CLSM with poorer resolution in Fig. 7 were proved to be the cross section of acicular crystals with the irregularly hexagonal shape.

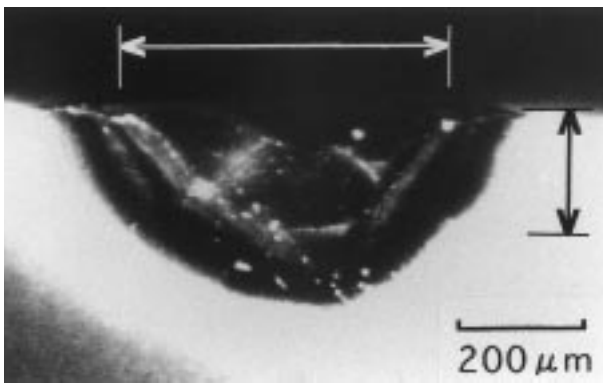

Figure 3 Cross section image of the cavity of Fig. 2B observed by CLSM using an autofluorescence light. Arrow indicates the size of the cavity.

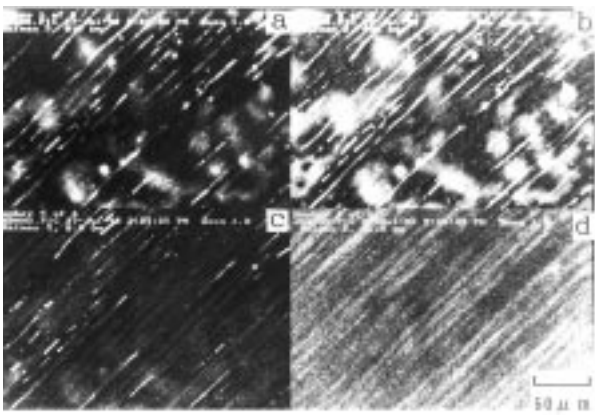

Figure 5 The thickness measurement of the splashes of Fig. 4 by CLSM. Sliced images at surface (a), 4 μm (b), 8 μm (c), 16 μm below surface (d), respectively.

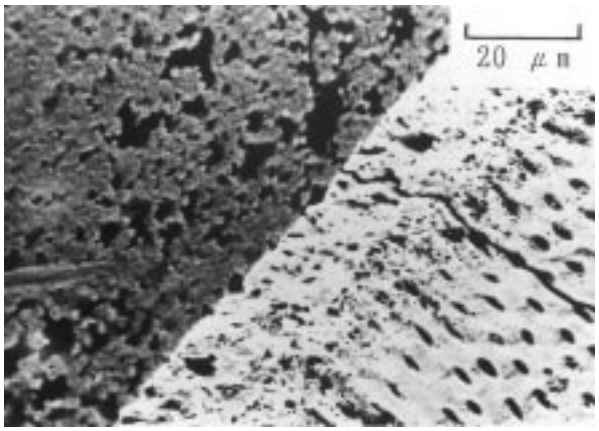

Figure 6 Cross section of cavity of Fig. 2B observed by SEM. Note the white contrast of the thermally deteriorated zone with the thickness about 15 μm at the inner surface.

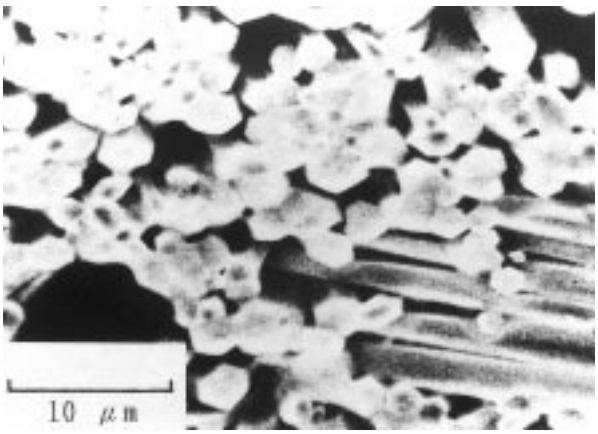

Figure 8 WET-SEM image of the inner surface at the bottom of the cavity of Fig. 2B.

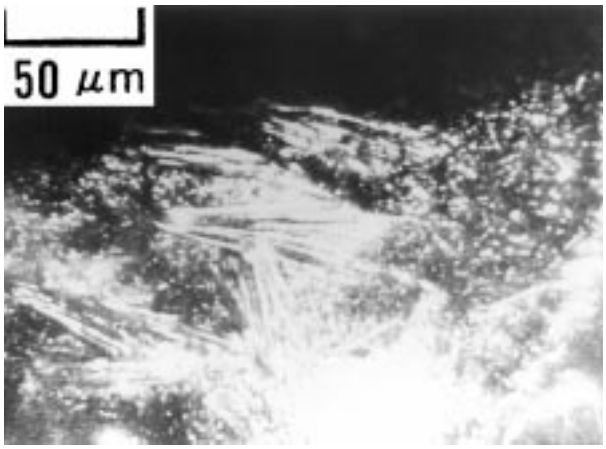

Figure 7 CLSM image of the inside of the cavity of Fig. 2B degenerated by CO_2 laser irradiation in dentin.

Fig. 9 is the AFM image of the non-irradiated enamel(a) and the bottom part of the cavity at the irradiated spot of Fig. 2B in dentin (b). In Fig. 9(a) the structure of about 50–150 nm is observed all over the specimen. Unirradiated dentin area was observed nearly in the same manner. Inside the cavity (Fig. 9(b)) the observed structure has much larger size. The crystals are grown up to the size of several hundreds nm. In some parts the regular shape of Fig. 9(b) was observed. It looks the same kind of morphology as an acicular shape observed by CLSM in Fig. 7 and by SEM in Fig. 8. The scale is, however, much smaller, of the order 1 μm or less. In other parts the structure of the irregular polygonal shape of the size 300–500 μm was observed all over. Some of them were in the irregularly hexagonal shape.

4. Discussion

4.1. Imaging with different microscopes

In the investigation of biological specimens, individual difference from specimen to specimen is often too large to be neglected. To solve this problem it is usual to use certain numbers of specimens and make statistical analysis. In this sense the use of the same spot of the same specimen throughout observation leads to the much simpler analysis and unambiguous conclusions. In this study three different imaging methods were applied. To keep using the same specimen it is inevitable to make no modification on the surface of specimens.

This is usually concerned to the observation by SEM, since it is necessary to make metal coating for non-conductive specimens. Under the low vacuum of $10^{-2} \sim 10$ torr electrocharging effect on non-conductive materials vanishes. This is due to the neutralization effect by the positively charged ions of residual gas with negative charge of electrons at specimen surface. WET-SEM operated in low vacuum can thus observe the water contained specimen without pre-treatment of fixing, dehydration and coating. This is suitable for the purpose of the present study. Since WET-SEM uses reflected electrons for imaging instead of secondary electrons to avoid the discharging effect and the reflectivity of injected electrons depends on atomic number of targets, the contrast gives rise to compositional image. This is another merit to give us the useful informations. The resolution of WET-SEM is typically the order of 10 nm.

Although the resolution is limited to the order of sub-micron due to the wave length of light, CLSM is the only one that can observe the inner structure of the three microscopes used in this study. The most significant feature is the observation of optically sectioned image of the inside structure of transparent or semi-transparent materials from the relatively wide area in several tens μm to mm range (Fig. 1, Fig. 5). It can also make imaging by selecting the lights with different wave length such as reflected light (Fig. 7) and fluorescent light (Fig. 1, Fig. 3, Fig. 5) collected simultaneously using multi channel detectors. In this study the intrinsic structure below surface of teeth was revealed in Fig. 1 using an autofluorescence light. In Fig. 5 the thickness of the drops splashed on dentin surface was estimated from the change of the sliced images from surface to underneath. For non-transparent specimens CLSM can provide the analysis of surface roughness by non-contact method with the resolution of larger than submicron. The depth profile of the cavity could be obtained with reflection light by this method.

AFM is suitable to show the features of morphology with high resolution and to make quantitative evaluation of surface roughness of the nm order in the relatively smaller area, a few tens nm to μm. AFM has the feature insensitive to atmosphere. It is possible to make high

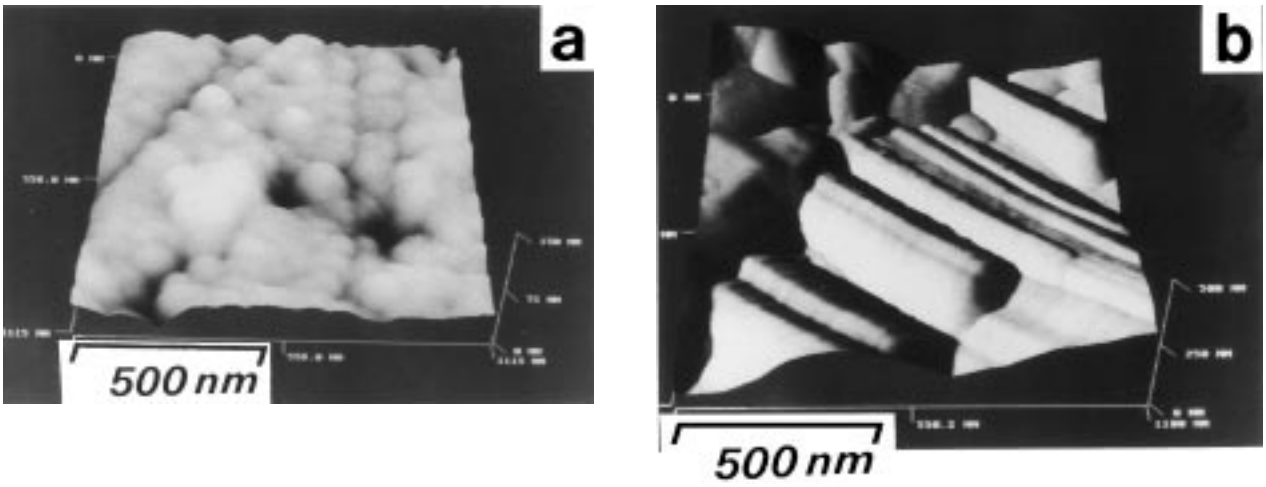

Figure 9 AFM images of the non-irradiated enamel (a) near Fig. 2A and the inside of the cavity of Fig. 2B in dentin formed by laser irradiation (b).

resolution observation in air, in water and even in liquid such as acid agent [8, 9]. The structure with 50 nm size of hydroxyapatite crystallites was easily observed everywhere on the surface of dentin and enamel as shown in Fig. 9(a). This order of resolution is nominally possible for SEM. However the high resolution SEM under high vacuum is preferable for the observation of this magnitude of structure. For WET-SEM it is difficult to attain the sufficient resolution because of the poorer signal/noise ratio under low vacuum.

4.2. Compositional informations

Reflection electrons give rise to the compositional contrast dependent on the atomic number of target specimens. This information is indisputable In the sense that the contrast has the origin in the intrinsic properties of atomic composition and different from artificial contrast by staining. Fig. 2 taken by WET-SEM distinguishes clearly the regions of enamel and dentin. The contrast of enamel is brighter than that of dentin, which suggests that the average atomic number of inorganic and organic components is higher in enamel. This is in a good accordance with the well-known fact that about 97% is inorganic, mainly hydroxyapatite including heavier atoms Ca, P, and organic matters, composed of light atoms C, N, O, H, are little in enamel. In dentin about 60% is inorganic, 20% is organic, mainly protein such as collagen and 10% is water. The ratio of heavier atoms is much smaller.

It is also noticed in Fig. 4 that the peritubular dentin showed higher contrast than intertubular dentin. This suggests the enrichment of heavier atoms in the vicinity of dentinal tubules, that is, Ca enrichment or high mineralization in peritubular dentin.

The contrast of the laser irradiated area in dentin (B, C, D in Fig. 2) is similar to that of enamel, while the cavity formed in enamel (Fig. 2A) shows the contrast almost indifferent from the unirradiated enamel area. This leads to the conclusion that inorganic compounds were enriched in the irradiated spot in dentin, which would be as a result of the evaporation away of organic compounds by laser irradiation. In the case of enamel the contained organic components are as little as 3%. Therefore the evaporation and elimination of organic compounds does not affect so much the composition and there was little change of contrast in compositional image.

In Fig. 4 the contrast of splashed drops is brighter than normal dentin area. In Fig. 6 the thermally deteriorated zone in the inner surface of the laser-induced cavity showed the white contrast compared with the normal dentin. All these suggest that they are results after the heavier inorganic constituents were enriched by melting of original dentin and evaporation of organic compounds.

The total thickness of the thermally deteriorated zone 15 μm and the transient zone 10 μm revealed by WET-SEM (Fig. 6) is in a good agreement with the estimation of the inner surface shell of the cavity 25 μm in the autofluorescent image by CLSM (Fig. 3).

4.3. Analysis of laser-induced cavity

With the power of laser shot the size of cavity became larger (Fig. 2). The laser energy irradiated to the spot of Fig. 2C was about 5 times that of D and that of B was 3 times of C. The size ratio of the irradiated area estimated from white contrast region of cavities in Fig. 2 is approximately proportional to the injected laser energy.

Before irradiation AFM showed the structures of the size 50–150 nm on enamel and dentin. These would be the crystallites of hydroxyapatite, main components of enamel and dentin. After CO_2 laser irradiation the long, acicular crystals and spots were observed by CLSM (Fig. 7) in the inner surface of cavity. Spots turned out irregular hexagons by WET-SEM (Fig. 8). The diagonal length of hexagons is about 3 μm . This is almost the same as the width of acicular crystals with the length 50 μm, which strongly suggests that both crystals are of the same origin. When acicular crystals are grown along inner surface, they can grow as long as 50 μm. When grown perpendicular or inclined to inner surface, the length of crystals is limited to the thickness of the deteriorated zone, approximately as 15–25 μm as observed by CLSM in Fig. 3 and by SEM in Fig. 6. The crystal looks a hexagon as cross section for this direction. The crystals with regular shape were observed by AFM (Fig. 9(b)) is probably the part of the surface morphology of an acicular crystal.

One possibility of newly grown acicular crystals is hydroxyapatite [10, 11], $Ca_{10}(PO_4)_6(OH)_2$ in stoichiometric chemical formula with the needle axis in *c* axis [0 0 1] of hexagonal structure. To identify these newly grown crystals it is necessary to apply microanalysis such as energy dispersive X-ray spectroscopy (EDS), micro-area FT-IR and micro-area X-ray diffraction using the same specimen.

5. Conclusion

Enamel and dentin of human teeth irradiated by CO_2 laser were observed and characterized by CLSM, WET-SEM and AFM using the same specimen without pretreatment. Optical tomographic imaging by CLSM, compositional imaging by WET-SEM, and high resolution observation of surface morphology by AFM were done for both the irradiated and non-irradiated area.

All the facts: the brighter contrast of the irradiated area in dentin in the reflection electron image, the cracks in the laser-induced cavity, the formation of acicular crystals of 50 μm length inside the cavity, the deteriorated zone surrounding cavity, the drops formed in the splashed manner from the center of cavity are in accordance to suggest that the instant thermal effect corresponding to heating to more than 1000 °C occurred at the surface of teeth by CO_2 laser shot. The surface of teeth was partially melted spontaneously, which caused the evaporation of organic compounds and then instant solidification. During this interval molten materials were recrystalized and grown to a large size of acicular crystals.

The reflection electron image in WET-SEM has the contrast derived from intrinsic origin and is useful to obtain the information about the compositional change induced by laser irradiation.

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