Chemical and morphological studies of the acquired pellicle formed subgingivally on dentin in vivo

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The morphological appearance and chemical composition of the subgingival pellicle were studied, using Auger analysis and scanning and transmission electron microscopy. A pellicle was formed on pieces of dentin (2 × 2 × 1 mm), prepared from freshly extracted teeth after root planing. The dentin slabs were inserted for 2 h into healthy gingival sulci. Control slabs cemented supragingivally were used for comparison. The results confirmed the presence of an organic film on the surface of all slabs. Auger analysis of the organic film showed the presence of Ca in the supragingival integument but not in the subgingival integument. The subgingival pellicle was in all cases thicker than the supragingival pellicle. The transmission and scanning electron microscopy observations confirmed the presence of a film essentially free of bacteria on the subgingival specimens and also indicated a possible morphological difference between the supra- and sub-gingival pellicle. □ *Auger analysis; electron microscopy; integument*

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The existence of an acquired integument covering the tooth surface, both supra- and sub-gingivally, has been established (1–3). The chemical composition and microscopic appearance of the supragingival pellicle have been extensively studied (4–9). These studies indicate that the basis of its formation is a selective adsorption of salivary proteins to the enamel surface. Further analyses of the acquired pellicle have shown that it consists mainly of salivary glycoproteins (8, 10, 11). A large number of the proteins have been identified by immunohistochemical methods. The most abundant were IgA, IgM, IgG, lysozyme, amylase, and C3 (12, 13).

The composition of the pellicle is probably dependent on the composition of the fluids surrounding the tooth and the chemical properties of the surfaces. The subgingival pellicle may therefore have a different chemical composition than the pellicle covering the enamel surface. Since initial bacterial adhesion may be influenced by the composition of the pellicle (14, 15), knowledge of its chemical composition could be of importance for the understanding of adhesion mechanisms of both bacteria and cells and for healing processes after periodontal therapy.

The aim of the present study was to compare some chemical and morphological properties of an experimental subgingival integument with those of a supragingival pellicle.

Materials and methods

Sampling procedure

Subgingival pellicle material was collected on small pieces of dentin placed in healthy gingival sulci for 2 h. The dentin slabs (2 × 2 × 1 mm) were prepared from the root surface of freshly extracted human teeth. Remnants of periodontal fibers and cementum were removed, to expose sound dentin. The specimens were polished with pumice and washed in saline before being placed in the gingival sulci. Interdental areas of the upper anterior region in a subject with clin-

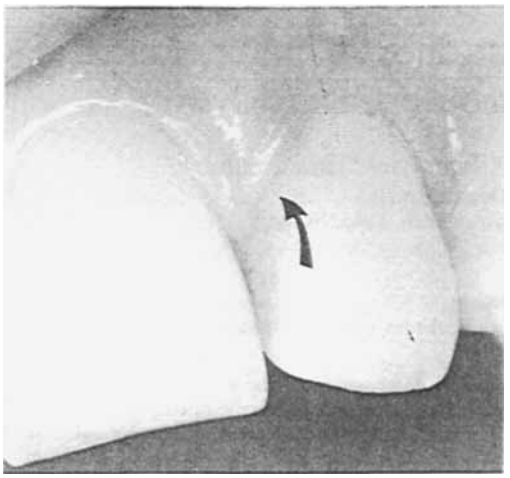

Fig. 1. A dentin slab placed inside the gingival sulcus of a subject with clinically healthy gingivae.

ically healthy gingiva (proximal sulcular depth of 2–3 mm) were selected as sampling sites. The selected areas were dried and isolated with cotton rolls. Eight dentin slabs were then inserted (one in each sulcus), with a pair of forceps (Fig. 1). The control slabs were cemented onto the labial tooth surface supragingivally by means of a zinc phosphate cement. Some specimens were also processed immediately after preparation. Experimental specimens were left for 2 h, during which no intake of food or drink and oral hygiene measures were allowed. The selected areas were again dried and isolated with cotton rolls before the slabs were removed. The surfaces of the specimens facing the gingival side of the sulcus were studied.

Auger analysis

The specimens were immersed in 2.5% gluteraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.2–7.4) for 48 h and then kept in 0.1 M Sorensen's phosphate buffer until further processing. The slabs were then dried in air and mounted. Care was taken to avoid contamination of the specimens. The surface integuments were analyzed by means of Auger electron spectroscopy (AES). The analyses were performed by ELAB (Electronics Research Laboratory, University of Trondheim, Trondheim, Norway.) AES was used in conjunction with argon ion sputtering to obtain the depth profiles of the integuments. The sequential sputtering times were 1/10 min and 1 min.

An argon ion beam of 3 keV energy and 100 $\mu A\ cm^{-2}$ ion current density was used. The presence of carbon, nitrogen, and oxygen was taken to indicate the existence of an organic film (16). The primary electrons were analyzed in the range of 15 to 1000 eV. The analysis was aimed at quantifying the relative contents of calcium, sulfur, phosphor, and nitrogen.

Scanning electron microscopy (SEM)

Sub- and supra-gingivally placed specimens were immersed in 2.5% gluteraldehyde in a 0.1 M Sorensen's phosphate buffer (pH 7.2–7.4) immediately after removal and left for 48 h. The slabs were then dehydrated in increasing concentrations of alcohol and acetone, dried in air, mounted on metal holders, coated with gold, and studied in a scanning electron microscope (Jeol JSM 50A).

Transmission electron microscopy (TEM)

The specimens were immersed in 2.5% gluteraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.2–7.4) for 48 h and postfixed in 1% osmium tetroxide in phosphate buffer, pH 7.2, at 4°C for 90 min. The specimens were dehydrated in increasing concentrations of alcohol and embedded in spur (low-viscosity resin). They were oriented so that thin sections could be cut with a diamond knife perpendicular to the slab surface. The sections were collected on a carbon-coated film on copper grids and stained with a saturated solution of uranyl acetate in 50% ethanol for 20 min, followed by lead citrate (17) for 5–8 min.

Results

Auger electron spectroscopy

Supragingival specimens. Carbon (C), nitrogen (N), and oxygen (O) were present

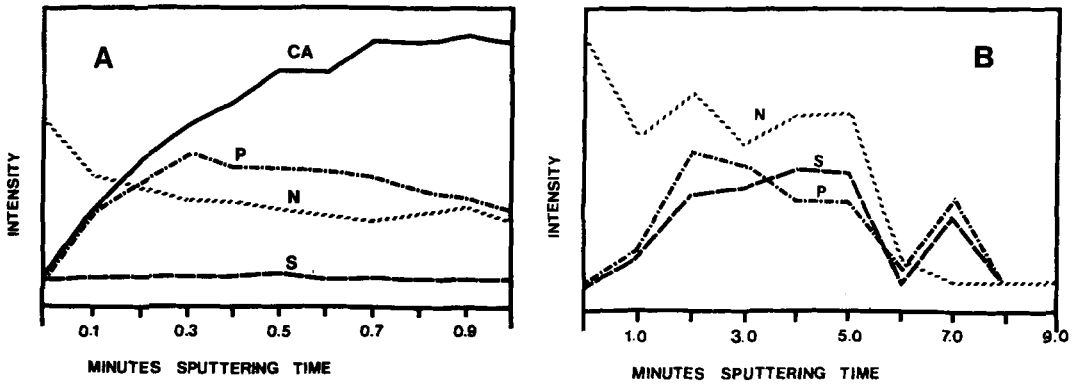

Fig. 2. Tracing of chemical depth profiles of the elements Ca, S, N, and P (intensity of Auger lines as a function of sputtering time) of integuments formed on specimens located supragingivally (A) and subgingivally (B).

on the surface and in the bulk of the integument. The elements were sputtered off in about 1 min. Ca was detected in increasing amounts towards the dentin surface. There was more phosphorus than sulfur on the spots analyzed. Depth profiles showing the relative intensity of electrons from Ca, S, P, and N in the integument formed on supragingival specimens, as a function of sputtering time, are depicted in Fig. 2A.

Subgingival specimens. C, N, and O were present on the surface and in the bulk of the integument. A sputtering time of more than 10 min was necessary to reach the dentin surface, indicating a thicker film than that found on spots analyzed on supragingival specimens. Ca was not detected on the surface or in the bulk of the integument (less than 0.1 atom %). Phosphorus and sulfur were found in equal amounts on the spots analyzed. Depth profiles of S, P, and N of the integument formed on subgingival specimens are shown in Fig. 2B.

Fig. 3. A freshly prepared specimen. Scratches in the surface (arrows) are due to instrumentation. (SEM; × 1100; bar = 10 μm).

Scanning electron microscopy

Micrographs of unused specimens showed a surface without an organic film. The picture revealed a dentin surface with scratches caused by instrumentation (Fig. 3).

Supragingival specimens. The SEM micrographs of the supragingival specimens showed a globular type of pellicle with scattered spherical particles on its surface (Fig. 4).

Subgingival specimens. A relatively 'smooth surface pellicle' that covered almost all the surface of the specimens (Fig. 5) was present. Spherical particles similar to those supragingivally were not seen on the subgingival pellicle surface in the present experiment.

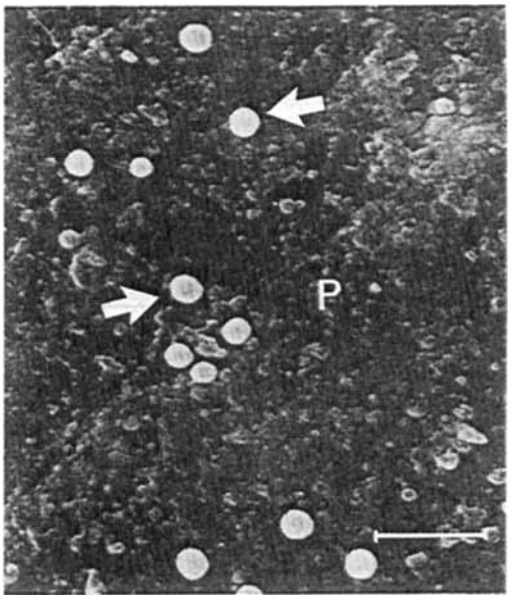

Fig. 4. Spherical particles (arrows) on top of a globular pellicle (P). Supragingival specimen. (SEM; × 3200; bar = 5 μm.)

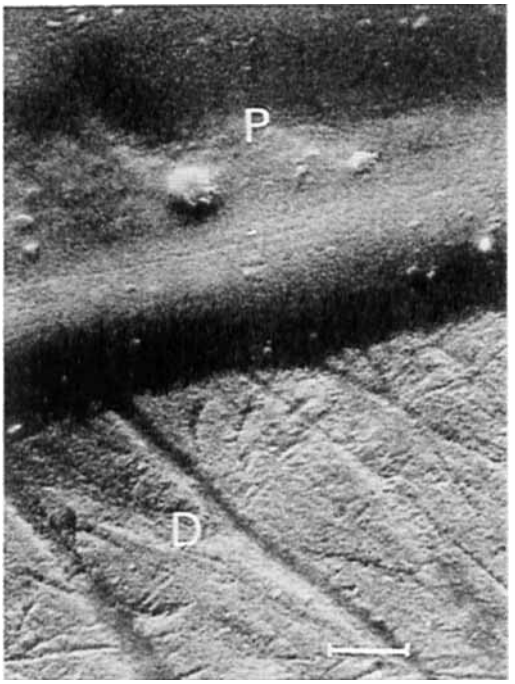

Fig. 5. A 'smooth surface pellicle' (P) formed on dentin surface. A piece of tape covered half of the specimen during the experiment (D = dentin); subgingival specimen. (SEM; × 1100; bar = 10 μm.)

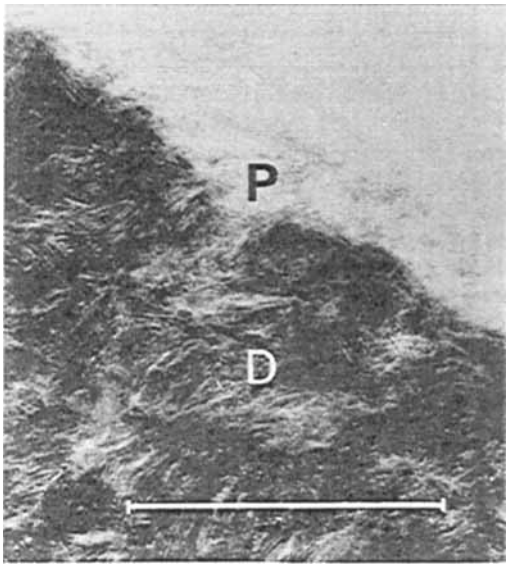

Fig. 6. Supragingival pellicle (P) formed on dentin surface (D). (TEM; × 80,800; bar = 0.5 μm.)

Transmission electron microscopy

The dentin slabs cemented supragingivally showed the presence of a thin film varying in thickness. No structures resembling bacteria were observed with this technique (Fig. 6). The dentin slabs placed subgingivally showed the presence of a thicker film (Fig. 7), which sometimes was covered by struc-

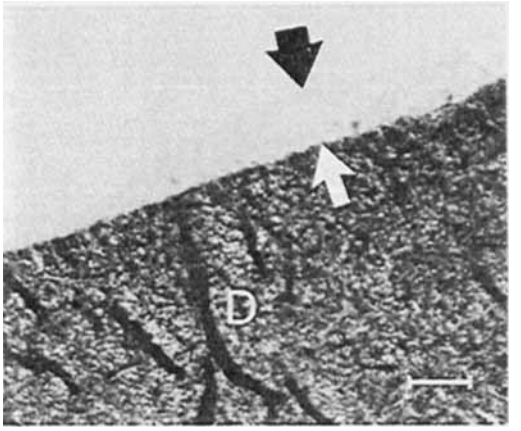

Fig. 7. Micrograph showing the thickness of the subgingival pellicle (arrows) (D = dentin). (TEM; × 8000; bar = 1 μm.)

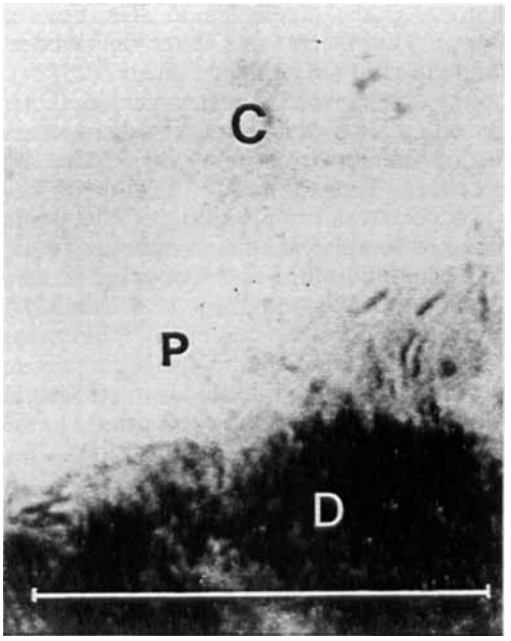

Fig. 8. Subgingival pellicle (P) formed on dentin surface (D), showing a cell (C) on top of the pellicle. (TEM; × 128,000; bar = 0.5 μm.)

tures resembling epithelial cells. Similar cells were also seen in contact with the dentin surface or embedded in the integument (Fig. 8). Fibrillar structures were also observed (Fig. 9).

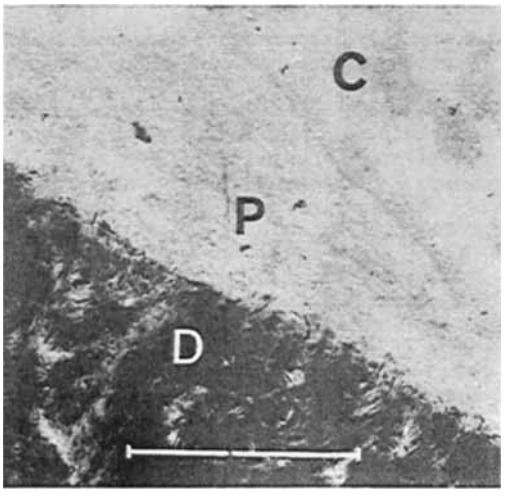

Fig. 9. Fibrillar structures in the pellicle material (P) (C = cell). (TEM; × 63,000; bar = 0.5 μm.)

Discussion

Dentin was used as a substrate in the present study to mimic the situation after periodontal therapy.

AES analyses have been widely used in metallographic studies. However, reports of its use in the analysis of biological films are scarce. Organic surfaces are essentially non-conductive, which creates the problem of charging the specimens by the primary electron beam. This was overcome by Skjørland (16) in his experiment by coating the specimens with a thin gold layer. This made it necessary to sputter off the gold before the analysis of the integuments could begin. Such a procedure may result in changes in the outermost layer. The non-conductive dentin slabs were therefore mounted to enable the primary electron beam to be directed almost tangentially to the convex surfaces. This procedure reduced the charging of the sample to be analyzed and therefore eliminated the need of gold coating. The AES analyses are based on the characterization of the secondary electrons emitted from the material. The depth from which the electrons can escape depends on their energy and, to some extent, on the density (molecules/cm^3) of the material to be analyzed (16). The molecular packing of the adsorbed organic material may vary from one surface to the next (18). The film thickness referred to in the present experiment is reflected in the sputtering time used to remove the layer—or layers—of the integuments to be analyzed. The sputtering time is not necessarily proportional to the thickness of the organic film. The time needed for sputtering off the subgingival integuments was approximately 10 times that required for removing the supragingival pellicle. This observation may be taken as an indication of a thicker subgingival pellicle and/or better packing or condensation of the organic material adsorbed. In the present study it is probably due to an approximately 10-fold thicker pellicle (Figs. 6 and 7).

Calcium was found in the supragingival pellicle, with the highest concentration near the dentin surface. This result is consistent with other observations (16). The presence

of sulfur in the supragingival pellicle is also in accordance with previous observations (8, 16) and may indicate the presence of sulfated macromolecules.

Phosphate buffer was used in fixation and storage of the specimens. Thus, phosphorus may have been incorporated in the pellicle material during processing, and the results should be interpreted with caution in this respect. The concentration of Ca in gingival fluid has been shown to be higher than that found in saliva (19). The observed difference in Ca concentration in sub- and supra-gingival pellicles in the present study does not contradict the view that Ca plays an active role in the formation of the supragingival pellicle (20, 21). The SEM micrographs of supragingival specimens showed the appearance of a globular pellicle, confirming previous observations (6, 9). The spherical bodies seen in the surface of the pellicle in Fig. 4 had a size corresponding to that of bacteria, but the shape differed from previously shown microorganism attached to pellicles (6, 9). The actual nature of these bodies cannot be ascertained by our experiment.

The subgingival pellicle displayed a smooth surface that covered almost the total surface of the specimens. No rounded bodies could be seen on the surfaces. Healthy gingival crevices are not entirely without bacteria (22); however, no particles compatible with the appearance of bacteria were seen adhering to the specimens placed subgingivally. There may be several reasons why the test slabs were apparently bacteria-free. Supragingivally, the initial adhesion of bacteria may start after approximately 30 min (6), and relatively few bacteria are found after 2 h (8). The initial adhesion may take a longer time in the gingival sulci, or there may be very few bacteria present in the environment.

Epithelial cells were occasionally seen bordering the pellicle surface or lining the dentin surface directly. This may indicate a kind of competition between cells and non-cellular matter for the retention sites on the dentin surface. The observation of fibrillar structures in the integument is in agreement with previous reports (9).

A likely source of the subgingival pellicle material is the gingival fluid. The flow of gingival fluid increases after mechanical stimulation of the teeth and gingiva (23, 24). In our experiment the mechanical irritation caused by the positioning of the slices within the gingival crevice probably has elicited an increase in gingival flow.

The chemical composition of the liquid phase in the gingival sulci is different from that of the liquid phase responsible for the formation of the supragingival pellicle (25). The indications of chemical differences between supra- and sub-gingival pellicles in our investigation are therefore in agreement with the concept that the composition of the liquid phases affects the composition of the integument.

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