Immunohistochemical and Electron Microscopic Observations of Rat Palatal Mucosa after CO$_2$ Laser Irradiation

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Purpose: The purpose of this study was to investigate the morphological changes of rat palatal mucosa after CO$_2$ laser irradiation.

Materials and Methods: The laser ablations were made with the CO$_2$ laser in continuous wave mode, 1 W, for 5 s. For light microscopic observations, animals were killed immediately, 0.5, 1 to 5 and 7 days after irradiation. The sections were stained immunohistochemically with primary antibodies: laminin, S100, polyclonal-keratin, HMB45 and CK20. For electron microscopic observation, tissues were fixed immediately and 12 h after irradiation.

Results: Immediately after irradiation, degeneration of the superficial layer of the epithelium was observed; however, the cells of the basal and para-basal layers were well preserved. Twelve hours after irradiation, the epithelium had degenerated; however, a laminin-positive reaction was still recognized at the basal membrane. The electron microscopy showed that, although the cell membranes had become unclear, desmosome-like structures were preserved. One day after the laser irradiation, the epithelium had separated from underneath the lamina propria. At 2 days after the irradiation, the irradiated areas were covered by fibrin materials without any visible damage of the connective tissue. At 7 days after the irradiation, the epithelium had regenerated completely without any cells positive for S-100, HMB45 or CK20.

Conclusion: These results suggest that the CO$_2$ laser affected binding proteins of the basal membrane without destroying the underlying connective tissue.

Keywords: CO$_2$ laser, oral mucosa, immunohistochemistry, electron microscopy.

Laser energy has become an important modality for surgical treatment. Depending on the wavelength of the light emitted by the laser medium, photocoagulation, photoevaporation or photodynamic effects are exerted on tissues.[1-3] Laser therapy has also found a place in clinical dentistry, including 1060-, 532- and 355-nm Nd:YAG,[4-7] 694-nm Q-switched ruby,[8-10] 488/514-nm argon,[8,11] and 10.6-μm CO$_2$.[2-15] These lasers have been recommended for various types of treatment in the oral cavity. At dental clinics, lasers are

Fig 1 CO_2 laser was applied on the surface of the rugae on the rat palatal mucosa.

used for dentin excavation,[16-20] pulp treatment before capping,[21] and root canal treatment.[22,23] Recently, CO_2 lasers have also been used for removal of melanin pigmentation of oral mucosa.[14,15,24-26,34]

The mechanism of ablation by CO_2 laser irradiation is that the laser energy is strongly absorbed by intra- and extracellular water, which turns to steam and bursts apart the cellular architecture.[1,3] At the point of impact, the cells are disrupted and carbonize, the structural proteins denature and adjacent small blood vessels and lymphatic channels occlude.[27-30] When the focal point of a laser beam is moved over a tissue surface, it will make an incision.[3]

Connective tissue healing is slower in laser-generated wounds than in scalpel-generated wounds. The healing characteristics at least partially account for the lack of scarring and contraction, which promotes excellent clinical results in terms of connective tissue healing.[27,31-33] On the other hand, laser treatment of pigmented lesions requires melanocytes to lie within the penetration range of the laser, and to contain melanin in order to absorb and convert the light energy into heat by photothermolysis. The wavelength of the CO_2 laser is almost completely absorbed in the superficial layers of tissues such as the epithelium, where melanocytes are located in the basal cell layer. There is little detectable effect on deep tissues, and although

several studies have been published on CO_2 laser wound healing, several aspects remain to be fully clarified.[14,34]

The purpose of this study was to investigate the ablation of epithelium in which melanocytes were present and examine the healing mechanism of various epithelial components such as keratinocytes, melanocytes, Merkel cells and Langerhans cells, after CO_2 laser irradiation of the rat palatine mucosa at the immunohistochemical and ultrastructural levels.

MATERIALS AND METHODS

Experimental animals

All animal experiments conformed to the Guidelines for the Experimental Animals Facility of the Tokyo Dental College. Sixty healthy adult male Sprague-Dawley rats, weighing about 200 g each, were used in this study. Animals were anesthetized with 2.5% sodium thiopental (Ravonal, Tanabe Pharmaceutical; Osaka, Japan). The animals were kept on a standard laboratory diet during the experimental time periods. Five animals each were used at each of the time periods (immediately after irradiation, and at 0.5, 1, 2, 3, 4, 5 and 7 days). Five normal rats were used as controls.

Laser irradiation

The lesions were made with a CO_2 laser (Panalas C05, Panasonic Dental; Osaka, Japan) applied at a wavelength of 10.6 µm, in continuous mode, at a 1 W setting with a defocused beam and a spot size of 0.15 cm in diameter for 5 s. The laser tip was placed in contact with the surface of the rugae of the palatal mucosa. Total laser energy was 282.49 J/cm^2. An air-flow system was provided at the tip of the handpiece (Fig 1).

Preparation of histological and immunohistochemical specimens

For histological observations, animals were killed with an overdose of sodium thiopental immediately, 0.5, 1, 2, 3, 4, 5 and 7 days after treatment and tissue specimens were excised and fixed with 10% neutral buffered formalin. Maxillae were removed and dehydrated before being embedded in paraffin. Paraffin sections, approximately 4 µm in thickness, were then cut at the laser irradiated sites. The control palatine mucosa was

cut perpendicularly to the midline of the palate. The sections were stained with hematoxylin and eosin.

For immunohistochemical staining, the Ventana system (Discovery Staining Module, Ventana Medical Systems; Tucson, AZ, USA) was employed. Paraffin sections were cut in exactly the same manner as described above. First, endogenous peroxidase activity was blocked with a 0.3% solution of hydrogen peroxide in methanol for 30 min. The sections were incubated with the following antibodies: a primary antibody to laminin which detects the basal membrane (E-Y Laboratories, San Mateo, CA, USA, diluted at 1:50), S100 which detects Langerhans cells (DAKO, Carpenteria, CA, USA, diluted at 1:100), polyclonal-keratin which detects keratinocytes (DAKO; diluted at 1:200), HMB45 which detects melanocytes (DAKO; diluted at 1:50), and CK20 which detects Merkel cells (Progen; Heidelberg, Germany, diluted at 1:100). Primary antibodies were diluted in normal goat serum and were reacted for about 1 h each at room temperature. Next, the sections were incubated for 30 min each with a biotinylated secondary antibody and streptavidin peroxidase reagents. Finally, the presence of peroxidase was visualized by diaminobenzidine reaction, with brown coloration indicating a positive reaction.

For electron microscopic observation, tissues were fixed immediately and at 12 h after irradiation with 2% glutaraldehyde in 0.1 M phosphate buffer for 24 h and post-fixed with 4-osmium tetraoxide in 0.1 M phosphate buffer for 2 h. Prior to dehydration, the tissues were stained en bloc with 2% uranyl acetate in 10% ethanol, dehydrated in graded alcohol and embedded in Epon 812. Ultrathin (75-nm) sections were cut, stained with uranyl acetate and lead citrate, and observed by transmission electron microscopy (H-7100, Hitachi; Tokyo, Japan).

RESULTS

Light microscopic observations

Immediately after laser irradiation, a degeneration and acantholysis of the superficial layer of the palatal stratified squamous epithelium showed intramucosal vacuoles in the epithelium. The cells of the basal and para-basal layers were, however, well preserved (Fig 2a). At 12 h after laser irradiation, all layers of the epithelium, from basal to keratinized layers, had degenerated. Basal membranes were swollen and indistinct in some areas. Cell-poor fibrous connective tissue located directly underneath the basal membrane was undamaged (Fig 2b). At 1 day after the laser irradiation, the epithelium was shed and fibrin-like material remained. However, connective tissue showed no remarkable changes. In sum, the CO_2 laser irradiation ablated the entire epithelium from the basal membrane (Fig 2c). At 2 days after laser irradiation, the epithelium had disappeared and the tissue was covered by fibrin material without any damage of the connective tissue (Fig 2d). At 3 days after laser irradiation, basal cells of the remaining epithelium at the periphery of the wound area had migrated onto the connective tissue surface (Fig 2e). Positive reactions for PK were observed in the regenerated epithelial cells (Fig 2f). At 5 days after laser irradiation, regeneration of the epithelium was observed and the basal to keratinized layers could be distinguished, as well as the basal membrane. Small amounts of fibrous connective tissue with a few small round cell infiltrations were observed under the regenerated squamous epithelium (Fig 2g). At 7 days after laser irradiation, stratified squamous epithelium with rete ridges covered the fibrous connective tissue, and the regeneration was almost complete (Fig 2h).

Immunohistochemical observations

In immunohistochemical staining of normal oral mucosa, a few cells were positive for either CK20 (Fig 3a), which detects Merkel cells, or for HMB45 (Fig 3b), which detects melanocytes in cells of the basal and para-basal cell layers. A few prickle cells were positive for S100 protein, which detects Langerhans cells (Fig 3c). The basal membrane of the epithelium and blood vessels were positive for laminin (Fig 3d).

Immediately and at 12 h after the laser irradiation, laminin-positive reactions were observed in the basal membrane in the same manner as the normal oral mucosa (Fig 3e). At 2 days after laser irradiation, the epithelium had disappeared and the expression of laminin could not be detected in the wound area except for the basal membrane of capillaries (Fig 3f). At 7 days after laser irradiation, a positive reaction for laminin could again be observed between the regenerative epithelium and the fibrous connective tissue, but this positive line was not continuous (Fig 3g). In the regenerated mucosa at 7 days after laser irradiation, no HMB-45 (Fig 3h), CK20 (Fig 3l), or S-100 (Fig 3j) positive cells could be observed.
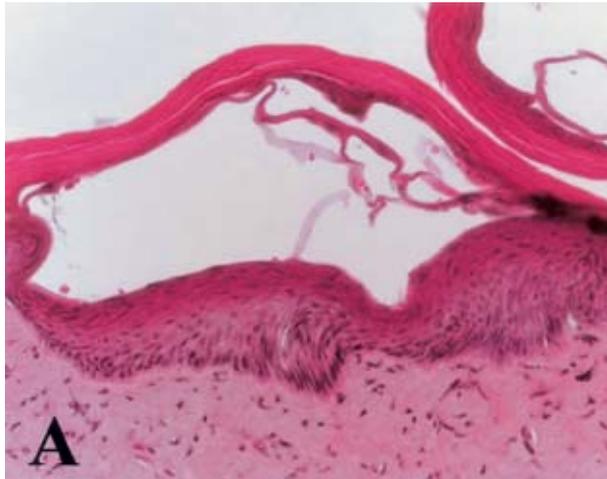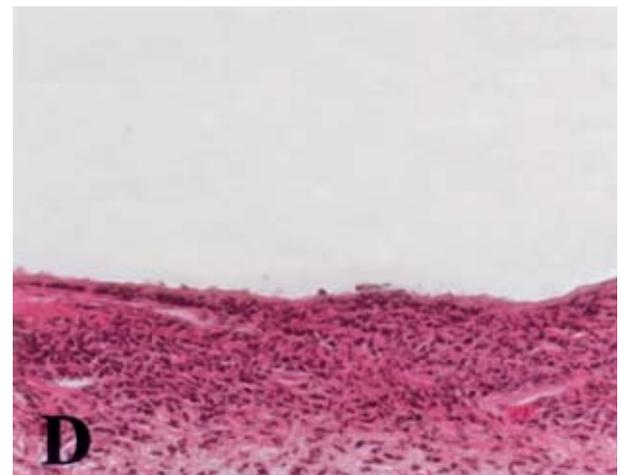
Fig 2a Histological micrograph of hematoxylin-eosin staining of rat palatine mucosa after irradiation with CO_2 laser (magnification = 200X). Immediately after irradiation, degeneration of the superficial layer and intramucosal vacuole of epithelium was observed, but the cells of basal and deep prickle cell layers were well preserved.
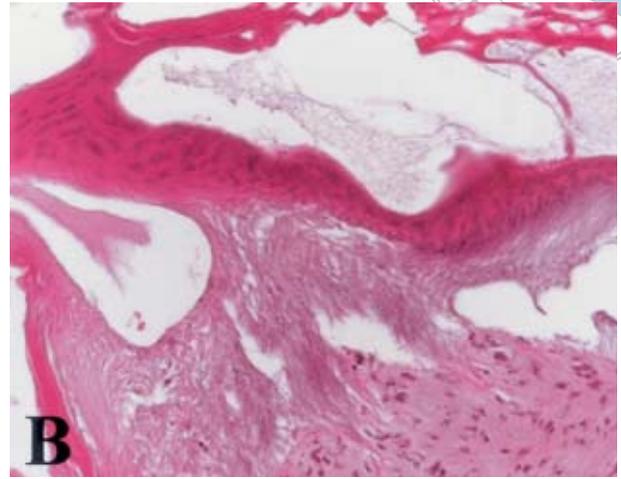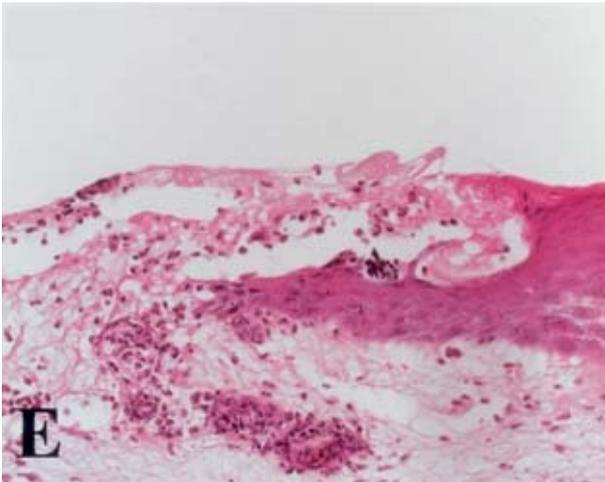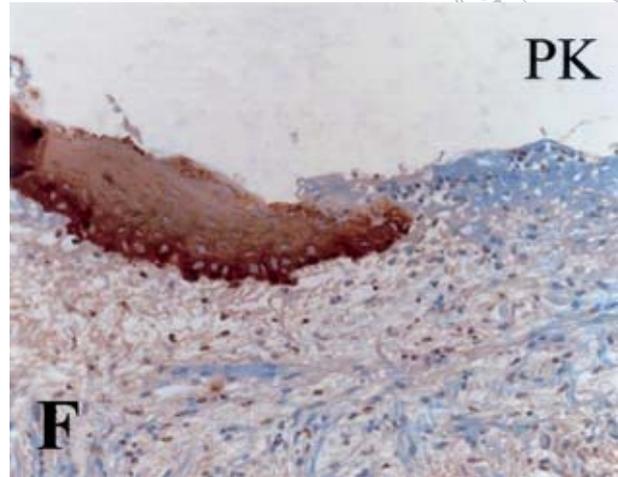

Fig 2b Histological micrograph of hematoxylin-eosin staining of rat palatine mucosa after irradiation with CO_2 laser (magnification = 200X). At 12 h after irradiation, epithelium is degenerated completely. Basal membrane is swollen and indistinct. Slight hyalinizing tendency is observed in the connective tissue located directly below the basal membrane.
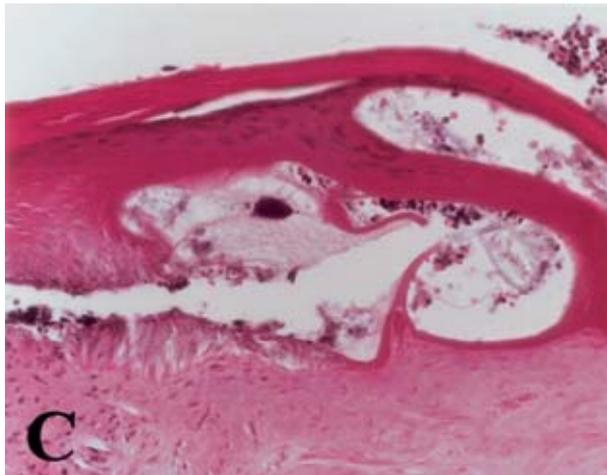
Fig 2c Histological micrograph of hematoxylin-eosin staining of rat palatine mucosa after irradiation with CO_2 laser (magnification = 200X). At 1 day after irradiation, the epithelium is shed and fibrinoid alteration is seen at the superficial layer of subepithelium. No organic change was observed in subepithelial connective tissue.
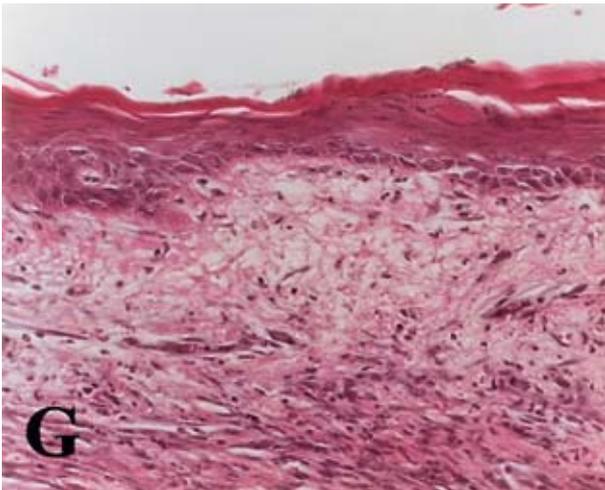

Fig 2d Histological micrograph of hematoxylin-eosin staining of rat palatine mucosa after irradiation with CO_2 laser (magnification = 200X). At 2 days after irradiation, the epithelium has disappeared and is covered by fibrin material without any damage to connective tissue.
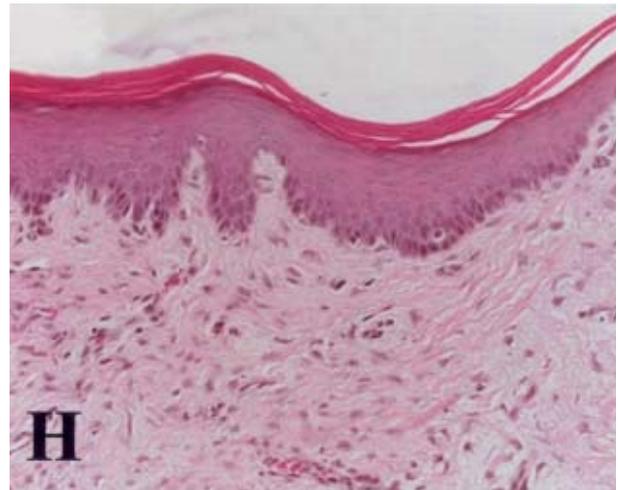
Fig 2e Histological micrograph of hematoxylin-eosin staining of rat palatine mucosa after irradiation with CO_2 laser (magnification = 200X). At 3 days after irradiation, cells of the basal layer migrated onto the wound surface.

Fig 2f Histological micrograph of hematoxylin-eosin staining of rat palatine mucosa after irradiation with CO_2 laser (magnification = 200X). At 3 days after irradiation, positive reaction for polyclonal keratin was observed in the regenerated epithelium.

Fig 2g Histological micrograph of hematoxylin-eosin staining of rat palatine mucosa after irradiation with CO_2 laser (magnification = 200X). Five days after irradiation, regeneration of the epithelium on the slightly inflamed connective tissue was observed.

Fig 2h Histological micrograph of hematoxylin-eosin staining of rat palatine mucosa after irradiation with CO_2 laser (magnification = 200X). At 7 days after irradiation, the epithelium was regenerated completely.
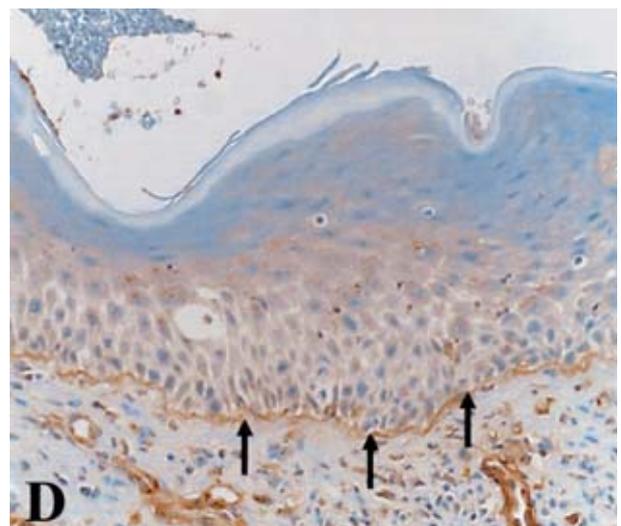
Fig 3a Immunohistochemical staining in the rat palatine mucosa. In normal mucosa, a few aggregated cells were positive for CK20 in the basal cell layer (arrows).
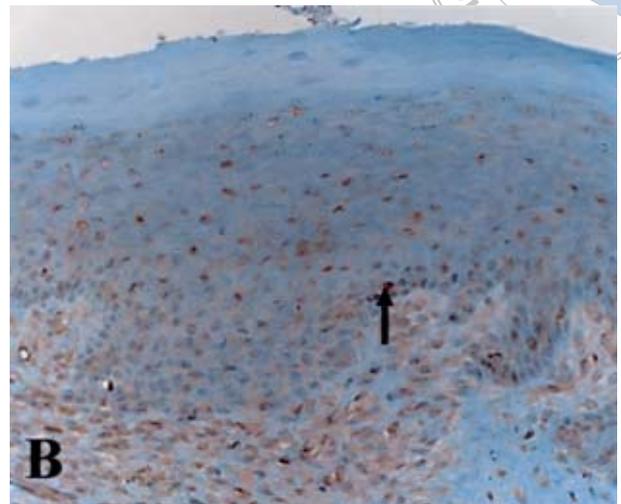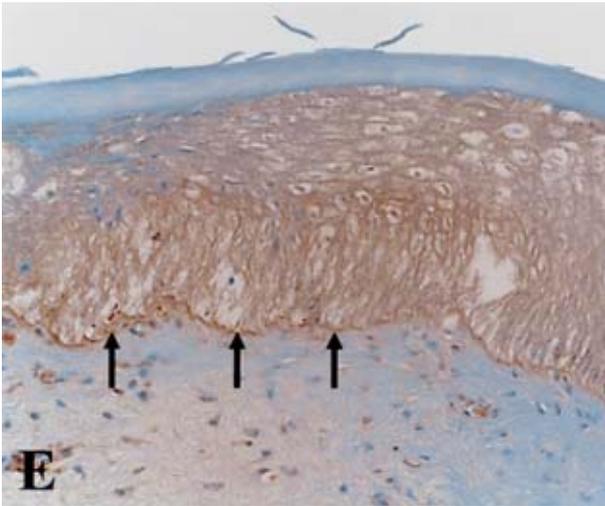

Fig 3b Immunohistochemical staining in the rat palatine mucosa. In normal mucosa, a few HMB45-positive cells can be detected in the basal cell layer (arrow).

Fig 3c Immunohistochemical staining in the rat palatine mucosa. In normal mucosa, a few cells were positive for S100 protein in cells of the basal and of the deep prickle cell layers (arrows).

Fig 3d Immunohistochemical staining in the rat palatine mucosa. Staining for laminin in the normal mucosa, the laminin-positive reaction is observed in the basal membrane (arrows).
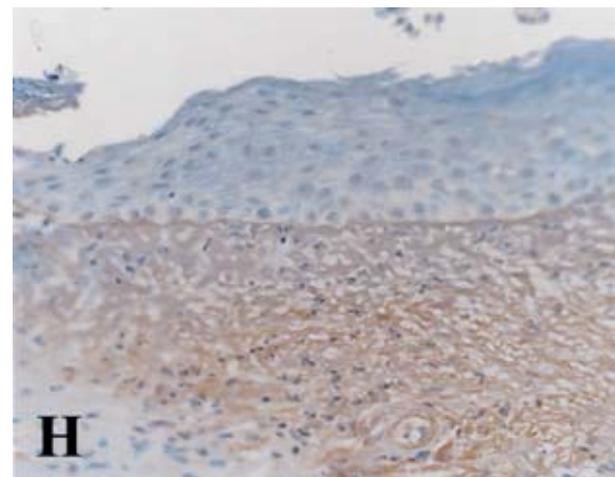
Fig 3e Immunohistochemical staining in the rat palatine mucosa. At 12 h after irradiation, positive reactions for laminin are still observed in the basal membrane (arrows).
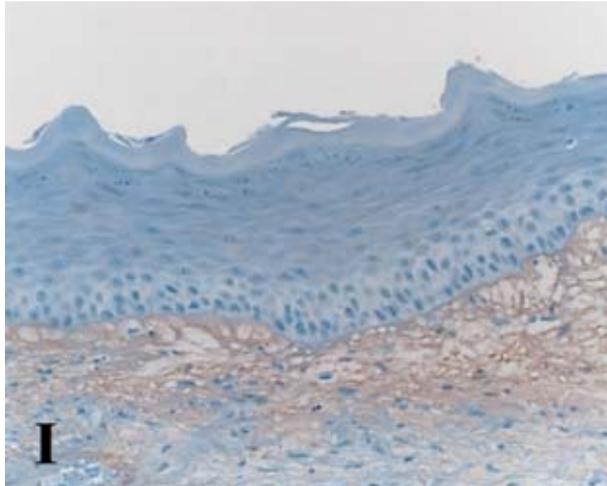

Fig 3f Immunohistochemical staining in the rat palatine mucosa. At 2 days after irradiation, expression of laminin was not detected in the wound area.

Fig 3g Immunohistochemical staining in the rat palatine mucosa. At 7 days after irradiation, positive reactions for laminin were again observed in the basal membrane of the regenerative epithelium (arrows), although it was discontinuous.

Fig 3h In regenerated mucosa at 7 days, HMB45-positive cells could not be seen.

Fig 3i Immunohistochemical staining in the rat palatine mucosa. In regenerated mucosa at 7 days after CO_2 laser irradiation, the expression of CK20 was not detected.

Fig 3j Immunohistochemical staining in the rat palatine mucosa. In regenerated mucosa at 7 days after CO_2 laser irradiation, the expression of S100 protein was not detected.

Electron microscopic observations

In the peri-basal cells of normal mucosa, many organelles and tonofilaments were observed in the cytoplasm of the basal cells. The basal lamina consisted of a clear lamina densa and a homogenous lamina lucida (Fig 4a). In intracellular spaces, many desmosomes contained desmoplastic plates with a central plate and anchoring filaments (Fig 4b).

Immediately after laser irradiation, almost all organelles in the basal cells were gone and a number of vacuoles were observed in the cytoplasm. Granulated hyper-electron dense structures were also observed around the nuclei (Fig 4c). Nuclear outlines were irregular and parts of the nuclear membrane were unclear. However, desmosomes were clearly observed in the intercellular spaces. The width of the lamina lucida was irregular and the basal lamina could not be detected in some areas (Fig 4d).

At 12 h after laser irradiation, the majority of cells in the epithelium were completely destroyed and only slight nuclear-like structures remained (Fig 4e). The cell membranes had become unclear, while desmosome-like structures and many tonofilaments floated in the degenerative tissue but not connecting cells (Fig 4f).

DISCUSSION

Many fundamental studies about wound healing after various types of laser irradiation, such as Nd:YAG or Er:YAG laser and CO_2 laser,[35-37] reported their extremely good clinical performance. Hidaka et al[38] reported that the wound healing process after incision of rat skin using a Nd:YAG laser produced sphacelus at the wound margin. This sphacelus formation by the Nd:YAG laser is conspicuous compared to that of the CO_2 laser. Because of that, the regeneration of the epithelium after Nd:YAG laser irradiation might be delayed. Matin et al[37] reported that destruction of the gingival organization in periodontal pockets of beagles irradiated by CO_2 lasers depended on the output power level. He concluded that regeneration of the gingival epithelium was almost complete at 7 days after laser irradiation and that connective tissue comprised of collagen fibers was formed. In particular, excellent clinical and histological results are provided when CO_2 lasers are used at power levels of 0.5 to 1.5 J.

CO_2 lasers generate far-infrared rays and are easily absorbed in tissue fluids of the body and the blood. As for the pyrexia action of CO_2 lasers, since they are applied locally, it is assumed that there is little influence or danger of pyrexia for the organism. In histological sections, Sharon et al[14] found that the CO_2 laser beam removed the epithelium in layers in a scalloping mode, accurately following the wavy surface until it reached
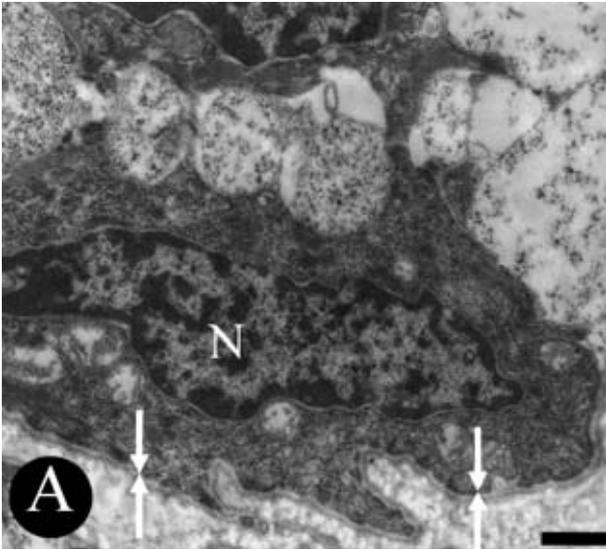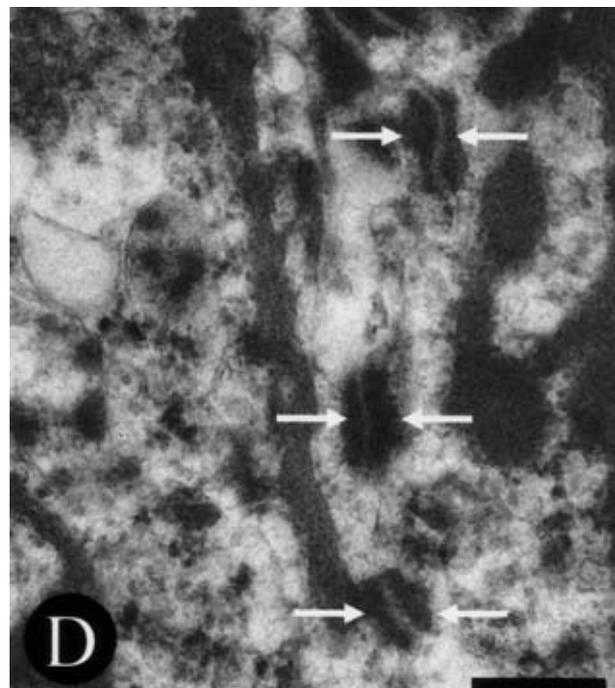
Fig 4a Electron micrograph of control rat palatine mucosa. The basal membrane in the basal lamina consisted of a clear lamina densa and homogenous lamina lucida (bar = 10 μm; original magnification 10,000X)
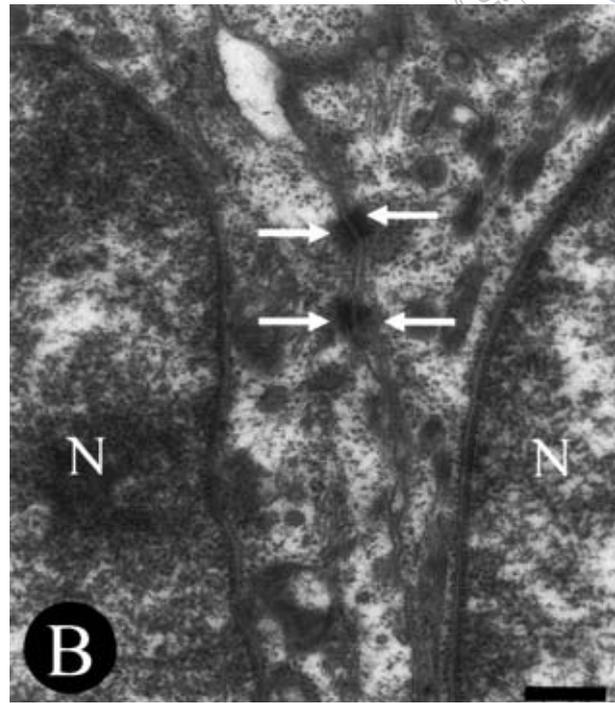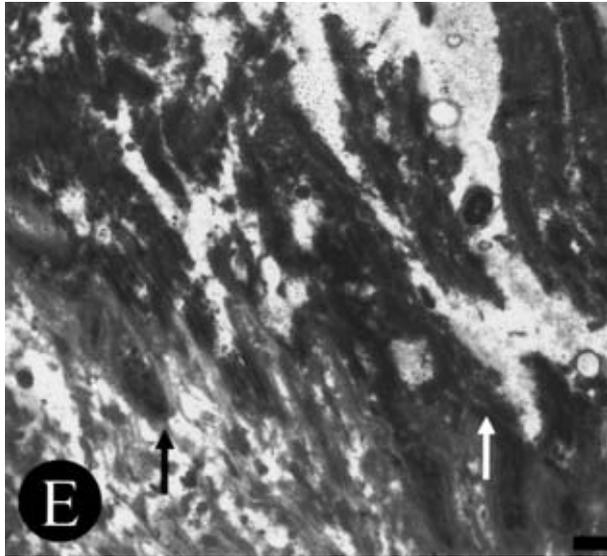

Fig 4b Electron micrograph of control palatine mucosa. In intracellular spaces, many desmosome-like structures contained desmoplastic plates which maintained the central plate; anchoring filaments are observed (bar = 5 μm; original magnification 17,000X).
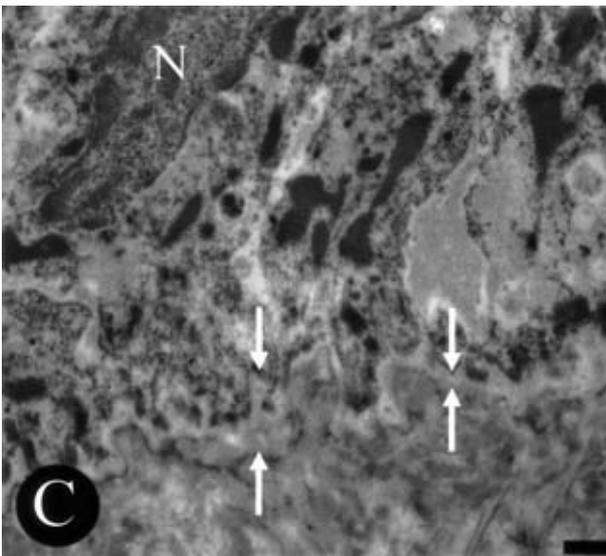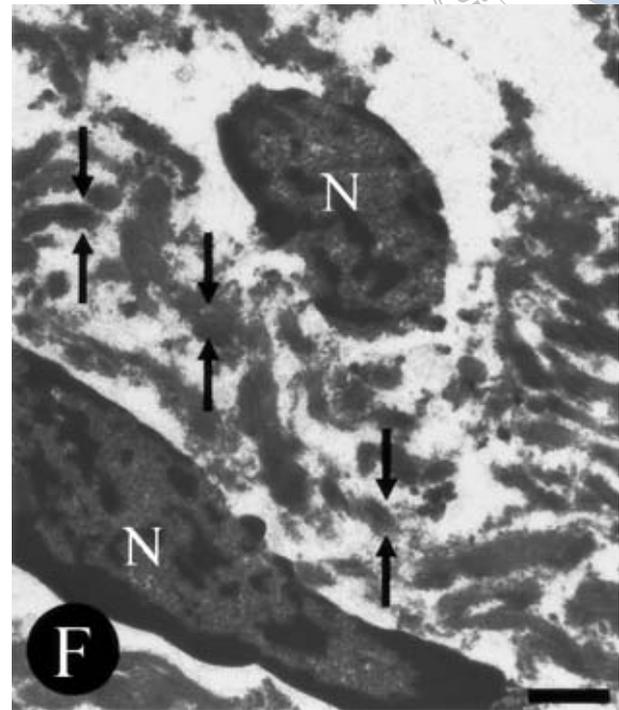

Fig 4c Electron micrograph of rat palatine mucosa after irradiation with the CO_2 laser. In the basal cell layer immediately after irradiation, the width of the lamina lucida is irregularly extended and basal lamina structures are partially destroyed (bar = 10 μm; original magnification 8000X).

Fig 4d Electron micrograph of rat palatine mucosa after irradiation with the CO_2 laser. In the basal cell layer immediately after the irradiation, desmosome structures in the intercellular space are occasionally found; conserved intercellular connections are apparent (bar = 5 μm; original magnification 30,000X).

Fig 4e Electron micrograph of rat palatine mucosa after irradiation with the CO_2 laser. At 12 h after irradiation, the majority of palatine mucosa cells are destroyed, forming large clefts (bar = 10 µm; original magnification 7000X).

Fig 4f Electron micrograph of rat palatine mucosa after irradiation with the CO_2 laser. At 12 h after irradiation, desmosome-like structures were suspended around the degenerative tissue. Many tonofilaments retain their structure (bar = 10 µm; original magnification 9000X).

the basement membrane, without touching the connective tissue. It is known that CO_2 lasers produce a carbonized layer which is subjacent to the exposed wound surface, and since it penetrates deeply into the tissue, it produces a slight inflammatory reaction, with no infiltration of inflammatory cells. Under the irradiation conditions of our experiments, the CO_2 laser irradiation was probably absorbed only to the depth of the epithelial layer, and cells of the basal layer and the basal membrane were not affected during or immediately after the laser irradiation. However, the influence of the CO_2 laser extends to the basal membrane afterwards, since the epithelium sloughs off without any damage to the underlying connective tissue. Luomanen et al[24] found that replacement and removal of the residual connective tissue matrix was slower in laser-induced than in scalpel-incision wounds. This could at least partially account for the lack of scarring and contraction, a frequently observed clinical trait in laser-treated tissues. However, unlike an electrosurgical

wound, a laser wound is not a burn; 10.6-µm photons are exponentially absorbed as a function of the amount of water, not as a result of resistance or conduction, and the CO_2 laser's air-cooling function is also highly beneficial. An important finding in their study was that laser treatment did not seem to inflict any great inflammatory damage on underlying connective tissue. In general, the speed and course of wound healing are influenced by events of coagulation, inflammation, and granulation in the connective tissue, but wound healing after CO_2 laser irradiation could bypass these healing mechanisms except for epithelial healing. The ultrastructural observations at the time of irradiation and immediately after irradiation with the CO_2 laser show that epithelial cell structure was completely destroyed, and only highly electron-dense structures remained, which may be aggregations of organelles. The width of the basal lamina had expanded irregularly in the basal membrane and disappeared in some areas at 12 h after laser treatment. Large vacuoles could be seen in the

majority of basal cells and deep prickle cells, and the basal membrane was destroyed. Only desmosome structures and tonofilaments could be recognized in the regenerating tissue. However, the elements of connective tissues were well preserved.

The effect of the CO_2 laser with the parameters used in this study extended to the basal membrane and disrupted binding proteins between epithelial cells and/ or the basal membrane. Regeneration of the epithelium occurred from adjacent normal basal cells composed of keratinocytes without any melanocytes, Merkel cells, or Langerhans cells. These results suggest that the CO_2 laser not only caused vaporization in the superficial epithelium, but also influenced degeneration of cell structure in basal and deep prickle layers. Therefore, the CO_2 laser should be effective for melanin ablation, although immediately regenerated epithelium is pathologically brittle.

ACKNOWLEDGMENTS

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