Morphological changes in oral mucosae and their connective tissue cores regarding oral submucous fibrosis

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Summary. Oral submucous fibrosis (OSF) is a chronic disease of the oral cavity characterized by an inflammatory reaction followed by severe fibro-elastic changes. The aim of the present study was to investigate the three-dimensional morphological changes in the connective tissue cores (CTCs) of the oral mucosa in OSF. The sample consisted of buccal mucosal biopsies from ten human subjects ranging in age from 40–45 years; five of them were clinically diagnosed as having moderate to severe OSF, and the remaining five served as unaffected controls. Half of each biopsy was formalin-fixed and paraffin-embedded for light microscopy, while the other half was fixed in a Karnovsky's solution, treated with HCl to exfoliate the epithelium, and processed for examination under a scanning electron microscope (SEM).

Oral submucous fibrosis biopsies exhibited heavily packed aldehyde fuchsin-positive fibers (i.e. elastic fibers) in the submucosa under the light microscope. Broad bundles of collagen fibers were seen in a concentrated manner in the deeper layers. Scanning electron microscopy of the buccal mucosa in OSF showed the finger-shaped CTCs to be attenuated beneath the epithelium at the initial stages of the disease. Patchy degenerative areas lacking the CTCs were observed in advanced cases. These degenerative areas increased gradually with the progression of the disease. Highly fibrosed cases showed severe degeneration of the CTCs, resulting in a smoothening of the connective tissue surface in the buccal mucosa.

Introduction

The oral mucous membrane is a unique tissue which is continuously exposed to various kinds of stressors, such as heat, cold, microorganisms, chemicals, and mechanical irritations in the process of food intake (Jorgensen 1981; Cox and Walker 1995; Davis *et al.*, 1998; Nahlieli *et al.*, 1999). In response to these stressors, both epithelial and connective tissue layers of the oral mucosa also exhibit acute and chronic reactive changes (Wallenius and Heyden 1972; Merne *et al.*, 2002).

Oral submucous fibrosis (OSF) is a chronic disease of the oral cavity, which is characterized by an epithelial/subepithelial inflammatory reaction followed by a fibro-elastic change in the submucosa (Shafer *et al.*, 1993). The disease is found commonly in people of Southeast Asia (Tsai *et al.*, 1999). Epidemiological studies have suggested that the habit of betel nut or quid chewing is a major aetiological factor of the disease (Murti *et al.*, 1995). Chang *et al.* (2001) experimentally demonstrated the cytotoxic effect of arecoline (the extract of arecanut) on buccal mucosal fibroblasts, resulting in an oversynthesis of collagen. However, the exact cause and the mechanism of the disease have not yet been properly established. On the other hand, structural changes in OSF have been studied in detail both at the light and the electron microscopic levels. Reichart *et al.* (1984) and Van Wyk *et al.* (1990) studied the patterns of distribution of different types of collagen in subjects with confirmed OSF. Ultrastructural findings of muscle degeneration in OSF were reported by el-Labban and Cannif (1985). However, it should be noted most of these studies investigating OSF were based on two-dimensional observations.

Scanning electron microscopic (SEM) observations of the three-dimensional structure of connective tissues have been carried out in the normal oral mucosa of the palate

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(Reichart and Althoff 1982; Paulsen and Thale, 1998; Watanabe *et al.*, 1999), tongue (Kobayashi and Iwasaki 1988; Kobayashi, 1992; Kobayashi *et al.*, 2001), and gingiva (Abe and Osawa, 1999). Similar studies by SEM are also available on the mucosa of the vocal folds and the intestine (Yamashita 1997; Ushiki, 1990). Furthermore, three-dimensional morphological changes in the connective tissues of the lining of the tongue and their lingual papillae due to ageing have also been reported (Kobayashi *et al.*, 2001). We have suspected that certain changes in the structure of the connective tissues would be also exhibited in the oral mucosa of OSF. Thus, the aim of the present study was to observe changes in connective tissues of the buccal mucosa in OSF using SEM.

Materials and Methods

The total sample consisted of 10 subjects: five of them were clinically diagnosed as having moderate to severe OSF, while the others serving as a control were unaffected healthy adult volunteers who underwent surgery for third molar impactions or road traffic accidents. The latter abstained from smoking, betel-nut chewing, or alcohol consumption. The age range of the sample subjects was 40–45 years. Punched biopsies of 5 mm in radius were taken from the buccal mucosae of each subject. Prior to starting the biopsy, informed consent was obtained from all of the subjects. The procedures for these investigations were approved by the ethical committee of the University of Peradeniya, Sri Lanka, prior to the start of these investigations. For light microscopy, one half of the biopsies were formalin-fixed and paraffin-embedded for hematoxylin-eosin (H-E) staining. Consecutive sections from the paraffin blocks were stained with aldehyde fuchsin for the identification of elastic fibers. The second half of the biopsy—used for the scanning electron microscopic study—was immediately rinsed with a cacodylate buffer and fixed in Karnovsky's solution (Karnovsky *et al.*, 1965).

The fixed biopsies were immersed in a 3.5N HCl solution for 2 to 3 weeks at room temperature (22–25°C). The HCl treatment caused the biopsies to exfoliate the epithelium from the underlying connective tissues precisely at the epithelial-connective tissue junction. The sections were washed with tap water and subsequently treated with a 0.5% tannic acid solution for 1 h at 4°C. The specimens were postfixed by immersion in 1% OsO₄ dissolved in a 0.1M phosphate buffer for 1 h at 4°C. The postfixed biopsies were dehydrated in graded series of ethanol and dried with *t*-butyl alcohol following a freeze-drying method (Inoue and Ohtake, 1988). The specimens were finally

examined under SEM after being coated with Pt and Pd (S-800, Hitachi-Hi-Technologies, Tokyo).

Results

Normal tissue

Light microscopy

In control specimens, the junction between the epithelium and the lamina propria ascended in peaks and descended into valleys forming numerous finger-like connective tissue cores (CTCs) (Fig. 1a). The arrangement, distribution, and density of the aldehyde fuchsin-positive fibers were regular and uniform. Numerous aldehyde fuchsin positive fibers (i.e., elastic fibers) ran towards the tips of the CTCs.

Electron microscopy

In tissues treated with HCl, the shape of the CTCs at the epithelial-connective tissue junction could be clearly observed by SEM because the epithelium was removed with this treatment. In tissue where the epithelium was exfoliated, finger-like projections of the connective tissue jutting out from the surface were apparent under the SEM (Fig. 1c). The arrangement of these projections was regular and dense. The distribution of the CTCs was even. Uniformly arranged woven connective tissue fibers were seen when the tissue profile was examined at a right angle plane to the CTC surface (Fig. 1b). The CTCs were approximately 100–350 µm long and 30–50 µm in diameter, with 500 CTCs per square millimeter in density.

Diseased tissue

Light microscopy

All the OSF biopsies and control samples were stained with the H-E and examined by light microscopy to confirm the diagnosis. The OSF sections exhibited an atrophic epithelium with a loss of rete-ridges. Bundles of collagen fibers extended downwards from the basement membrane. The deep connective tissue exhibited heavy fibrosis with dense collagen bundles occasionally extending into the underlying striated muscles. The density of the subepithelial collagen bundles varied between specimens due to differences in the grade of the disease. Infiltrations of chronic inflammatory cells were concentrated in the subepithelial connective tissue. All the experimental samples exhibited a unique arrangement of aldehyde fuchsin-positive fibers. Broad bundles were arranged in a

Fig.1. a: Light microscopic picture of the normal buccal mucosa of a healthy adult in the control group. The picture indicates the uniform distribution of aldehyde fuchsin positive fibers in the entire lamina propria. Bar = 100 μ m. **b:** Scanning electron microscopic picture of **a** after removal of the epithelium. Dense and uniformly woven connective tissue fibers are seen in the profile. Bar = 100 μ m. **c:** Tissue profile of the epithelial exfoliated CTCs shown in **b**. The distribution of the finger-like protrusions is even and regular on the surface. Bar = 50 μ m

Fig. 2. Photograph of buccal mucosal biopsy in OSF. The section was stained for elastic fibers using aldehyde fuchsin. Broader bundles of aldehyde fuchsin positive fibers are seen in the deeper layers (arrows). Fiber distribution is scanty in the superficial area (asterisks). Bar = 200 μ m

rather concentrated manner towards the deep portion of the connective tissue layers in contrast to the superficial area, where the density of aldehyde fuchsin positive fibers was fine and sparse (Fig. 2, 4a). The epithelium was not deeply anchored to the lamina propria in any of the diseased tissue that was observed, and an arcade appearance of the dermal papilla was lacking.

One of the diseased cases showed inflated capillaries and lymphatic ducts associated with infiltration (Fig. 2). Most of these inflated capillaries were lined with pericytes consisting of aldehyde-fuchsin positive fibers. Other cases (Fig. 4a) exhibited a thick but homogeneous appearance situated on the superficial part of the lamina propria. In the hematoxylin-eosin and masson-trichrome stained sections, this layer appeared nearly homogenous and looked hyalinated — although thin aldehyde-fuchsin positive fibers were sparsely scattered throughout this region.

Electron microscopy

Patchy attenuations of the CTCs were evident at the initial stages of the disease when the tissue profiles of the mild cases were examined (Fig. 3c). Uniformly arranged woven bundles of collagen fibers were visible, in a plane cut at a right angle to the CTC surface (Fig. 2b). However, in advanced cases of OSF, patchy degenerations lacking the finger-like CTCs were observed on the surface of the connective tissue (Fig. 4c). The CTCs around the degenerated patches and throughout the affected area were attenuated and significantly diminished both in size and number. The CTCs were approximately 40–300 μ m long and 25–50 μ m in diameter, with twenty CTCs per square millimeter in density distributed on the degenerated patches (Fig. 3c). Rod-like CTCs disappeared from the central area of the degenerative patches (Fig. 3c). The degenerative patches were distributed irregularly throughout the affected areas. However, the distribution of these patches, the degree of diminution in their size, and

Fig. 3. a: Scanning electron photomicrograph showing the surface appearance of the buccal epithelium in OSF. Bar = 50 μm .

b: Tissue profile of the epithelial exfoliated CTCs (when looking at a plane cut at a right angle to the CTC surface, in the mild cases of OSF). Bar = 100 μm . **c:** Degenerated patches on the CTC surface and attenuated CTCs around the patches. Bar = 500 μm

the density of the CTCs varied according to the severity of the disease. The CTCs were approximately 40–140 μm long and 40–50 μm in diameter, with five CTCs per square millimeter in density distributed on the degenerated patches (Fig. 4c). In advanced cases where the fibrosis was heavy and homogenous by light microscopy, the appearance of the surface after removing the epithelium was highly degenerative by SEM, in that finger-like CTCs were completely absent. Only numerous horizontally running ridges were seen on the flat smooth surface. No uniformly arranged woven texture of collagen fibers was observed when the tissue profile was examined in the surface cut at a right angle to the CTC surface (Fig. 4b). The disturbance in the uniformity of the woven architecture of the collagen bundles may be due to heavy fibrosis.

Discussion

The present investigation revealed that SEM observation of the CTCs proved very informative for a three-dimensional morphological evaluation of the mucosal connective tissue. This method was previously used in comparative anatomical studies of the lingual papillae (Yoshimura *et al.*, 2000, 2002; Silva *et al.*, 2002).

Studies of three-dimensional morphological changes of the mucosal connective tissues in OSF should aid in understanding further aspects of the disease. Our SEM observations is the first to clearly demonstrate morphological changes in the CTCs beneath the epithelial layer of the lining mucosae affected by OSF; a gradual attenuation of the CTCs was often observed in OSF, and patchy degenerations lacking the CTCs were also scattered over the affected area. Our findings also revealed that the patchy degenerative areas may increase gradually and

Fig. 4. Legend on the opposite page.

spread throughout the affected area as the disease becomes more advanced, thus finally displaying a flat surface with horizontally running ridges on the surface of the connective tissue. Structural deviation in OSF may be a response in the disease process, which is induced through chronic chemical irritants. The ultrastructural changes in the CTCs lying beneath the epithelium in OSF have not been previously reported, while similar degenerative changes in the CTCs have been noted in the lingual mucosa of human subjects as a result of the aging process (Kobayashi *et al.*, 2001; Yoshimura, unpublished data). However, the degeneration observed in the CTCs of the lingual mucosa has been irregular in contrast with that found in the aged mucosa (Kobayashi *et al.*, 2001; Yoshimura, unpublished data). There have been some hypotheses that this disease is caused by combinations of several factors such as chemical irritants, and not only arecoline but also i.e. capsaicin (Sirsat and Khanolkar, 1960), nicotine (Chang *et al.*, 2001), or other etiologic factors, i.e. nutritional factors (Pillai *et al.*, 1992), auto-antibodies (Canniff *et al.*, 1986). Betel nut chewing used to be frequently associated with tobacco leaf and limestone. Furthermore, Chang *et al.* (2002) analyzed metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) in OSF-derived cultured cells and showed that arecoline acted not only as an inhibitor on gelatinolytic activity of MMP-2 but also as stimulator on TIMP-1. Gelatinases (a.k.a., MMP-2; type IV collagenases) may be especially important for the development of tissue fibrosis (Chang *et al.*, 2002). A synergistic effect among several etiologic factors may be affected to this inconstant degeneration.

Though the structural changes were found at the CTCs on the bearing surface, no remarkable electron microscopic differences were observed between the epithelial surfaces of the normal buccal mucosa and the mucosa in OSF. Based on the pathological textbook by Shafer *et al.* (1993), epithelial atypia sometimes may be present on typical OSF. Reichart *et al.* (1984) reported the presence of some pitted cell surfaces and microridges on the epithelium — although

the morphological changes were rather minor in their studies. Extensive research using more numerous samples from different grades of the disease is needed to confirm the findings in the present study.

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Fig. 4. a: Photomicrograph of the buccal mucosa severely affected with OSF. The section was stained with aldehyde fuchsin to demonstrate the distribution of densely packed thick bundles of aldehyde fuchsin positive fibers in the deep layers and sparsely arranged thin bundles in the superficial layers of the lamina propria. Bar = 100 μ m. **b:** Scanning electron microscopic picture of the buccal mucosa in OSF after removing the epithelium. Both the CTC surface and the right angle plane are demonstrated in the picture. Heavily packed fibers give a homogeneous appearance to the superficial layers of the lamina propria. Bar = 50 μ m. **c:** Scanning electron microscopic picture demonstrating highly degenerative patches on the CTCs surface of the epithelium-removed buccal mucosa in advanced OSF. Bar = 50 μ m

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