Age and the architecture of oral mucosa

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Abstract Age changes affect the oral mucosa (the protective lining of the oral cavity), but few of these have been studied objectively. The aim of this study was to quantitatively analyse a number of morphometric parameters of the ageing oral mucosa. The fractal dimension of the epithelial connective tissue interface (ECTI) was estimated in 42 samples of normal buccal mucosa to correlate any changes in their irregularity to the age of the individuals. Morphometric parameters extracted from theoretical cell areas computed programatically were also analysed. Results showed no significant change in ECTI complexity associated with age; however, there was

indication that epithelial cells tended to become larger and flatter with age. Interestingly, while some parameters did not show significant differences case wise, cluster analysis showed that the data clustered the cases into three main age groups: one representing the first two decades of life, another group represents adult life (21–50 years) and the last group representing the ageing population (50–90 years).

Keywords Ageing · Oral mucosa · Fractal · Tissue architecture · Morphology

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Introduction

With the advances in medicine and extended average human life expectancy, there is a tendency for the number of geriatric individuals to increase. It is therefore important to understand the ageing process of human tissues to aid in the care of that sector of the population.

The lining of the oral cavity provides protection from the forces of mastication and other potentially noxious effects. The ageing of the oral mucosa has so far been characterized mostly in relation to changes in the oral epithelium such as less prominent rete ridges, decreased mean thickness (Shklar 1966; Scott et al. 1983; Williams and Cruchley 1994), decreased cell density (Hill 1988), decreased mitotic activity and consequently a slowdown in tissue regeneration and healing rates (Barakat et al. 1969; Karring and Loe 1973; Hill et al. 1994).

Unfortunately, few studies have concentrated on quantitative morphological changes associated with the ageing of the oral mucosa. Therefore, the aim of this work is to quantitatively and objectively study the architecture and morphology of histological sections representing normal oral mucosa (from biopsies with no mucosa-related pathology) from different age groups, to elucidate any associated age changes. Characterisation of the architectural tissue changes associated with ageing may help to understand the mechanisms leading to these tissue alterations and facilitate their monitoring.

Materials and methods

Samples

Forty-two normal human buccal mucosa samples were obtained from the Oral Pathology archives of the Graduate School of Medical and Dental Science, Niigata University, Japan. The cases represented nine different age groups (each group representing a decade in life ranging from the first to the ninth decade). Twenty-four cases were from female patients (mean age, 52 ± 19 years), while the remaining 18 cases were from male patients (mean age, 53 ± 26 years). The distribution of cases along different age ranges is shown in Table 1.

Imaging

Forty-two digital images of the cases were captured at $\times 4$ magnification (resolution, $1.61 \mu\text{m}$) using an

Eclipse E800 Microscope (Nikon, Japan) with a DXM1200C high-definition cooled colour digital camera (Nikon, Japan). Images representing three different age groups are shown in Fig. 1. Twenty-six images representing the same age groups were captured at $\times 20$ magnification (resolution, $0.32 \mu\text{m}$).

Image analysis

The image processing and analysis was conducted using the popular ImageJ platform (Rasband 1997–2010). Images were analysed at two levels of organisation:

(A) The tissue level

The $\times 4$ images were processed to isolate the epithelium from the underlying connective

Fig. 1 Haematoxylin and eosin (H&E)-stained normal oral buccal mucosa sections, $\times 4$ objective. **a** Mucosa from a 4-year-old patient, **b** from a 48-year-old patient and **c** from an 81-year-old patient

Table 1 Mean box fractal dimension and the number of cases for different age groups

Age range (years)	Mean box fractal dimension	Number of cases
0–10	1.1069 ± 0.03267	2
11–20	1.1117 ± 0.08987	2
21–30	1.1414 ± 0.07095	4
31–40	1.1290 ± 0.03822	4
41–50	1.0933 ± 0.05661	5
51–60	1.1373 ± 0.05657	8
61–70	1.0992 ± 0.04456	9
71–80	1.1260 ± 0.04751	5
81–90	1.0903 ± 0.01675	3

tissue and the background. This was achieved using a multilevel thresholding procedure (Otsu 1979) applied to the green channel of the images. Profiles of the epithelial connective tissue interface (ECTI) were then extracted using a Laplacian edge detector filter. Further details of the procedures used here have been published elsewhere (Abu Eid and Landini 2003). The complexity of the ECTI reflects the degree of the rete pegs' prominence. This was estimated using fractal geometry (Abu Eid and Landini 2003) which provides different methodologies to estimate the degree of complexity of objects by means of fractal dimensions (a number that represents space-filling properties of objects). The box counting method was used in this study to estimate the global fractal dimension of the ECTI.

(B) The cellular level

The $\times 20$ images were processed to segment the epithelial compartment into theoretical cell areas (virtual cells or v-cells). The epithelial compartments were first isolated using a combination of multilevel thresholding (Otsu 1979) and manual separation (in cases where the staining properties of the samples provided little contrast between different tissues). The v-cell borders were determined automatically by localization of nuclei based on the optical density of the haematoxylin stain after applying a colour deconvolution algorithm (Ruifrok and Johnston 2001). Subsequently, the nuclei were converted into nuclear “seeds” using *greyscale reconstruction* (Landini and Othman 2003), and finally, a watershed transform (Vincent and Soille 1991) was applied to the result of the reconstruction to segment the epithelial compartment into areas of influence unique to each nuclear seed. An example of such analysis is shown in Fig. 2.

Cellular morphologic properties were extracted from the segmented images and included: v-cell perimeter, area, radius of the inscribed circle centred at the centre of mass, radius of the enclosing circle centred at the centre of mass, largest axis length (Feret's diameter), breadth, convex hull, area of the convex hull polygon, radius of the minimal bounding circle, aspect ratio, roundness, area equivalent diameter,

Fig. 2 **a** $\times 20$ H&E image of normal oral epithelium, **b** haematoxylin component of **a** obtained by colour deconvolution. **c** Segmented epithelial compartment after applying the watershed transform. **d** Segmented epithelial compartment after performing the logical AND operation between images **a** and **c**. The inter-cell space is one pixel wide, but it has been widened via a morphological erosion for visualisation purposes

perimeter equivalent diameter, equivalent ellipse area, compactness, solidity, concavity, convexity, shape, R-factor, modification ratio, sphericity and rectangularity (Landini 2010).

Statistical analysis

Statistical analysis of the data was performed using SPSS version 17 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to compare the means of the morphological parameters across the different age groups; post hoc Tukey's honestly significant difference test was used to disclose how homogenous subset data relate to the actual age groups, and Ward's cluster analysis was performed to reveal any natural clustering of the data. Significance of the statistical test was considered for values of $p < 0.05$. Cluster analysis was done using R version 2.11.0 (R foundation for statistical computing, <http://www.r-project.org/foundation/>).

Results

The mean box fractal dimension for each age group is shown in (Table 1). No significant trends in the ECTI complexity values with age group were found (one-way ANOVA, $p>0.05$).

A total of 52,507 v-cells from a selection of cases ($n=26$) were analysed for various morphological parameters. Despite some of the parameters analysed (case-wise) not showing statistically significant differences across the different age groups (one-way ANOVA, $p>0.05$), cluster analysis showed that two main clusters exist with different average ages; the first cluster had a mean age of 42.5 years, which is 22.2 years younger than the second cluster (mean age, 64.7 years). Figure 3 shows the dendrogram of clustered data.

Comparing the cellular morphological parameters cell wise (rather than averaging the parameters per

case) showed statistically significant differences between the v-cells of different age ranges (one-way ANOVA, $p\leq 0.001$).

The homogeneous subsets in the data (post hoc Tukey's honestly significant difference test, $p>0.05$) based on different parameters clearly suggested that morphological differences were present between three main age ranges; the first included cases from the first two decades of life (0–20 years), the second included cases between 21 and 50 years of age and the third included cases over 50 years of age.

Table 2 shows the changes in some of the parameters across these three main age groups. Parameters measuring the size of the v-cells (area, perimeter, radius, breadth, etc.) clearly displayed an increase in v-cell size with age. Concavity also increased with age, while convexity decreased. Roundness, circularity and sphericity also tended to decrease with age, indicating that the v-cells became flatter with ageing.

Fig. 3 Dendrogram (Ward's method) of the cellular parameters. *Numbers in italics* represent the mean age for each cluster

Table 2 Mean values for some of the cellular parameters for the three main age groups

Age range (years) parameter	0–20	21–50	51–90	Change with age
Area (pixel ²)	1,070	1,215	1,332	Increase
Perimeter (pixels)	135.34	148.08	152.19	Increase
Feret's diameter (pixels)	48.9	52.1	54.7	Increase
Breadth (pixels)	32.8	34.5	35.3	Increase
Aspect ratio	1.51	1.53	1.56	Increase
Circularity	0.65	0.64	0.63	Decrease
Roundness	0.52	0.52	0.51	Decrease
Compactness	0.71	0.71	0.70	Decrease
Concavity (pixel ²)	160	206	233	Increase
Convexity	0.942	0.937	0.936	Decrease
Sphericity	0.44	0.43	0.42	Decrease
Rectangularity	0.577	0.578	0.576	Increase then decrease

The table also notes the trend changes these parameters undergo with ageing

Discussion

There have been various age-related changes of the oral mucosa described in the literature, but few of those studies have used quantitative approaches. Most work has concentrated on changes of the oral epithelium such as decrease of the prominence of rete ridges (Scott et al. 1983), decreased mean thickness (Shklar 1966; Scott et al. 1983; Williams and Cruchley 1994; Stablein and Meyer 1988; Montagna 1965; Christophers and Kligman 1965; Squier et al. 1975; Giacometti 1965) which affects the physical barrier that the epithelium provides (Powell 1981), decreased cell density (Hill 1988), decreased mitotic activity and cell proliferation (Cameron 1972; Leith 1978; Whiteley and Horton 1963) and a slowdown in tissue regeneration and healing rates (Barakat et al. 1969; Karring and Loe 1973; Hill et al. 1994; Engeland et al. 2006).

Most of these observations have been understood as a progressive atrophy of the oral mucosa; however, other studies found no change in the oral epithelial thickness (Cameron 1972; Loe and Karring 1971; Lavelle 1968) or in the proliferation rate of epithelial cells (Hornstein and Schell 1975) with some even reporting increased epithelial thickness (Ryan et al. 1974) and increased rate of cell renewal (Andrew and Andrew 1956; Toto et al. 1975).

In our study, 42 cases of normal oral mucosa were analysed at the tissue and cellular levels. The samples included cases from the first nine decades of life. When the fractal analysis data were analysed based on nine groups (each representing a decade of life), the

results did not show any particular trends of change. However, cell-wise analysis revealed more obvious age changes particularly in three age groups that were found as separate homogenous subsets (post hoc Tukey's honestly significant difference test), the first representing the growth spurt (the first two decades of life), the second representing the adult life (20–50 years) and the third group representing the ageing adult (51–90 years). Classification of patients into three age groups was also reported by Zimmermann and Zimmermann (1965), who divided their sample into three age groups, after using six groups failed to display any definite patterns on their data. However, they had different age ranges (10–29, 30–49, 50 years and older). Cowpe et al. (1985) divided patients into four age groups: <21, 21–40, 41–60 and >60 years. In both these studies, however, the methods through which the age groups were decided were unclear and appear to be somewhat arbitrary, while in our study, the age groups we analysed were based on the natural grouping of the data, hence eliminating any bias caused by predetermining the age groups.

In the current study, we found no change in the irregularity of ECTI profiles with advanced age. While these results agree with the findings of Hill (1988), they disagree with those of previous studies reporting a decrease in the rete ridges' surface area of lingual epithelium and a continuous atrophy and simplification of structure throughout the adult life (Scott et al. 1983). Less prominent rete ridges have also been described in the ageing skin (Montagna and Carlisle 1990; Burns et al. 2004); however, the methodology that leads to this description is not clear.

In our study, the complexity of the ECTI profiles was determined objectively using a reproducible methodology based on fractal geometry principles. The reduced complexity and decreased convolution of the rete ridges have been attributed to a reduction in the number of basal cells which determine the shape of the ECTI profiles. Some authors found no change in basal cell numbers with age (Hill 1988; Karring and Loe 1973; Cameron 1972) which would seem to agree with our results (with no consequential change in ECTI complexity). Others however reported a decrease in the number of basal cells per millimetre length of epithelium (Sharav and Massler 1967). Interestingly, no indications have been made on the relation between epithelial atrophy and the rate of remodelling of the underlying connective tissue. While epithelium atrophy could in principle take place without the remodelling of the lamina propria, for modulation of epithelial thickness and rete peg prominence to keep the same pace, it would require a re-building of the fibrous collagenous structure of the connective tissue compartment. In this sense, it is possible that these two events (epithelial thickness and rete peg prominence) are modified at different rates and by different amounts over time, thus giving a possible explanation to the contradicting reports.

In our study, we used the fractal dimension as a measure of irregularity for ECTI profiles. This method has been used previously to describe changes in ECTI complexity in normal, dysplastic, malignant and pseudomalignant oral lesions (Abu Eid and Landini 2003, 2005a, 2006, 2005b). The mean box fractal dimension for ECTI profiles from the buccal mucosa in this study (1.16) was comparable to that in a previous study (1.07) (Abu Eid and Landini 2003). Other studies however used different methods to describe the change in ECTI irregularity such as measuring the rete ridges' surface area (Scott et al. 1983) or measuring the length of the basement membrane using a sonic digitizer (Hill 1988).

A wide range of cellular morphometric features was described in this study. Our findings suggest that epithelial cells become larger with age as measured by cell area, perimeter, Feret's diameter and breadth, among others. These results contradict those of Pappelis et al. (1973), who did not detect any significant variation in nuclear and cell size of buccal squames with ageing.

In their study, Cowpe et al. (1985) found a significant difference in nuclear area with ageing,

but no difference in cell area. It is important to point out, however, that Pappelis et al. (1973) and Cowpe et al. (1985) calculated the size of squames from smears; our data were generated from tissue sections. Squames may not necessarily represent the true shape and size of all epithelial cells as they belong to a particular stage of maturation. In addition to that, in their study, Pappelis et al. (1973) determined the optical path difference of selected nuclei using a transmitted light interference microscope. Photomicrographs of the nuclei were then projected and manually traced, and the nuclear dry mass was then calculated using the optical path difference, the nuclear area and the refractive index of nuclear proteins. In our study, however, we calculated the cell area directly from tissue sections, and we measured the cellular area of all the cells in each image rather than select some. No change in cell size with age has also been reported by Hill (1988) in the mouse oral mucosa, although they found an increase in the ear and footpad mean epithelial cell size.

We also found that epithelial cell shape changes with increasing ageing. Our findings suggest that the cells become flatter with age as measured by convexity, concavity, roundness, circularity and sphericity. This might be attributed to changes in the level of maturation of the cells as suggested by Dhawan and Toto (1965) in relation to the reduction of cells in the population which might allow them to remain in the epithelium for a longer time and to mature further. They suggest that this accounts for the increased number of cells in the granular and keratin layers and the reduction of cells in the prickle cell layer.

The flattening of the cells coupled with the increase in their size could explain the apparent reduction in both epithelial thickness and cell proliferation reported in the literature. Flatter cells occupy less space than normal-shaped cells, and although they are larger in size, they are less numerous, giving the overall effect of reduced epithelial thickness. Further studies are needed to confirm any relations between cell size, shape and overall epithelium thickness since our analysis is based on 2D sections of a 3D tissue. More fine-grained analysis, such as layer-by-layer analysis, may also uncover other changes in the ageing oral mucosa that currently may be masked by the pooling of all cell maturation stages.

Preliminary quantitative results obtained in this study indicate that ageing of the oral mucosa does not seem to affect significantly the irregularity of the epithelial connective tissue interface in the range of scales investigated. Mean (case-wise) morphological cellular features extracted from theoretical cell constructs also showed no tendency to change with the ageing process when considered alone, but when submitted to post hoc Tukey's honestly significant difference test, natural groups with different average ages emerged.

When morphological features were compared cell wise, statistically significant differences were found to exist between three main age ranges; 0–20 years, 21–50 years and 51–90 years.

Further studies are needed to look into the association of these variables and further morphological features of epithelia in different age groups preferably using large sample sizes. Animal models might also provide further insights into the problem. Studies of the ageing mucosa at the molecular level will also provide information which can be coupled with morphometric studies to help further understanding of these changes.

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