Morphometric analysis of atrophic changes in human lingual epithelium in iron deficiency anaemia

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SUMMARY A stereological analysis of epithelial structure at the lateral surface of the tongue showed that iron deficiency anaemia was associated with reduced epithelial thickness despite the absence of overt mucosal abnormalities. The epithelial atrophy was entirely due to a reduction in the size and number of cells in the maturation compartment. By contrast, the progenitor cell compartment was increased in thickness due to an increase in the number of cells. This hyperplastic reaction may be a trophic response to the overall loss of epithelium in this condition.

Although the association of clinical atrophy of the oral mucosa with iron deficiency anaemia has long been recognised at a descriptive or semiquantitative level,^{1–5} only recently have systematic quantitative analyses been made of the histological changes.^{6,7} The morphometric studies to date, however, have been confined to the buccal mucosa, and little is known of the quantitative changes developing in the lingual mucosa. This is despite the fact that the tongue is the site most commonly and severely affected by the intraoral lesions of iron deficiency anaemia.⁸ Furthermore, not all cases of iron deficiency anaemia give rise to clinically discernible oral lesions,^{2,8} and it is not known if microscopic changes occur in those cases of iron deficiency anaemia that are free of overt mucosal abnormalities.

In view of these uncertainties concerning human lingual epithelium in iron deficiency anaemia we took the opportunity provided by a recent programme of investigations into the influence of age, sex, alcohol, and smoking on epithelial structure in clinically normal tongues^{9,10} to examine an additional group of cases with haematological evidence of iron deficiency anaemia. This paper, therefore, compares various stereologically determined variables of epithelial structure at the lateral surface of the tongue between a series of cases of iron deficiency anaemia and a large series of non-anaemic, iron sufficient cases, which served as a control.

Material and methods

All samples of lingual mucosa were obtained from

necropsies performed within 48 hours after death. Criteria for selecting necropsies were identical between the two groups save for the presence or absence of iron deficiency anaemia. Three general exclusions were applied to eliminate conditions known to affect the oral mucosa. These were systemic diseases—namely, leukaemia, lymphoma, rheumatoid disease, diabetes mellitus, and megaloblastic anaemia; therapeutic history—namely, radiation to the head or neck, and immunosuppressive or cytotoxic drugs; and local pathological changes—namely, macroscopic abnormality, histopathological change, and autolytic change. All the tongues were macroscopically normal in appearance. Age and sex were recorded along with a detailed history of smoking habits and alcohol consumption over the preceding five years, which was obtained by interrogation of close relatives. Whenever this information was absent or unreliable the case was deleted from the study. All cases were of sudden death and, therefore, were generally without the complications associated with chronic illness and the administration of drugs. The selection and composition of the control series of necropsies has been described fully elsewhere.¹⁰

Iron deficiency anaemia was diagnosed by the twin criteria of a low mean corpuscular haemoglobin in blood samples at necropsy (<27 pg/cell) and the absence of stainable iron in samples of sternal marrow treated by Perl's method.¹¹ Those that gave contrasting results were eliminated from both series of necropsies, as were cases of megaloblastic anaemia diagnosed from examination of the sternal marrow by the method of Berenbaum.¹² After all exclusions had been made there were 34 cases in the

series with iron deficiency anaemia and 161 in the control series.¹⁰

Standardised samples of mucosa, measuring $2 \times 1 \times 1$ cm, were carefully dissected from the middle third of the lateral surface of the tongue midway between the lateral circumvallate papilla and the tip. Fixation was by formol acetic methanol for 24 hours followed by storage for several days in buffered formol saline. The blocks were routinely processed for paraffin embedding and carefully orientated for sectioning perpendicular to the epithelial surface. Five sections of $5 \mu\text{m}$ thickness were cut from each of five levels throughout each block. By random numbers one section was selected from each level for the stereological analyses and stained by haematoxylin and eosin.

STEREOLOGICAL ANALYSES

All measurements were made by stratified random sampling over the five sections from each block using an automatic stage microscope and a televised system of projection, which allowed the superimposition on to the monitor screen of the magnified image of the section and the various measuring grids. The methods used are given in detail by Scott *et al.*⁹

The mean epithelial thickness and the thickness of epithelial compartments were estimated by the method of Eveson and MacDonald.¹³ The compartments comprised the maturation layer and the progenitor layer. The layers were distinguished by the level at which cells became wider than their height.⁹

Mean cell numbers were calculated by counting the numbers of nuclear profiles in each compartment within a standard counting column $35 \mu\text{m}$ wide and taking the mean of at least 35 counts. The mean cell sizes were assessed as mean cell areas, obtained by dividing the previously estimated areas of the progenitor layer and maturation layer by the number of cells in each, which had been determined from nuclear counts. The nucleocytoplasmic ratio was obtained from simple estimations of area by differential point counting with the Holmes correction.¹⁴ The determination of nuclear diameter was made by direct measurement of nuclear profiles using a video position analyser (VPA 1000, Leitz Instruments Ltd) and correcting by Abercrombie's method,¹⁴ all as previously described.⁹

Comparative estimates of the surface areas of the upper and lower boundaries of the epithelium were made by calculating the mean number of intersections for every $500 \mu\text{m}$ of surface length made by a Merz alternate semicircles grid,¹⁴ measuring at least 35 fields for each sample.

Means and variances were determined for each variable and the differences between the iron defi-

ciency anaemia and the control series were tested by Student's *t* test with significance accepted at the 5% level of probability.

Results

As our previous studies on the series of cases without iron deficiency anaemia had already indicated that age, sex, and increasing levels of alcohol consumption and tobacco smoking affect epithelial structure^{9 10} each of these factors was tested for its association with iron deficiency anaemia by χ^2 analysis (Table 1). This showed a significant degree of association between iron deficiency anaemia and female sex but not with the other independent variables. For this reason the results were treated separately for each sex.

MEAN EPITHELIAL THICKNESS, THICKNESS OF MATURATION LAYER, AND THICKNESS OF PROGENITOR LAYER

From the Figure it can be seen that the mean epithelial thickness was reduced in cases of iron deficiency anaemia in both men and women. Only in men, however, was the difference significant. In men in the control series the mean epithelial thickness was 266 (SEM 6.61) μm , and this underwent reduction of 27% in the iron deficiency anaemia series to 194 (20.20) μm . For this difference $t = 3.36$ ($p < 0.01$). In women the mean epithelial thickness was 228 (7.80) μm in the control series and 206 (7.83) μm in the iron deficiency anaemia series. The Figure also shows the contribution made to the mean epithelial thickness by each of the epithelial cell layers, maturation layer and progenitor layer: the thickness of the progenitor layer was higher in iron deficiency anaemia in both men

Table 1 Association between independent variables and presence of iron deficiency anaemia

Iron deficiency anaemia and:	χ^2	df	p
Age (years):			
16-55	0.09	1	0.76
56-98			
Sex:			
Men	4.54	1	0.03
Women			
Alcohol consumption (units/day)*:			
0	5.20	3	0.16
<4			
4-6			
>6			
Tobacco consumption (cigarettes/day)†:			
0	2.43	3	0.49
<11			
11-24			
>24			

*One unit of alcohol = $\frac{1}{2}$ pint of beer = 1 glass of wine = 1 measure of spirits.

†Five cigarettes = two pipes = one cigar.

Mean epithelial thickness of lateral tongue mucosa in men (M) and women (F) in series of necropsies in cases of iron deficiency anaemia and in control series without iron deficiency anaemia. Column heights show mean epithelial thickness divided into progenitor layer and maturation layer. Differences in mean epithelial thickness and thickness of maturation layer between iron deficiency anaemia and control series were significant in men only. Differences in progenitor layer were significant for both sexes.

and women. In men the thickness of the progenitor layer increased from 32.7 (0.43) μm to 35.9 (1.13) μm , for which $t = 2.69$ ($p < 0.05$). In women the thickness of the progenitor layer increased from 31.6 (0.72) μm to 36.7 (1.25) μm , for which $t = 3.59$ ($p < 0.01$). Expressed as a percentage of mean

epithelial thickness, the thickness of the progenitor layer increased from 12.2% to 18.5% in men and from 13.9% to 17.8% in women. Clearly, the reductions in mean epithelial thickness shown in the Figure were entirely due to reductions in the thickness of the maturation layer. Only in men was this reduction significant: in the control series the thickness of the maturation layer in men was 233 (6.60) μm and this reduced to 158 (19.26) μm ($t = 3.67$, $p < 0.01$), while in women the thickness of the maturation layer in the control series was 195 (7.83) μm and this reduced to 170 (14.78) μm in the iron deficiency anaemia series.

NUMBERS OF CELLS IN THE PROGENITOR AND MATURATION LAYERS

Table 2 (men and women, respectively) summarises these results. It can be seen that the number of cells in the progenitor layer was significantly higher in the iron deficiency anaemia series in both men and women by a difference of 14.2% in men and 12.3% in women. By contrast, in the maturation layers the numbers of cells showed a reduction in the iron deficiency anaemia series, compared with the control values, amounting to 16.4% in men and 12.6% in women. For each sex the difference was significant (Table 2).

SIZES OF CELLS IN THE PROGENITOR AND MATURATION LAYERS

The size of cell in the progenitor layer remained unchanged in both series in men and women (Table 2). The size of cell in the maturation layer was significantly reduced, however, in the iron deficiency anaemia series in men (Table 2) but not in women (Table 2).

MEAN NUCLEAR DIAMETER AND NUCLEOCYTOPLASMIC RATIOS IN THE PROGENITOR LAYERS

The mean nuclear diameter remained virtually unchanged at ≈ 8.0 μm (range of group means

Table 2 Mean (SEM) numbers and sizes (areas) of cells in progenitor and maturation compartments of epithelium from lateral surface of tongues of men and women in presence or absence of iron deficiency anaemia

Variable	Iron deficiency anaemia series		Non iron deficiency anaemia series t			
	Men (n = 12)	Women (n = 22)	Men (n = 92)	Women (n = 69)	Men	Women
No of cells in progenitor layer	12.9 (0.55)	11.9 (0.30)	11.3 (0.13)	10.6 (0.18)	2.74*	3.59†
Size of cells in progenitor layer (μm^2)	99.6 (3.66)	106 (2.71)	102 (1.39)	105 (2.49)	0.48	0.24
No of cells in maturation layer	14.3 (1.03)	13.9 (0.83)	17.1 (0.44)	15.9 (0.57)	2.47*	2.00*
Size of cells in maturation layer (μm^2)	380 (34.1)	420 (18.7)	490 (13.4)	444 (17.1)	2.99*	0.96

*Significant at $p = 0.05$.

†Significant at $p = 0.001$.

7.96–8.05 μm) irrespective of sex or the presence or absence of iron deficiency anaemia. The nucleocytoplasmic ratio, however, was significantly higher in iron deficiency anaemia in both men and women. In men the nucleocytoplasmic ratio was increased from 0.28 (0.005) μm to 0.32 (0.012) μm ($t = 2.78$, $p < 0.05$). In women the nucleocytoplasmic ratio was increased from 0.27 (0.01) μm to 0.31 (0.01) μm ($t = 3.63$, $p < 0.01$).

MEAN NUMBERS OF INTERSECTIONS AT THE UPPER AND LOWER EPITHELIAL BOUNDARIES

At the lateral surface of the tongue the upper boundary is normally almost flat, so that the mean number of intersections at this surface is low and relatively small increases in surface area produce significant increases in the mean number of intersections. In the control series the mean number of intersections (upper) in men was 5.5 (0.03) μm and in women 5.4 (0.03) μm . In the iron deficiency anaemia series these values were significantly increased. In men the mean number of intersections (upper) was 5.7 (0.03) μm ($t = 3.90$, $p < 0.01$) and in women it was 5.6 (0.04) μm ($t = 2.72$, $p < 0.05$). At the lower epithelial boundary there was evidence of a flattening of the rete structure associated with iron deficiency anaemia, but this occurred only in men: the mean number of intersections (lower) reduced from 7.3 (0.15) μm to 6.7 (0.20) μm ($t = 2.39$, $p < 0.05$). In women, however, this variable was unchanged in the control series (7.1 (0.19) μm) and the iron deficiency anaemia series (7.1 (0.22) μm).

Discussion

We used low mean corpuscular haemoglobin as a criterion for anaemia because, unlike the estimation of whole blood haemoglobin, this variable is unaffected by the separation of blood constituents that occurs after death. We validated this use of mean corpuscular haemoglobin at necropsy by comparing the values of mean corpuscular haemoglobin shortly before and after death in five randomly chosen cases that came to necropsy. There was a mean difference of under 4% between mean corpuscular haemoglobin values before and after death. Low mean corpuscular haemoglobin is itself an indication of hypochromic anaemia, but in each case in our iron deficiency anaemia series there was additional supporting evidence of depleted iron stores in the marrow. The use of the two criteria together plus the rigorous exclusion of equivocal cases enabled us confidently to ascribe cases to separate groups on the basis of the presence or absence of iron deficiency anaemia.

Age and smoking levels were evenly distributed between the iron deficiency anaemia series and the non-iron deficiency anaemia series (Table 1) and we have reported our results separately for sex. The value of χ^2 (Table 1) for the levels of alcohol consumption in cases of iron deficiency anaemia, however, although not significant at the 5% level, suggests that an association between these two independent variables may have influenced our results. Close examination of our data showed that this association was negative for iron deficiency anaemia: there were marginally higher levels of alcohol consumption in the control series. As alcohol is associated with epithelial atrophy in the tongue,¹⁰ it follows that the small imbalance between our two series diminished rather than exaggerated the differences due to iron deficiency anaemia which have been reported here. The relatively small number of cases in the iron deficiency anaemia series precluded any analysis of the effects of interactions between more than any two of the five independent variables examined.

The stereological methods used here were based on the techniques for epithelial measurement described by Franklin and Craig¹⁵ and Eveson and MacDonald.¹³ No corrections were made for shrinkage of tissue during fixation or processing, and no account was taken of intercellular spaces, particularly in the estimations of sizes of cells. Moreover, cell size was estimated as an area rather than volume.¹⁵ Furthermore, the definition of the progenitor layer that we used, although convenient and reproducible, probably exaggerates the true extent of this layer. For these reasons the variables of lingual epithelial structure reported here cannot be regarded as absolute and should be used cautiously in any comparison with values reported from other laboratories. Nevertheless, they provide a valid and useful basis for comparisons within our study and allowed us to quantify the extent of differences between our two series of cases.

This investigation, therefore, has provided quantitative data to support the notion that lingual epithelial atrophy occurs in human iron deficiency anaemia. It is important to note that the histological changes reported occurred in the absence of overt clinical abnormality of the oral mucosa. These findings suggest, therefore, that the painful, erythematous glossitis that typically develops in some 30–40% of patients with iron deficiency anaemia^{2,8} has its origin in an underlying generalised atrophy of the lingual epithelium.

Our results indicate that the reduced epithelial thickness in iron deficiency anaemia is due to a reduction in the thickness of the maturation layer (Fig.), which in turn is caused by loss and shrinkage

of cells in this epithelial compartment in iron deficiency anaemia. These changes in the tongue, therefore, closely parallel changes in the structurally similar but thicker stratified squamous epithelium in the cheek recently reported in patients with iron deficiency anaemia by Rennie *et al.*⁶ In the buccal mucosa the reduction attributed to iron deficiency anaemia, uncorrected for age or sex differences, was 46% of the control value.⁶ By comparison, the reduction associated with iron deficiency anaemia in lingual epithelium for men and women combined, in this study, although significant, was only 19%. The reason for this much smaller reduction may be partly due to the uneven distribution of the high levels of alcohol consumption in the iron deficiency anaemia and non-iron deficiency anaemia series. This factor may also underlie the fact that the differences in mean epithelial thickness and thickness of the maturation layer in women did not achieve significance, although this could also be due to a smaller magnitude of changes occurring in the epithelium of women as the mean epithelial thickness is already 16% thinner than that of men.⁹

The results of this study also indicate that reactive changes occur in the progenitor cell compartment in iron deficiency anaemia in both men and women (Fig.). Not only was the progenitor layer some 13% thicker in iron deficiency anaemia (both sexes combined) but also the nucleocytoplasmic ratio in the progenitor layer was increased by about 12%, which, together with the finding of an unchanged nuclear diameter, suggests that there may have been a small reduction in the size of cells in the progenitor layer, despite the failure to show this by direct estimation of area (Table 2). An increase in nucleocytoplasmic ratio, resulting from a reduced cytoplasmic diameter and an increased nuclear diameter, was found by Monto *et al.* in desquamated cells from the dorsum of patients with iron deficiency anaemia and accompanying glossitis.⁵ The different site and the presence of inflammatory changes, however, suggest that these findings may not be strictly comparable with those of the present study.

A possible explanation for the hyperplasia in the progenitor layer associated with iron deficiency anaemia in this investigation may be a trophic effect consequent on the reduced epithelial thickness. The smaller size and reduced number of cells in the maturation compartment of the epithelium in iron deficiency anaemia could result in lower concentrations of inhibitory chalone, which in turn could lead to a proliferative response in the progenitor layer. Consistent with this notion is the evidence from studies on cell kinesis carried out on the ventral tongue

mucosa of hamsters,¹⁶ which showed increased rates of production of cells in animals with iron deficiency anaemia. Moreover, the argument in favour of a general mechanism for the increase in the progenitor layer, rather than as a specific effect of iron deficiency anaemia, is supported by our observations on the effects of alcohol and smoking on human lingual epithelium,¹⁰ where reductions in mean epithelial thickness were accompanied by hypertrophy of the progenitor layer.

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