ACTA STEREOL 1983; 2/1: 109-117
PAPER

STEREOLOGICAL ASSESSMENT OF THE EPITHELIAL-CONNECTIVE TISSUE JUNCTION IN EXPERIMENTAL ORAL CANCER

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ABSTRACT

In the present report, morphological changes at the epithelial-connective tissue junction have been evaluated in carcinomas induced by the application of DMBA to hamster cheek pouch mucosa. Stereological intersection counting procedures were used to quantify hemidesmosomes (HD) and lamina densa (LD) in relation to the basal plasma membrane (BM). Parameters characterising both relative surfaces ($S_{S_{HD,BM}}$; $S_{S_{LD,BM}}$) and number/unit surface ($N_{S_{HD,BM}}$) were significantly reduced in cheek pouch carcinomas when compared with normal mucosa, whereas individual hemidesmosomal dimensions remained unaffected. Stereological methods can be used to provide valuable objective information on structural aspects of malignant transformation, and these techniques may be usefully extended to determine the specificity of these alterations.

INTRODUCTION

Ultrastructural reports have described a complex organization at the epithelial-connective tissue junction in skin and oral mucosa, in which a number of structurally identifiable components are responsible for providing the mechanism of attachment between the two tissues (Briggaman and Wheeler 1975). There are several reports which have documented structural alterations at this junction during malignant transformation (Frei 1962; Frithiof 1969; Woods and Smith 1969; White et al. 1981) and more recently some of these have been examined using morphometric methods (McNutt 1976; Frei 1978; White and Gohari 1981 b, c). In the present report we use stereological methods to

evaluate the changes in hemidesmosomes and lamina densa in carcinomas produced in hamster cheek pouch mucosa using the chemical carcinogen 7,12 dimethylbenz(α) anthracene (DMBA).

MATERIALS AND METHODS

The cheek pouches of young (4-6 weeks old) male Syrian golden hamsters were treated with a 0.5% solution of DMBA in liquid paraffin for 10 weeks; after 16 weeks, specimens were obtained by multiple biopsy of the treated areas and processed for electron microscopy. Details can be found in White and Gohari (1981a) and White et al. (1981). Semithin sections were prepared and stained with toluidine blue and these were used to assign individual blocks to the category of carcinoma. In order to warrant this diagnosis, the epithelium had to possess atypical features (Smith and Pindborg 1969) and to demonstrate histological evidence of invasion of the adjacent lamina propria. 5 blocks with these features were obtained from each of five animals. From each block, ultrathin sections (c. 70 nm) were cut and stained with lead citrate and uranyl acetate. Eight micrographs were recorded at random from the epithelial-connective tissue junction at a final magnification of 18,750 on an AEI EM6B electron microscope at an accelerating voltage of 60 kv and calibrated with a diffraction grating replica. The details of the sampling procedures are provided in Table 1.

Table 1

Stereological Sampling Procedures for Ultrastructural Analysis of the Epithelial-Connective Tissue Junction

Exptl. stages	No. of Animals	No. of blocks/ animal	No. of sections/ block	No. of mics/ section	Total no. mics/ stage
2 (Normal, Carcinoma)	5	5	1	8	200

Micrographs were quantified using intersection counting procedures. From each micrograph, the following primary data were obtained: number of intersections with hemidesmosomes (I_{HD}), basal plasma membrane (I_{BM}) and lamina densa (I_{LD}) and the number of hemidesmosomes (N_{HD}).

This enabled the generation of several secondary stereological parameters. These have been discussed in detail in a previous publication (White et al. 1982).

The relative surface areas of basal plasma membrane associated with either hemidesmosomes ($S_{S_{HD,BM}}$) or lamina densa ($S_{S_{LD,BM}}$) were determined using the following equations

$$S_{S_{HD,BM}} = \frac{I_{HD}}{I_{BM}}$$

$$S_{S_{LD,BM}} = \frac{I_{LD}}{I_{BM}}$$

Mean hemidesmosomal trace length $\bar{B}_{HD}$ was calculated from the relationship

$$\bar{B}_{HD} = \frac{\pi}{2} \times \bar{I}_{HD} \times h$$

where h is the spacing of the test lines. Mean hemidesmosomal diameter ($\bar{\Delta}_{HD}$) was obtained following correction by Abercrombie's (1946) method i.e.

$$\bar{\Delta}_{HD} = \bar{B}_{HD} \times \frac{4}{\pi}$$

The numerical density of hemidesmosomes present on unit surface of basal plasma membrane ($N_{S_{HD,BM}}$) was determined from

$$N_{S_{HD,BM}} = \frac{N_B}{\bar{\Delta}_{HD} + (\frac{4}{\pi})\ t}$$

where N_B is the number of hemidesmosomes present on a unit length of basal plasma membrane and t is the section thickness.

The primary data were pooled for each animal and the secondary parameters calculated to provide a mean (n = 5) for each group. Data was analysed using a Student's t test.

RESULTS

In normal hamster cheek pouch mucosa, the epithelial-connective tissue junction was regular and the basal plasma membrane was gently undulating (Fig. 1). Localised densities were present on the inner leaflet of this membrane, corresponding to the attachment plaques of the hemidesmosomes. Bundles of fine tonofilaments could be seen entering these plaques from the epithelial cytoplasm. A dense sheet, the lamina densa, lay in close relationship to the basal plasma membrane, separated from it by a relative clear space, the lamina lucida. In the subjacent lamina propria, dense bundles of collagen fibrils were present.

In carcinomas, (Fig. 2) lamina densa breaks were observed, through which protruded extensive cytoplasmic processes or pseudopodia. These contained ribosomes, and occasional vesicles and membrane-bound dense bodies. These latter were probably primary lysosomes. Hemidesmosomes were absent from these structures. The adjacent lamina propria was usually disorganized, and often collagen fibrils were entirely absent.

The results of the quantitative analysis are presented in Table 2.

Table 2

Results of the Stereological Analysis of the Epithelial -Connective Tissue Junction

		Normal	Carcinoma	
$S_{S_{HD,BM}}(\%)$	$\bar{x}$	40.1	13.3	($p<0.001$)
	S.E.M.	1.3	3.2	
$S_{S_{LD,BM}}(\%)$	$\bar{x}$	98.1	42.5	($p<0.001$)
	S.E.M.	1.1	6.7	
$\bar{\Delta}_{HD}$ (μm)	$\bar{x}$	0.229	0.204	(N.S.)
	S.E.M.	0.004	0.007	
$N_{S_{HD,BM}}(\mu m^{-2})$	x	8.1	3.2	($p<0.001$)
	S.E.M.	0.7	0.6	

Results of the statistical analysis are present in the right hand column. N.S. = not significant.

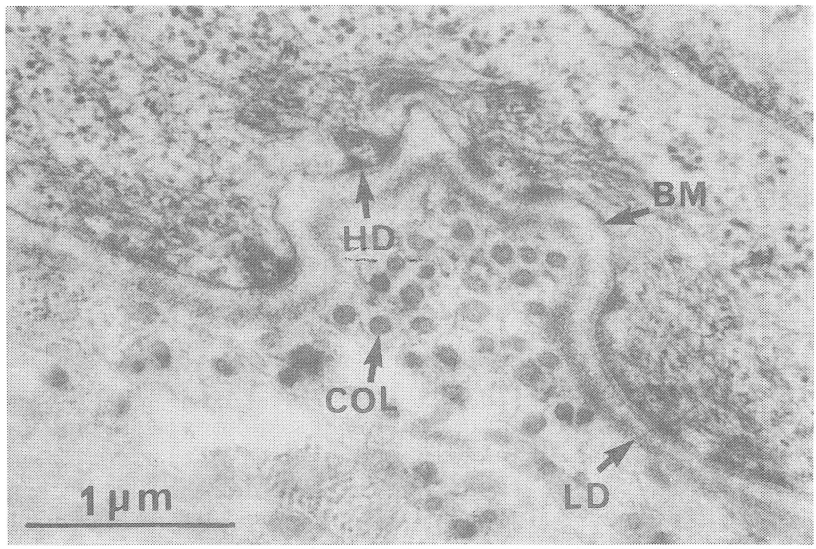

Fig. 1. Normal epithelial-connective tissue junction. Basal plasma membrane (BM), hemidesmosomes (HD), lamina densa (LD) and collagen fibrils (COL) are clearly visible.

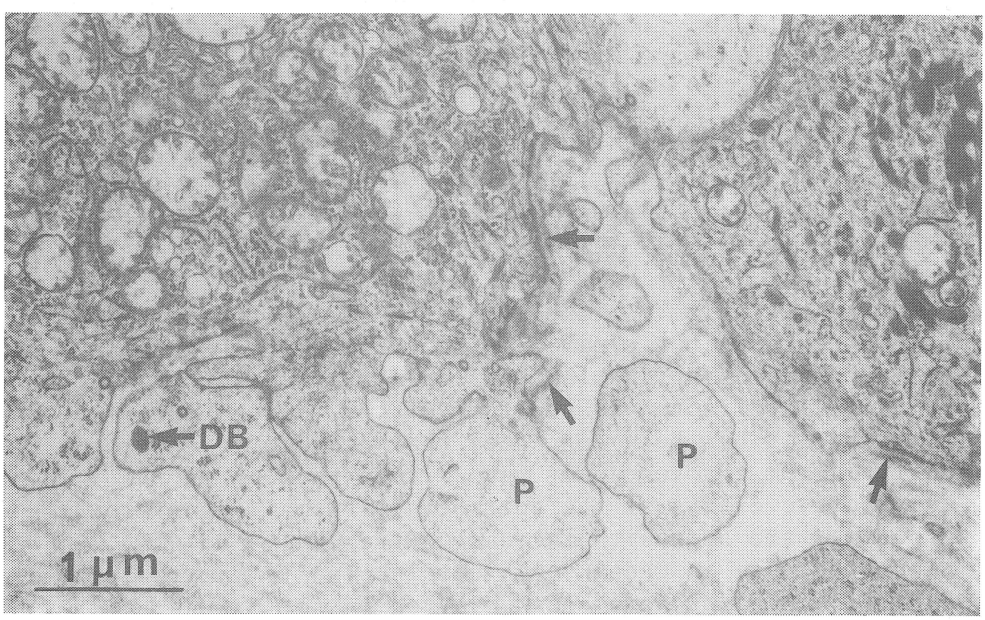

Fig. 2. Epithelial-connective tissue junction from a hamster cheek pouch carcinoma. Cytoplasmic pseudopodia (P) pass into the lamina propria through breaks in the lamina densa (→). Some contain membrane-bound dense bodies (DB).

The value for $S_{S_{LD,BM}}$ in carcinomas was less than one half the value obtained in the normal tissue, and the parameter $S_{S_{HD,BM}}$ was reduced to one third of the value present in the normal group. Mean hemidesmosomal diameter $\bar{\Delta}_{HD}$ was slightly, but not significantly, lower in carcinomas whereas $N_{S_{HD,BM}}$ data indicated that there were about 8 hemidesmosomes/μm^2 in normal tissues but that this decreased to only about 3 in carcinomas.

DISCUSSION

The findings of the present report describe significant reductions in the relative surface areas of basal plasma membrane occupied by hemidesmosomes and lamina densa in carcinomas when compared with the tissue of origin. Further, the reduction in hemidesmosomal surface is due to a decrease in their frequency, but individual hemidesmosomes have similar dimensions in both groups.

The application of stereological methods to this experimental carcinogenesis system has objectively described changes which occur in carcinomas. It is thought that the prime function of hemidesmosomes and lamina densa at the epithelial-connective tissue junction is that of attachment (Briggaman and Wheeler 1975). The reductions in relative surface area and frequency of these structures in carcinomas suggest that there is a less firm attachment between the two tissues and it is likely that there is a removal of restriction of movement of the malignant epithelial cells in order for them to invade the lamina propria.

The mechanism for the loss of hemidesmosomes and lamina densa is not clear. These alterations may arise from an increasing inability of the transforming epithelial cells to produce them or they may be actively removed. It has been suggested that hydrolytic enzymes derived from lysosomes may have a role to play in the destructive events at the epithelial-stromal interface. Lysosomes are seen with increasing frequency and of increased size in cheek pouch carcinogenesis (Smith 1972; Gohari 1978), and have been observed in the pseudopodia (White and Gohari 1981 b, c and see Fig. 2). A marked inflammatory infiltrate inevitably accompanies carcinogenesis, within which macrophages are particularly prominent (Gohari and Johnson 1974). Macrophages possess

lysosomes and these may also contribute to the alterations at the junction. In vitro studies have been performed in which enzymatic separation of epithelial and connective tissues has resulted in the production of pseudopodia as well as removal of hemidesmosomes and lamina densa (Fukuyama et al. 1974; Scaletta and MacCallum 1974). Enzymatic methods could be used in conjunction with stereological analyses to investigate the turnover of hemidesmosomes and lamina densa.

The pathogenesis of these changes could also be evaluated in more detail, for example by quantifying lesions induced at earlier stages of carcinogenesis. There is also a requirement for establishing whether the changes are specific to malignancy or whether they occur as a secondary phenomenon, for example in response to inflammation. This will require the analysis of a variety of non-neoplastic inflammatory conditions in order that the contribution of inflammation to these junctional alterations can be assessed. If specificity is detected, similar analyses could be carried out on human material, and it may eventually prove possible to develop stereological tests for the evaluation of human premalignancy.

Another possible line of investigation is related to studies directed towards reversal of these changes. Both hemidesmosomes and lamina densa are synthesised by epithelial cells and can be considered as products of epithelial differentiation. Carcinogenesis may be considered as a process involving dedifferentiation and loss of hemidesmosomes and lamina densa might reflect this process. It has been suggested that carcinogenesis may be inhibited or reversed by using substances such as vitamin A analogues which promote epithelial differentiation (Bollag and Hanck 1977; Sporn and Newton 1979) and stereological analyses of carcinomas treated with vitamin A compounds may provide useful insights into the biology of tumour regression.

ACKNOWLEDGEMENTS

This work was funded in part by the Medical Research Council, the Wellcome Trust (F.H.W.) and the Yorkshire Branch of the Cancer Research Campaign (K.G.). We are grateful for the support and advice received from Professor C.J. Smith and Professor R. Barer, and for the

excellent technical assistance of Maurice Rudland, Neil Cameron, David Thompson, Ray Codd and Maureen Tune.

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