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PAPER

# VASCULARITY IN EXPERIMENTAL ORAL NEOPLASIA: A STEREOLOGICAL APPROACH

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ABSTRACT

The effects of topical applications of liquid paraffin and the chemical carcinogen DMBA on the blood vessels in the lamina propria of hamster cheek pouch mucosa were investigated using stereological methods. Liquid paraffin and DMBA were applied for 10 weeks and samples of mucosa were obtained after 10 and 16 weeks respectively after the commencement of applications. A systematic stratified sampling procedure was used to obtain stereological estimates of volume ($V_V$) and length ($L_V$) densities, mean transverse sectional area ($\bar{A}$) and number of profiles per unit area ($N_A$). Significant increases in $V_V$, $L_V$, $\bar{A}$ and $N_A$ were found in both liquid paraffin and DMBA groups when compared with untreated mucosa; values for $V_V$ and $\bar{A}$ were higher in the DMBA group than in the liquid paraffin group. The vascular alterations found in DMBA-treated mucosa deserve further evaluation, and are probably responsible for providing an adequate nutritional supply for a rapidly proliferating transforming cell population.

## INTRODUCTION

Stereological methods are being increasingly applied to a variety of pathological problems, and this has resulted in a better understanding of the pathogenesis of several diseases. Our own studies deal with defining the structural alterations which occur in malignancy, with the aim of improving knowledge with regard to the development of neoplasia, and eventually, of establishing whether some particular stereological parameters may be of value in the detection of premalignant lesions.

We have previously used electron microscopy to describe quantitative morphological alterations in the hamster cheek pouch mucosa model treated with the chemical carcinogen 7,12 dimethylbenz(α)anthracene (DMBA) (White et al., 1980; White & Gohari, 1981, a,b,c). In the present report, we consider some quantitative alterations at the histological level which occur in the blood vascular system in the lamina propria of hamster cheek pouch mucosa in response to the application of DMBA.

## MATERIALS AND METHODS

The medial aspects of cheek pouches of male Syrian golden hamsters were treated with thrice-weekly application of 0.5% DMBA in liquid paraffin (White et al., 1981) for 8 weeks. 2 control groups were also used, one of which was left untreated and the other received thrice-weekly applications of liquid paraffin for 10 weeks. Samples of mucosa were obtained from 5 animals from the untreated groups and from each of those treated with liquid paraffin after 10 weeks and with DMBA after 16 weeks (DMBA-16). Animals were anaesthetised with sodium pentobarbitone and tissue was removed from the medial aspects of pouches. Specimens were fixed in 10% buffered formol saline, routinely processed for histology and embedded in paraffin wax.

Sampling procedures for stereology are summarised in Table 1.

Table 1

| Exptl groups | No. animals/ group | No. sections/ animal | No. fields/ section | Total no. fields/ group |
|---|---|---|---|---|
| 3 (normal, liquid paraffin, DMBA-16) | 5 | 10 | 4 | 200 |

One tissue block was selected from each animal and serial sections 5μm thick were obtained. Every tenth section was removed and mounted on a glass slide. These sections were stained with Van Gieson and a total of 10

sections obtained from each animal. The sections were examined on a Zeiss microscope fitted with an epidiascope attachment which was arranged to project the image onto the measuring tablet of a MOP AMO3 (Kontron) semi-automatic image analysing system. A total of 10 randomly selected histological fields was measured on each section at a magnification of x104.

Using a light cursor, the areas of blood vessels ($A_{BV}$) and of the lamina propria ($A_{LP}$) were measured on each microscopical field, as were the number of blood vessel profiles ($N_{BV}$). For the purposes of this investigation, blood vessels were defined as those structures lined by endothelial cells which contained erythrocytes. No attempt was made to distinguish between arteries, veins and capillaries.

From this primary data, the following stereological parameters for blood vessels were determined for the untreated and for each of the experimental groups, with the lamina propria as the reference unit.

Volume density, $V_V = \frac{A_{BV}}{A_{LP}}$

Number/unit section area, $N_A = \frac{N_{BV}}{A_{LP}}$

Length density, $L_V = 2 \times N_A$

Mean transverse sectional area, $\bar{A} = \frac{V_V}{L_V}$

For statistical analysis, data were pooled on an individual animal basis and a one-way analysis of variance followed, where required, by a Student's t-test was performed.

## RESULTS

Normal hamster cheek pouch mucosa consists of a thin stratified squamous keratinising epithelium, beneath which lies a fibrous lamina propria containing fibroblasts, moderately dense bundles of collagen and occasional blood vessels and nerves (Fig. 1). The application of DMBA to the cheek pouch produces a series of well-documented changes (Smith, 1972, White et al., 1981) which in the epithelium

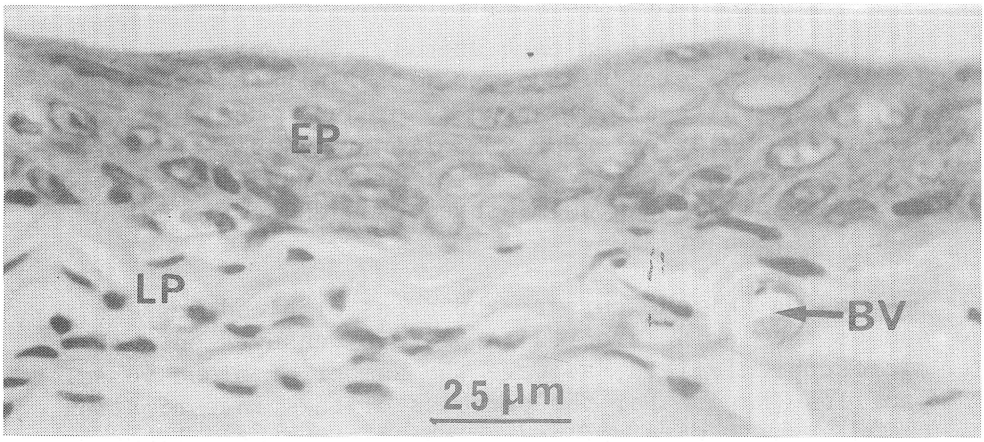


Fig. 1. Normal hamster cheek pouch mucosa. A thin stratified squamous epithelium (EP) overlies a fibrous lamina propria (LP) in which occasional small blood vessels (BV) are found.

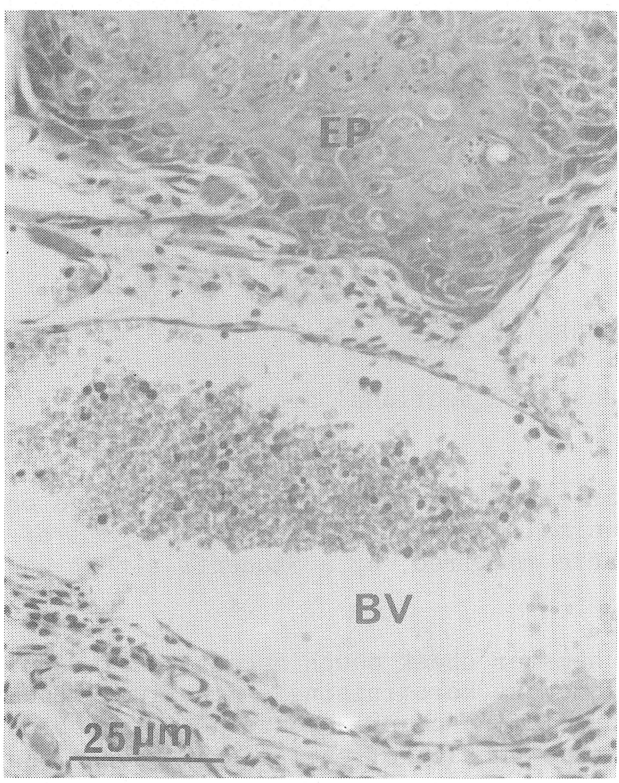


Fig. 2. Hamster cheek pouch mucosa - DMBA-16. Extremely large blood vessels (BV) are found in close association to dysplastic epithelium (EP).

have been classified as hyperplastic, dysplastic and carcinomatous. Papillomatous lesions developed at about the 10th week of application and these subsequently underwent malignant change. The adjacent lamina propria also showed marked changes, which were particularly prominent in the later stages of treatment. After 16 weeks of DMBA treatment, the mucosa exhibited variable appearances (Fig. 2). Multiple papillomas, some with early invasive characteristics, were present and extensive areas demonstrated hyperplastic features with marked epithelial atypia. The changes in the lamina propria included diffuse inflammatory cell infiltrates, including both lymphocytes and macrophages, loss of staining intensity of the collagen fibres with Van Gieson and increases in the dimensions of the blood vessels. Liquid paraffin treated tissues showed no marked deviation from untreated material, other than the presence of occasional foci of chronic inflammatory cells.

The results of the quantitative analysis are presented in Table 2 and of the statistical analysis in Table 3.

Values for $V_V$ increased fivefold in the liquid paraffin group when compared with the normal group ($p<0.01$) and the value for the DMBA-16 group was approximately eighteen times larger than the normal group ($p<0.01$).

Table 2

Stereological Parameters characterising Blood Vessels in Cheek Pouch Carcinogenesis

| | | Normal (N) | Liquid Paraffin (LP) | DMBA-16 |
|---|---|---|---|---|
| $V_V$ | $\bar{x}$ | 0.0056 | 0.0327 | 0.1085 |
| | SEM | 0.0026 | 0.0058 | 0.0367 |
| $L_V$ ($\mu m^{-2}$) | $\bar{x}$ | 0.00038 | 0.00137 | 0.00114 |
| | SEM | 0.00007 | 0.00019 | 0.00018 |
| $N_A$ ($\mu m^{-2}$) | $\bar{x}$ | 0.00019 | 0.00068 | 0.00057 |
| | SEM | 0.00004 | 0.00010 | 0.00009 |
| $\bar{A}$ ($\mu m^{-2}$) | $\bar{x}$ | 12.6 | 26.2 | 86.4 |
| | SEM | 3.3 | 2.9 | 20.5 |

Table 3

Results of Statistical Analysis

| comparison / parameter | N vs LP | N vs DMBA-16 | LP vs DMBA-16 |
|---|---|---|---|
| $V_V$ | $p<0.01$ | $p<0.01$ | $p<0.02$ |
| $L_V$ | $p<0.01$ | $p<0.01$ | N.S. |
| $N_A$ | $p<0.01$ | $p<0.01$ | N.S. |
| $\bar{A}$ | N.S. | $p<0.02$ | $p<0.05$ |

$L_V$ and $N_A$ values were obtained from the same primary data and demonstrated the same trends. Values for both parameters demonstrated approximately three-fold increases in both liquid paraffin and DMBA treated tissues ($p<0.01$). The mean transverse sectional area of blood vessels, $\bar{A}$, doubled in the liquid paraffin treated tissues, whereas in the DMBA-16 group, the value was seven times that found in the untreated control group.

## DISCUSSION

The results of this study have defined in quantitative terms the extent of the vascular alterations which occur in response to the chemical carcinogen, DMBA, and its vehicle liquid paraffin. The application of stereological techniques has enabled an accurate objective assessment of the vascular changes to be made. Qualitative examination of the tissues revealed marked changes in the blood vessels in the DMBA-treated group, whereas in the liquid paraffin-treated group, no changes were detected. The quantitative data obtained have enabled us to suggest that in both experimental groups, volumetric and length density increases occur. Further, the methods afford means of detecting the nature of these alterations with more precision. Thus the data for blood vessel parameters characterising their number and mean area enable us to conclude that the increases in vascular density occur as a result of both increased frequency and increased individual size. Hence both experimental treatments result in the production of more blood vessels and further these are more dilated in comparison with those present in untreated tissue.

In previous work on experimental cheek pouch carcinogenesis, it has been assumed that application of the liquid paraffin vehicle produces no discernible alteration in the tissue (Smith, 1972). In the present quantitative study, we have clearly demonstrated that significant vascular changes occur in response to its application, and this result emphasises the value of using appropriate controls when stereological analyses are being performed. This aspect of the work is also important in that it illustrates how previously unsuspected changes can be brought to light by quantitative analysis. Where a change is suspected, as in the DMBA-treated tissue, stereological techniques can be used to determine its extent.

It is now established that at least some malignant cells are able to secrete a substance known as tumour angiogenesis factor (TAF). This induces vascular proliferation and ensures the viability of the proliferating tumour cells by providing them with an adequate blood supply (Folkman, 1974; Folkman & Cotran, 1976). Although TAF has not yet been isolated from tumours of the hamster cheek pouch, it seems likely that such a mechanism operates, and the results of this report would support this hypothesis. However it is also known that neoplastic transformation is inevitably accompanied by a variable inflammatory response and one of the features of inflammation is increased vascular permeability and dilatation. It would thus seem to be important to determine the specificity of the changes which we have detected by using appropriate non-neoplastic inflammatory controls.

There is a requirement for the analysis of mucosa treated after shorter periods of time, in order to determine the way in which these changes develop. It would also be valuable to adopt an approach in which the sampling procedure is modified in order to take into account the histopathological changes present in the overlying epithelium. The possibility of applying stereological techniques with a view to establishing an aid for the diagnosis of premalignant lesions also requires evaluation, and recently Ferguson & Smillie (1979) have suggested that DMBA induced lesions which appear clinically vascular are more likely to progress to malignancy than those which do not. The present report suggests that stereological methods may eventually prove to be of considerable value in studies

which are directed towards the pathogenesis and diagnosis of epithelial malignant lesions.

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