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Ploidy and Proliferative Characteristics of Sheep Epidermal Squamous Cell Carcinoma Determined by Flow Cytometric DNA Analysis

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Abstract—*Multiple biopsies from each of 22 primary sheep epidermal squamous cell carcinomas were analysed by flow cytometry to determine the G_0/G_1 modal DNA content ("ploidy") and cell cycle characteristics within each tumour. Ten of 12 tumours where aneuploidy was present demonstrated uniform intra-tumour aneuploid populations regardless of the site of biopsy. Increasing tumour volume (from stage I/II to stage III/IV lesions) was associated with increased histological variability and ultimate heterogeneity of G_0/G_1 DNA content, whilst the mean numbers of S phase cells decreased. These features were consistent with the effects of variable tissue hypoxia seen with changes in effective vascularity in developing tumours. Decreasing histological differentiation was associated with an increase in numbers of cells synthesising DNA within 44 biopsies with measurable S phase, and, in stage I/II biopsies, correlated with an increased incidence of aneuploidy.*

INTRODUCTION

SHEEP epidermal squamous cell carcinoma (SCC) is a naturally occurring neoplasm physically and histologically analogous to squamous carcinoma of skin in man [1]. Lesions usually arise from hyperkeratotic skin, developing into nodular or ulcerated carcinomas (Fig. 1), and metastasise to regional lymph nodes and lung [1–3]. Observations on the aetiological and pathological features of this carcinoma have been documented [1–3], along with immunotherapy studies to evaluate tumour enhancement [4] and suppression of the lymphocyte blastogenic response [5], and study of the effects on the immune system of tumour removal [6] and cyclophosphamide therapy [7].

The proliferative features of this carcinoma have not, however, been determined using flow cytometric (FCM) estimation of cellular DNA content. This paper reports a comprehensive study of the cell cycle stage and ploidy characteristics of sheep epidermal SCC, and the relationship of these parameters to tumour histology and volume.

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MATERIALS AND METHODS

Animals

Twenty-two Merino ewes bearing epidermal SCC (17 auricular, four nasal, and one involving the lower lip) had been obtained from central New South Wales, Australia. Animals were housed under cover in standard concrete enclosures, the lesions being kept clean with surgical pad dressings. The study was carried out with the approval of a Sydney University Animal Ethics Committee.

Tumour sampling

Following intravenous thiopentone induced anaesthesia, tumour volume was estimated *in vivo* as a product of the cross-sectional area by depth for non-conical lesions, or as one-third the base area by height for conical lesions. Tumour volume ranged from 2.8 to 840 cm^3 (Table 1). Half the tumours were stage I/II lesions (tumour volume < 5 cm^3 and 5–25 cm^3, respectively), the remainder being stage III/IV lesions (tumour volume 26–40 cm^3 and > 40 cm^3, respectively), using the classification of Jun *et al.* [5]. Two to ten biopsies, at least 1 cm apart, were taken from the tumours *in*

Table 1. Table showing tumour: identification, volume, biopsy number, histological degree of differentiation, S phase cell numbers, and the DNA content of aneuploid populations.

Tumour	Volume (cm^3)	Biopsy 1	Biopsy 2	Biopsy 3	Biopsy 4	Biopsy 5	Mean %S Phase (± S.E.M.)
Stage I/II							
A	2.6	well[a]	well				
		9.8%S[b]	10.1%S				10.0 ± 0.1%[c]
B	4.5	well	well				
		2.2n(61%G_1)[d]	2.2n(31%G_1)				–
C	5.0	mod	mod				
		14.7%S	19.0%S				16.9 ± 2.1%S
D	5.2	well	well				
		22.9%S	24.0%S				23.5 ± 0.5%S
E	7.9	poor	poor				
		2.8n(46%G_1)	2.8n(40%G_1)				–
F	8.3	poor	poor				
		20.4%S	24.2%S				22.3 ± 1.9%S
G	12.3	poor	poor				
		3.2n(30%G_1)	3.2n(16%G_1)				–
H	12.5	well	well				
		10.2%S	8.4%S				9.3 ± 0.9%S
I	13.8	poor	poor				
		4.4n(29%G_1)	4.4n(16%G_1)				17.8 ± 0.9%S
		18.7%S	16.8%S				
J	15.0	well	well				
		2.9n(18%G_1)	2.9n(16%G_1)				–
K	19.0	well	well	mod			
		12.1%S	9.6%S	12.1%S			11.4% ± 0.9%S
Stage III/IV							
L	31.5	well	well	well			
		2.6n(18%G_1)	2.6n(18%G_1)	2.6n(19%G_1)			–
M	33.8	well	well	well			
		5.4%S	5.8%S	7.3%S			6.2 ± 0.6%S
N	54.7	mod	poor	mod			
		6.7%S	7.2%S	7.2%S			7.0 ± 0.2%S
O	56.0	mod	mod	mod	mod		
		12.3%S	10.3%S	13.5%S	7.7%S		11.0 ± 1.3%S
P	61.4	mod	poor	poor	mod		
		2.2n(15%G_1)	2.2n(21%G_1)	2.2n(61%G_1)	2.2n(15%G_1)		–
Q	155.7	well	well	well	well		
		2.3n(28%G_1)	2.3n(16%G_1)	2.4n(19%G_1)	2.3n(13%G_1)		–
R	161.0	well	well	mod	mod		
		2.2n(55%G_1)	2.2n(12%G_1)	2.2n(13%G_1)	2.3n(8%G_1)		–
S	160.3	mod	mod	mod	mod	well	
		4.1n(9%G_1)	4.1n(8%G_1)	4.1n(7%G_1)	4.1n(13%G_1)	4.1n(11%G_1)	
		6.7%S	6.7%S	15.4%S	19.1%S	14.3%S	12.4 ± 2.5%S
T	163.0	well	well	well	well	well	
		16.2%S	11.7%S	10.6%S	12.3%S	11.7%S	12.5 ± 1.0%S
U	255.9	well	well	well	well	well	
		4.3n(7%G_1)	12.6%S	2.5n(16%G_1)	2.5n(7%G_1)	9.2%S	
		11.4%S					10.2 ± 0.6%
V	840.0	[e](1) mod	(2) mod	(3) mod	(4) well	(5) well	
		2.4n(19%G_1)	2.4n(25%G_1)	11.1%S	10.9%S	2.4n(22%G_1)	
		(6) well	(7) well	(8) well	(9) well	(10) mod	8.7 ± 0.8%S
		8.6%S	7.1%S	7.3%S	6.9%S	2.4n(21%G_1)	

[a] Histological differentiation: well = well differentiated; mod = moderately differentiated; poor = poorly differentiated.

[b] The number of S phase cells expressed as a percentage.

[c] The mean percentage S phase (±S.E.M.) of diploid biopsies, or biopsies where two clones of cells were present and the DNA distributions of these subpopulations did not overlap.

[d] A hyperdiploid population of cells with the proportion of cells with abnormal DNA content expressed as a percentage of the total G_0/G_1 cells. All aneuploid biopsies contained a 2n diploid population of cells.

[e] Arabic numerals, in brackets, denote the 10 biopsies taken from tumour V.

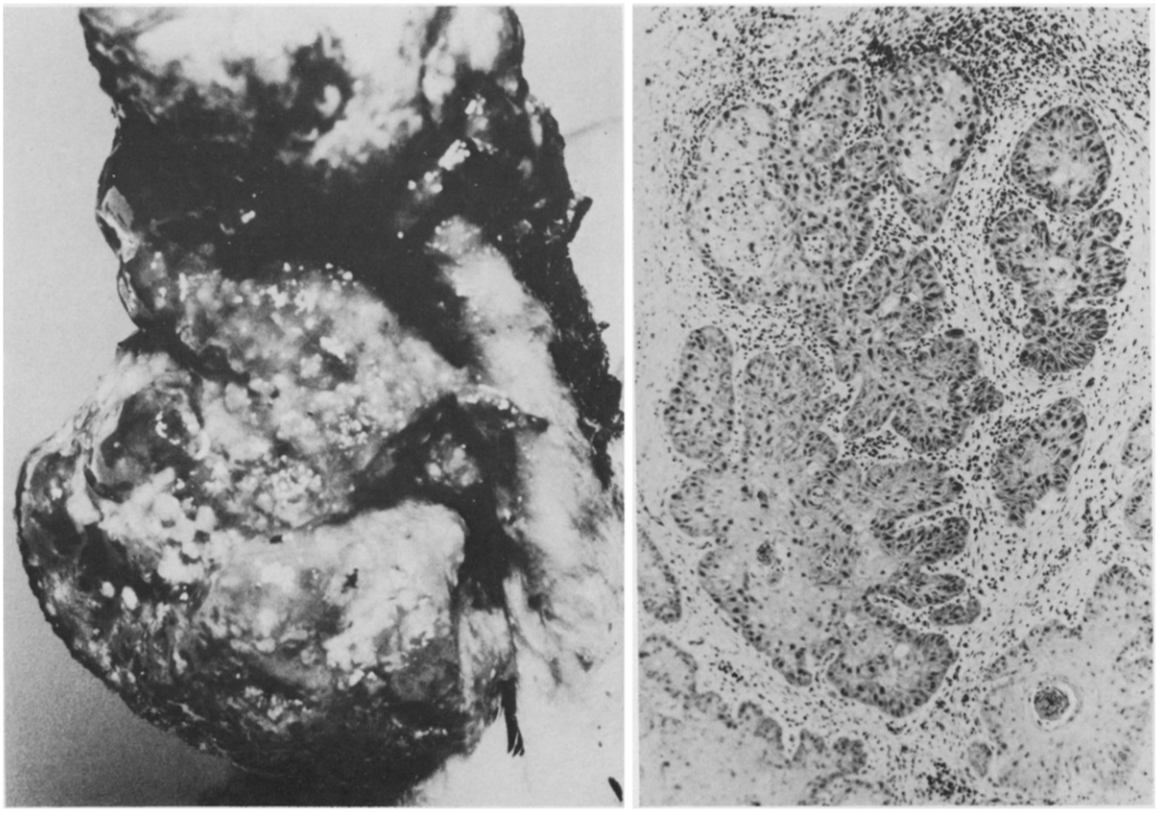

Fig. 1. ***Sheep epidermal squamous cell carcinoma.*** (left) ***Typical sheep epidermal squamous cell carcinoma, a nodular lesion arising from the ear.*** (right) ***Histology of the tumour showing islands of malignant epidermal cells, "keratin pearl" formation, and mononuclear cell infiltration of the stroma.***

situ. Samples up to 0.75 cm^3 were excised and debrided of any obviously necrotic material using a scalpel, the biopsy sites being padded with gauze to control any bleeding. The sheep were then sacrificed by thiopentone overdose, the whole tumour removed, and geographic location of the biopsies mapped.

Flow cytometry

Biopsies from 11 tumours were immediately placed in Hank's Balanced Salt Solution (HBSS) and processed for FCM analysis using a modification of the method of Taylor *et al.* [8]. Briefly, following coarse mincing with scissors in a solution containing 1% (V/V) of Triton X-100 (Packard Instrument Company Incorporated, Downer's Grove, Illinois), HBSS and RNA-ase A-grade (Calbiochem Behring, La Jolla, California) at a final concentration of 1 mg/ml, propidium iodide (Calbiochem, San Diego, California) was added to obtain a final concentration of 50 μg/ml. The fragments were then gently agitated for 1 min, and this suspension filtered through paper with a nominal pore size of 30μ to remove residual tumour fragments. Experience has shown that this method gives a high yield of single, intact tumour cells suitable for FCM. Histograms were obtained on a minimum of 5×10^5 propidium iodide stained cells using a Cytofluorograf model FC–200 or a Cytofluorograf 50H cell sorter (Ortho Instruments, Westwood, Massachusetts).

Estimation of cellular DNA content of the remaining 11 tumours was performed retrospectively on paraffin embedded tumour sections, a well-described technique which allows FCM analysis of archival tumour tissue [9]. Briefly, 30μ sections from a paraffin embedded tumour block were dewaxed in xylene, rehydrated in a sequence of 100, 95, 70 and 50% ethanol, then washed with distilled water. The tissue was then resuspended in 0.5% pepsin (Sigma Chemical Company, St. Louis, Missouri) in a water bath at 37°C for 30 min. Cells were stained by suspension in DAPI (4, 6-diamidina-2-phenylindolichloride, 1 mg/ml: Boehringer Mannheim, West Germany) in RPMI 1640 tissue culture medium. Cellular DNA content was measured using an ICP22 flow cytometer (Ortho Instruments, Westwood, Massachusetts).

In assessing the results chromosome studies were not performed because of the frequency of bacterial contamination, and the difficulty of obtaining metaphases, so evidence for clonal relationships rests in the similarity of the DNA content of G_0/G_1 cells. Unfixed chicken red blood cells were added to all fresh samples studied as an internal DNA standard [10]. The coefficient of variation (CV) of the G_0/G_1 peaks from all biopsies ranged from 2.2 to 5.8, with a mean (±S.E.M.) of 4.0 ± 0.1%. The proportion of cells in the DNA synthetic (*S*) phase of the cell cycle was estimated using a planimetric modification of the Hillen integration method for samples analysed using the FC-200 or 50H Cytofluorograf [11], and by computer analysis for samples evaluated by the ICP22 flow cytometer [12]. *S* phase estimations could not be performed where two clones of cells were present and the DNA distributions of these populations overlapped.

Histology

One portion of each biopsy sample was fixed in formalin and processed for histopathologic examination. All biopsies were examined by one observer, independently of the FCM analysis, and confirmed as being SCC. The samples were graded into well, moderately or poorly differentiated, depending on the degree of keratin formation, using established criteria [13]. For each section examined, the tumour was classified according to the least differentiated appearance [14].

Statistical analysis

Tumour stage was correlated with degree of differentiation, histological heterogeneity and incidence of aneuploidy, and histological differentiation related to aneuploidy, by applying the chi-square formula. The relationship between tumour stage and numbers of cells synthesising DNA was assessed by a mean/standard deviation based *t* test. Tests of linear and quadratic trend for groups with unequal sample size were used to correlate *S* phase cell numbers with histological differentiation.

RESULTS

Histology

The degree of differentiation found in 73 biopsies from 22 tumours is shown in Table 1. Two biopsies were taken from 10 tumours with uniform histology seen in lesions A, B, D, H and J (well differentiated), lesion C (moderately differentiated), and lesions E, F, G and I (poorly differentiated). Where three biopsies were taken, two tumours were uniformly well differentiated (lesions L and M), whilst tumour K varied from well to moderately differentiated, and tumour N from moderately to poorly differentiated. Four biopsies were taken from four tumours: lesions O and Q were histologically uniform (moderately and well differentiated, respectively), however, lesion P was moderately to poorly differentiated, and tumour R well to moderately differentiated. Where five samples were excised, tumours T and U were uniformly well differentiated, whilst tumour S was moderately differentiated in four sites and well differentiated elsewhere. Within lesion V, six biopsies were well differentiated and four moderately differentiated. Overall,

these carcinomas displayed a high degree of histological differentiation, with 41 biopsies (56%) being well differentiated: within the remaining samples 21 (29%) were moderately differentiated and 11 (15%) were poorly differentiated. Ten of 16 carcinomas with uniform intra-tumour histology were well differentiated (63%), with two being moderately differentiated and four poorly differentiated.

With regard to tumour stage, a significant difference was demonstrated between the degree of differentiation of 23 biopsies from stage I/II lesions and 50 biopsies from stage III/IV lesions ($P < 0.01$). In the former group 12 biopsies (52%) were well differentiated, three (13%) moderately differentiated and eight (35%) poorly differentiated, compared with 29 (58%) well differentiated, 18 (36%) moderately differentiated and three (6%) poorly differentiated biopsies in the latter group.

Six of the 22 tumours (27%) were characterised by intra-tumour histological variability, five of these (lesions N, P, R, S and V) were greater than 40 cm^3 (stage IV), whilst the sixth (lesion K) was 19 cm^3. There was a significant correlation between the number of histologically heterogeneous stage IV lesions compared with tumours less than 40 cm^3 ($P < 0.05$). Where heterogeneity occurred, histology ranged from well to moderately differentiated, or moderately to poorly differentiated, with no lesion demonstrating well to poorly differentiated biopsies.

DNA content of G_0/G_1 *peaks:*

Fifty-one per cent of biopsies displayed G_0/G_1 peaks with abnormal DNA content (aneuploidy), the overall incidence of aneuploid tumours being 55%. Ten carcinomas (lesions B, E, G, I, J, L, P, Q, R and S) showed aneuploidy in all biopsies (Table 1), whilst in the case of tumours U and V, three of five and four of 10 biopsies, respectively, were aneuploid. The remaining 10 lesions showed diploid ($2n$) populations in all biopsies. All samples with abnormal DNA content were hyperdiploid or hypertetraploid, and also showed a proportion of diploid cells. For all tumours the degree of aneuploidy ranged from $2.2n$ to $4.4n$, whilst the proportion of G_0/G_1 cells with abnormal DNA content ranged from 7 to 61%. Uniformity of aneuploid clones was found in each of two biopsies from lesions B, E, G, I and J, in three of three biopsies from lesion L, in four of four biopsies from lesions P, Q and R, in five of five samples from lesion *S*, and in four of 10 biopsies from lesion V. In tumour U, three of five samples were aneuploid, however, two distinct aneuploid clones of cells, characterised by different degrees of ploidy ($2.5n$ and $4.3n$), were present. The geographic distribution of the aneuploid populations in lesions U and V is shown in Fig. 2. In lesion U, the three aneuploid biopsies (biopsies 1, 3 and 4) came from adjacent sites, whilst in lesion V, 3 of the aneuploid biopsies came from adjacent regions of the tumour (biopsies, 1, 2 and 5) but the fourth (biopsy 10) was geographically well removed.

When detection of abnormal DNA content within samples was related to tumour stage, 10 of 23 stage I/II biopsies (43%), and 27 of 50 stage III/IV biopsies (54%), were aneuploid. The correlation between tumour stage and aneuploid biopsies was not significant ($P > 0.05$).

S *phase cell numbers*

The proportion of *S* phase cells in 44 biopsies with either no detectable aneuploidy (36 biopsies), or with the DNA distributions of an aneuploid and a diploid population not overlapping (eight biopsies), ranged from 5.4 to 24.2%, with a mean *S* phase (±S.E.M.) of 12.0 ± 0.8%. Within these biopsies, the mean proportion of *S* phase cells in 15 biopsies from stage I/II tumours was 15.6 ± 1.5%, and 10.1 ± 0.6% in 29 biopsies taken from stage III/IV tumours. The correlation between tumour stage and mean number of *S* phase cells was significant ($P < 0.001$).

In 14 carcinomas it was possible to compare the *S* phase fractions of two or more biopsies (Table 1). Within 11 of these lesions (tumours A, C, D, F, H, I, K, M, N, U and V) regional differences between the proportions of *S* phase cells were negligible. In the remaining three tumours (stage IV lesions O, S and T), the intra-tumour differences in *S* phase cell numbers were 5.8, 12.4 and 5.6%, respectively.

Histology and FCM analysis

Within 73 biopsies, from all tumours, 41 were well differentiated with 18 (44%) being aneuploid, 21 were moderately differentiated with 11 (52%)

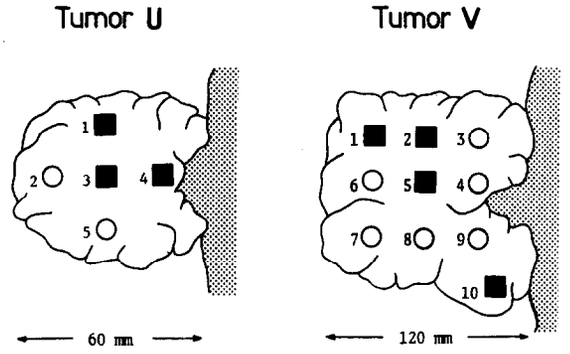

Fig. 2. Location of aneuploid and diploid populations in multiple biopsies of tumours U and V. These are diagrammatic maps of tumours U and V showing the biopsy sites. Both were cauliflower-like lesions arising from and enveloping the ear. The shaded areas represent the skin of the scalp and the base of the ear remnant. Squares indicate where aneuploidy was present coexistent with a diploid population (■), circles indicate biopsies in which only diploid cells were identified (○).

being aneuploid, and 8 of 11 poorly differentiated biopsies (73%) were aneuploid. Within 23 biopsies from stage I/II tumours, 12 were well differentiated of which four (33%) were aneuploid, three moderately differentiated biopsies were entirely diploid, and six of eight poorly differentiated biopsies (75%) were aneuploid. Of 50 biopsies from stage III/IV carcinomas, 29 were well differentiated with 14 (48%) demonstrating abnormal DNA content, 18 were moderately differentiated with 11 (61%) being aneuploid, and two of three poorly differentiated biopsies (66%) were aneuploid. The correlation between degree of differentiation and aneuploidy was marginally significant within stage I/II biopsies ($P = 0.05$), and not significant for stage III/IV biopsies ($P > 0.05$) or when all biopsies were considered irrespective of tumour stage ($P > 0.05$).

Tests of trend conducted across the three degree of differentiation groups showed a significant linear correlation with S phase cell numbers when 44 biopsies, with measurable S phase, were analysed irrespective of tumour stage ($P < 0.01$), whilst quadratic trend was not significant ($P > 0.05$). There were no significant linear or quadratic trends observed within either stage I/II tumours ($P > 0.05$), or stage III/IV tumours ($P > 0.05$).

DISCUSSION

The evaluation of sheep epidermal SCC permits an opportunity to determine the extent to which this spontaneously occurring neoplasm represents a homogeneous population of cells with respect to the existence of aneuploid cell clones, and to the cytokinetic characteristics of diploid populations. The carcinomas studied displayed a remarkably homogeneous intra-tumour G_0/G_1 DNA content, assuming that cells with similar DNA content belonged to the same population. Of 12 tumours where aneuploidy was present, 10 demonstrated consistent aneuploid populations regardless of the site of biopsy, albeit in different proportions. Such homogeneity has been reported in some human tumours [15, 16], particularly gynaecological cancer [17]. Two lesions (tumours U and V), however, provide evidence that the heterogeneity of DNA content found in those tumours was due to the coexistence of cell clones and not merely to random differences. In tumour U, two different aneuploid clones ($2.5n$ and $4.3n$) were found within neighbouring regions of the tumour, whilst entirely diploid populations were found in other locations. The aneuploid samples in tumour V all contained what appeared to be the same clone ($2.4n$) coexistent with diploid populations elsewhere. As three of the four aneuploid biopsies were adjacent to each other, but the fourth was geograpically separate, it is interesting to speculate whether this was the result of migration of cells within the tumour, or an independent mutational event producing the same result. The concomitant presence of more than one malignant clone is consistent with the "clonal evolution" hypothesis of tumour growth. As lesions U and V were by far the largest, and probably the oldest, carcinomas studied, it is possible that heterogeneity of cellular DNA content occurs late in the development of sheep epidermal SCC. Heterogeneity of human tumour cell DNA content has been described in lung [18, 19], colo–rectal [20, 21], and head and neck carcinoma [22].

The mean number of S phase cells in sheep SCC biopsies (12.0% S phase) compares with the mean (8.0% S phase) reported for human epidermal SCC [23]. In stage III/IV tumours, the significant decrease in mean S phase cell numbers, and associated increase in the number of tumours with variable intra-tumour S phase cells, compared with stage I/II tumours, may reflect the effects on proliferating malignant cells of variable oxygen tension. After initial growth of blood vessels, the relative vascularity of tumours is known to decrease, and vascular stasis and variability in flow through capillaries to increase, with increasing tumour volume. This combined effect severely hinders nutrient exchange and results in the formation of regions of relative hypoxia [24]. Decreases in both the labelling index and growth fraction, with increasing tumour size, have been found to depend on tissue oxygen tension [25, 26]. A factor which may further retard the growth of advanced tumours, such as lesions U and V, is the gradual development of clonal heterogeneity within tumours which may lead to mutual extinction of tumour subpopulations hindering aggressive proliferation [27].

Sheep SCCs display a high degree of cellular differentiation irrespective of tumour stage. The significant correlation between tumour volume and histological differentiation reflects the greater number of poorly differentiated biopsies compared to moderately differentiated biopsies in stage I/II lesions, and the reversal of this trend in stage III/IV lesions: the relevance of this finding remains obscure. The significant occurrence of histological variability within the stage IV tumours studied, compared with all other stages, again appears to implicate the effects of regional tissue hypoxia associated with increasing tumour volume.

Studies of human epidermal SCC have suggested that a correlation exists between decreasing degree of differentiation, and abnormal cellular DNA content and S phase cell numbers [23, 28, 29]. Such a relationship was demonstrated in stage I/II tumour biopsies, with a significant increase in the incidence of aneuploidy seen from well to

poorly differentiated samples. With only 3 of 50 stage III/IV biopsies, and 11 of 73 overall biopsies, being poorly differentiated, it is possible that a relative lack of numbers of biopsies in this differentiation category contributed to the lack of a significant correlation between aneuploidy and histology within these two groups.

As increasing histological differentiaton may lessen the likelihood of detecting cell clones with abnormal DNA content, the high degree of cellular differentiation characteristic of sheep SCC could have contributed to the relatively low incidence of aneuploid tumours (55%) compared to human epidermal SCC. Whilst no comprehensive studies of the incidence of aneuploidy in human skin cancer, as determined by FCM DNA analysis, have been reported, small series [23, 28] have suggested an incidence of 75–80%, which falls within the established range for human solid tumours in general [15]. In addition, the range of ploidy in sheep SCCs were not quite as extensive (diploid to hypertetraploid) as the range found in equivalent human lesions (diploid to hyperoctoploid) [23, 28, 29].

A significant linear trend across differentiation grades, when biopsies with measurable *S* phase cell numbers were considered irrespective of tumour stage, was related to the higher mean %*S* phase of poorly differentiated biopsies compared with well and moderately differentiated biopsies. This suggests that decreasing histological differentiation is associated with increasing proliferative activity in sheep epidermal SCC. That this relationship was not demonstrated within biopsies from tumours of similar stage again may reflect the low numbers of poorly differentiated biopsies, particularly in stage III/IV lesions.

The analogous aetiologically and histological features of sheep epidermal SCC and human skin cancer, and the occurrence of substantial lesions on the body surface enabling ease of observation and biopsy, enhance the suitability of this naturally occurring tumour model for investigation. We conclude that the majority of sheep SCC exhibit intra-tumour uniformity of cellular G_0/G_1 DNA content in all but the largest lesions. Increasing tumour volume (stage I/II to stage III/IV lesions) is associated with increasing histological variability, and ultimate heterogeneity of cellular DNA content, whilst the mean numbers of *S* phase cells decrease. These features are consistent with the effects of variable tissue hypoxia seen with changes in effective vascularity in developing tumours.

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