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Proliferation kinetics of streptozotocin-induced renal tumours in mice

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Abstract Renal tumours were induced in female mice 132 days after intravenous administration of streptozotocin. The tumours exhibited papillary and/or solid architecture with papillary tumours showing no histological evidence of malignancy. Malignant behaviour, manifest as infiltration of adjacent renal tissue and lymphatic infiltration, was noted for tumours with solid architecture. Intermediate architectural forms exhibiting dual papillary and solid architecture were identified. Proliferation kinetics were evaluated by enumeration of silver-staining nucleolar organizer regions and proliferating cell nuclear antigen expression. The results showed a stepwise progress in cell proliferation between histologically normal renal tubule epithelium [untreated animals, mean AgNOR score (MAS) 2.32, mean PCNA index (MPI) 0.53%; treated animals, MAS, 2.44; MPI 0.99%], dysplastic tubule epithelium (MAS, 4.15; MPI 1.65%), papillary tumours (MAS, 5.90; MPI 3.89%) and solid tumours (MAS, 6.94; MPI, 6.80%). Solid tumours as a group were significantly larger than papillary tumours and were associated with a significantly longer mean post-injection survival interval. The findings suggest that at least some solid tumours evolve from tumours exhibiting papillary architecture.

Key words Mouse · Renal neoplasia · Streptozotocin Nucleolar organizer regions · Proliferating cell nuclear antigen

Introduction

The behaviour of small renal parenchymal tumours is the subject of conjecture with current opinion suggesting that tumours exhibiting a papillary architecture are likely to follow a benign course [10]. This behaviour is difficult to confirm by clinical studies on human subjects as such tumours cannot be confidently differentiated from small overtly malignant tumours on clinical grounds and ethical constraints would necessitate prompt surgical management of any potentially malignant tumours.

The observation that some of the renal tumours induced in experimental animals by chemical carcinogenesis have a similar morphology to human renal cell carcinoma (RCC) provides a model for the study of tumour evolution and may facilitate the identification of morphology with a potential for malignant behaviour.

Several studies have confirmed that the diabetogenic antibiotic streptozotocin induces parenchymal tumours in rat kidneys which have been noted to have a similar histology to human RCC [1, 3, 9, 13, 16]. Similar tumours have also been noted in a small series of mice injected with streptozotocin following pancreatic isograft transplantation [12]. The histological features of streptozotocin-induced renal tumours in mice have been described by Hard [8], who found a high frequency of tumours in his series following administration of a single dose of streptozotocin and speculated that the model would be suitable for studies on renal carcinogenesis.

The present study was undertaken to evaluate the carcinogenic effect of streptozotocin on the mouse kidney, to examine the morphology and behaviour of streptozotocin-induced tumours and to determine tumour proliferation kinetics by evaluation of silver-staining nucleolar organizer region (AgNOR) numbers and expression of the cell proliferation-related proliferating cell nuclear antigen (PCNA).

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Materials and methods

Approval for this study was obtained from the Ethical Committee for Animal Experimentation of the Wellington School of Medicine and the principles of laboratory care as outlined in NIH publication 85-23 (1985) were followed.

One hundred and sixty female CBA T_6 T_6 mice aged between 30 and 162 days of age (mean age 82.3 days) were weighed and injected intravenously, via the tail vein, with a single bolus of 2.5% streptozotocin (Sigma S-0130) in 0.9% sodium chloride solution. The streptozotocin solution was prepared immediately prior to injection and was administered in a dosage of 250 mg streptozotocin/kg mouse body weight.

Following administration of streptozotocin solution, the animals were housed in the Animal Facility, Wellington School of Medicine and were monitored daily for toxic effects of streptozotocin. Those animals showing any signs of distress manifest as decreased spontaneous movement, apparent lethargic response to stimulation, failure to groom and faecal soiling were sacrificed by cervical dislocation. All surviving animals were sacrificed by cervical dislocation at the termination of the study.

Excepting those animals rendered unsuitable for examination due to cannibalism, all mice that were found dead or were sacrificed were subjected to post-mortem examination. The kidneys from each mouse were dissected free and were sectioned longitudinally at intervals of approximately 1 mm.

Renal slices were embedded in paraffin and sections were stained with haematoxylin and eosin. A 3-μm section was cut from each block of renal tissue identified as containing a neoplastic lesion and this was stained for AgNORs using a modified silver-colloid method as previously described [5]. Further, 4-μm sections were also cut from each of these blocks for immunohistochemical detection of PCNA using PC10 (Dako M879) diluted 1:100 in TRIS-buffered saline as the primary antibody. Rabbit anti-mouse antibody (IgG2a, kappa isotype, Dako Z014) 1:300 in TRIS-buffered saline was used as a cross-link. This was followed by incubation in biotinylated swine anti-rabbit antibody, 1:200 in TRIS-buffered saline. Sections were then incubated in streptavidin biotin complex and the reaction was visualised by 3,3' diaminobenzidine substrate. Sections were counterstained in Mayer's haematoxylin. Sections of mouse colon and human tonsillar tissue were used as positive controls. TRIS-buffered saline was substituted for the primary antibody as a negative control.

AgNOR scores were derived by counting discrete argyrophilic foci within each nucleus. Careful focusing was employed to visualise individual AgNORs within aggregates in addition to satellite AgNORs distributed throughout the nucleus [5]. A mean AgNOR score was derived for each tumour following evaluation of 100 nuclei. An eyepiece integration grid was used to ensure that nuclei were evaluated once only. Only those nuclei that appeared to be sectioned at, or close to, the equatorial plane were counted. In lesions containing fewer than 100 nuclei in section, the AgNOR score was derived from counting all nuclei within the lesion in the section examined.

PCNA indices were derived by examining 1000 cells, with the index being the percentage of positively staining nuclei. Only those nuclei that showed clear granular staining for PC10 were considered positive for counting purposes [6]. Where lesions contained fewer than 1000 cells, the PCNA index was derived following examination of all cells of the lesion present within the sectioned examined and again an integration grid was employed to ensure that each nucleus was evaluated once only.

Morphometric measurements of tumour diameter were undertaken using a Zeiss Intergrationsplatte I integration grid. The grid was calibrated for the various microscopic objective magnifications (2.5×, 10×, 16×, 25× and 40×) employed using a Zeiss stage micrometer containing a 5-mm scale divided at 1-mm intervals and a further distance of 1 mm divided into 10-μm intervals.

Results

Fifty-five mice died spontaneously from 0 to 132 days after injection of streptozotocin and the remainder of the animals were sacrificed between 9 and 555 days after injection. The median post-injection survival period for all animals in the series was 144 days. Four mice that died spontaneously 5, 9, 16 and 26 days after administration of streptozotocin were severely cannibalised and tissues from these animals were not available for histological examination. Tissue from all the remaining 156 mice was examined by light microscopy.

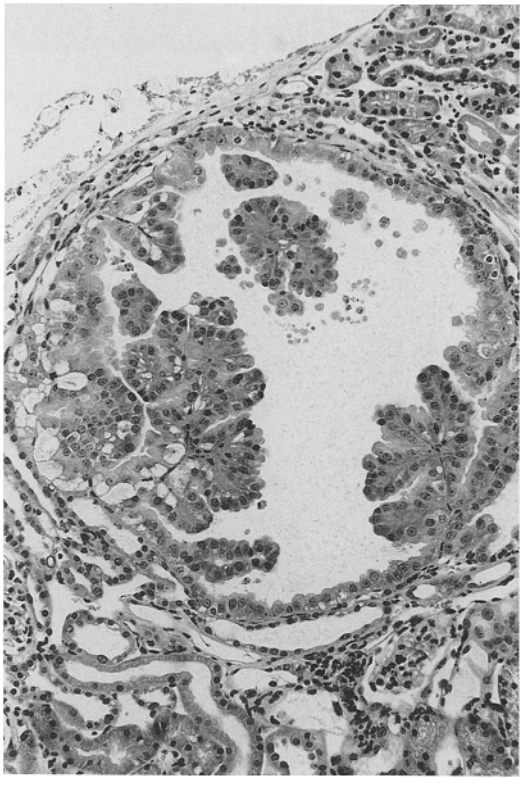

Fig. 1 Streptozotocin-induced renal tumour showing papillary architecture. H & E, ×175

A total of 225 individual tumours were identified in 61 tumour-bearing mice. The number of tumours per mouse ranged from 1 to 10 and the number of tumours per kidney ranged from 1 to 6. Tumours varied in size from 0.14 to 15 mm.

Smaller tumours lacked an investing pseudocapsule; however, pseudocapsules formed by condensation of adjacent host renal tumour were often seen surrounding many of the larger tumours. Light microscopic examination of the renal tumours showed them to be composed of epithelial cells arranged in a variety of architectural patterns varying from cystic and papillary to solid (Figs. 1, 2). Small tumours were confined to the renal cortex, while some larger tumours had a cortical component but also showed extension into the renal medulla.

The majority of smaller tumours were acinar, having the appearance of dilated renal tubules with papillary structures projecting into the lumen (Fig. 1). The tumour cells of the tubular component were simple cuboidal to columnar, exhibiting a mild to moderate degree of nuclear pleomorphism. The papillary excrescences were also composed of cuboidal to columnar neoplastic cells. These were arranged in stratified and pseudostratified aggregates along a thin branching fibrovascular stroma. The

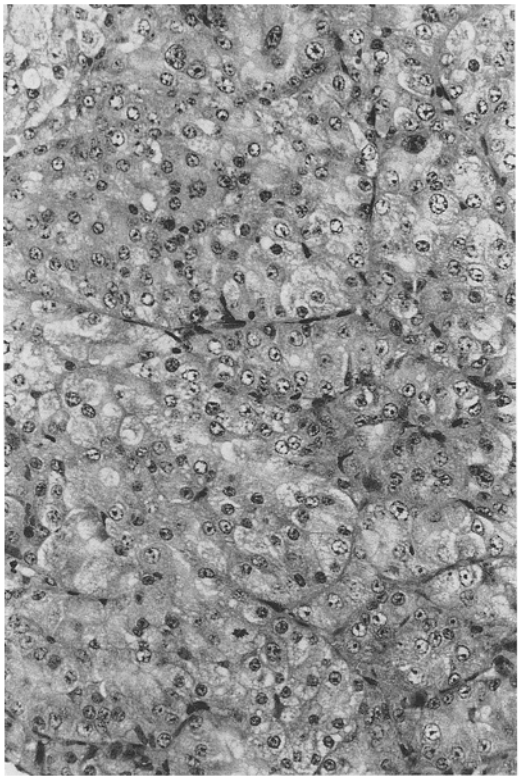

Fig. 2 Streptozotocin-induced renal tumour showing solid architecture. H & E, ×310

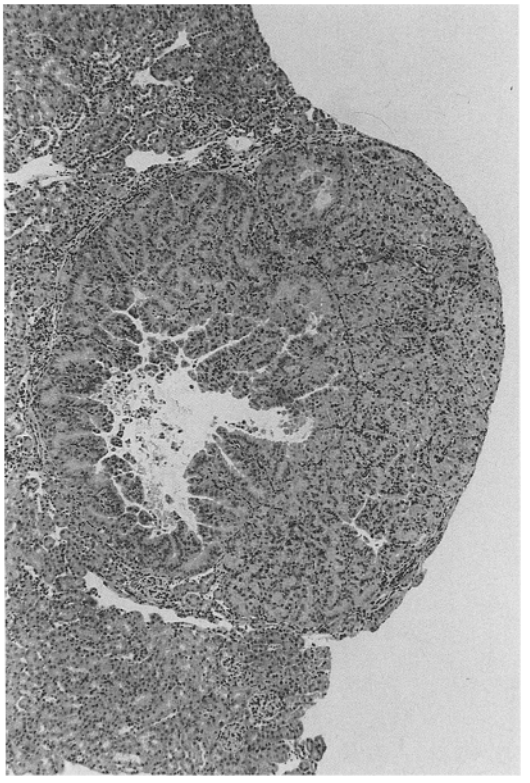

Fig. 3 Streptozotocin-induced renal tumour showing mixed papillary and solid architecture. H & E, ×63

cells contained abundant cytoplasm which was predominantly granular and eosinophilic. Occasional tumours contained foci of cells that exhibited varying degrees of cytoplasmic vacuolation which, where marked, imparted a clear cell appearance to the tumour. Rare tumours were composed almost entirely of these vacuolated cells.

Solid tumours were composed of cells similar in character to those of the cystic and papillary tumours (Fig. 2), although foci showing more pronounced nuclear pleomorphism and areas of confluent necrosis were frequently seen. In some tumours the cells were arranged in a trabecular pattern exhibiting both a papillary and solid architecture (Fig. 3).

Some solid tumours showed direct infiltration into adjacent renal tissue. In one tumour (555 days post-streptozotocin injection) lymphatic infiltration within the perirenal fat was noted. Both the primary and metastatic tumour in this instance exhibited a solid pattern.

In sections taken from kidneys containing cystic/papillary and/or solid neoplasms and in some kidneys where tumours were not identified, foci of renal tubular dysplasia were seen within the renal cortex. These tubules were dilated, and consisted of dysplastic simple or pseudostratified cuboidal to columnar epithelium. There was a mild degree of nuclear pleomorphism and the cytoplasm had an eosinophilic granular appearance similar to that seen in both cystic/papillary and solid tumours.

Dysplastic foci within renal tubules were first observed at 120 days post-injection and the earliest papillary tumour was seen in a mouse 132 days post-injection. Papillary and/or solid tumours were identified in all 50 animals that survived 220 or more days following streptozotocin administration.

Sufficient tissue was available from 165 tumours (92 papillary; 2 mixed papillary and solid; 71 solid architecture) and from 65 dysplastic tubules, for histological assessment of AgNORs and PCNA expression. All sections stained satisfactorily using the silver-colloid method for AgNORs (Fig. 4) and the streptavidin biotin immunohistochemical method for PCNA. Occasional PCNA-stained sections showed minimal background staining; however, PCNA-positive cells were easily discerned by the presence of granular, sharply localised, nuclear-specific staining. AgNOR scores and PCNA indices for renal cortical tubule epithelium were also derived from sections of renal cortex from 10 untreated control mice and from sections of histologically normal renal cortex from 20 streptozotocin-injected mice (mean post-injection interval 326.6 days; range 198–524 days).

The mean AgNOR score and PCNA index for each of the histological categories is shown in Table 1 and the distribution of AgNOR scores and PCNA indices for individual specimens in each histological category is shown in Fig. 5.

Analysis of data using Student's unpaired *t*-test showed a significant difference between mean AgNOR scores and PCNA indices for dysplastic tubules, papillary tumours and solid tumours, and between AgNOR

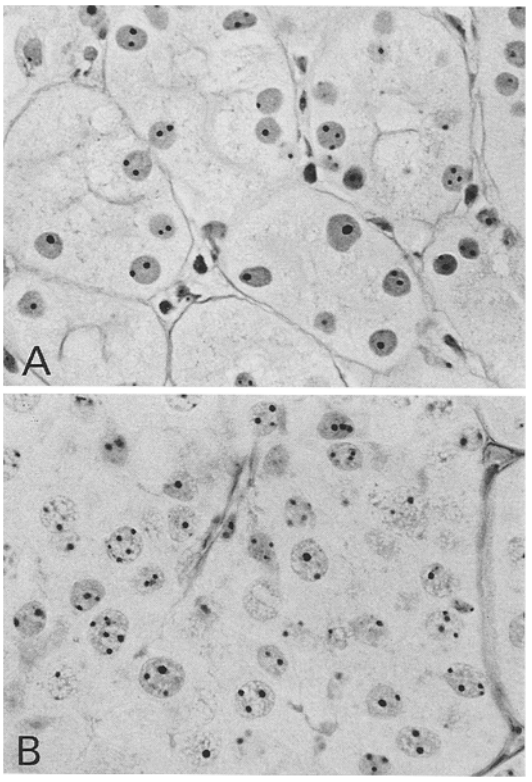

Fig. 4 AgNORs in histologically normal tubules in streptozotocin-treated mouse (**A**) and renal tumour of solid architecture (**B**). Silver-colloid stain, ×830

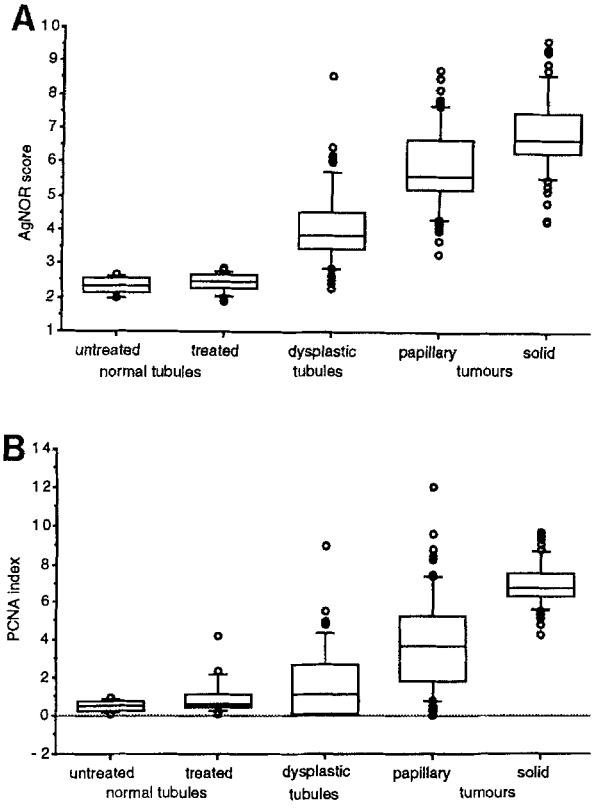

Fig. 5 Mean AgNOR scores (**A**) and PCNA indices (**B**) for each histological category. *Boxes* show 25th, 50th and 75th percentiles and *bars* show 10th and 90th percentiles

Table 1 Mean AgNOR scores and PCNA indices for histologically normal renal tubules, dysplastic renal tubules and renal tumours (mixed papillary-solid tumours (n = 2): AgNOR scores 7.83 and 12.10, PCNA indices 7.33 and 15.40)

Category	Numbers examined	Mean AgNOR score (SD)	Mean PCNA index (SD)
Histologically normal tubules, untreated mice	10	2.32(0.26)	0.53(0.32)
Histologically normal tubules, treated mice	20	2.44(0.28)	0.99(0.94)
Dysplastic tubules	65	4.15(1.16)	1.65(1.85)
All tumours	165	6.37(1.27)	5.26(3.51)
Papillary tumours	92	5.90(1.16)	3.89(2.54)
Solid tumours	71	6.94(1.18)	6.80(3.67)

scores for these groups and histologically normal tubules in treated and untreated animals. PCNA indices showed significant difference between histologically normal tubules (treated and untreated animals) and both architectural types of tumour, but not between dysplastic tubules and either group of histologically normal tubules. Neither AgNOR scores nor PCNA indices differentiated between histologically normal tubules in treated and untreated mice (Table 2). There was weak correlation between AgNOR scores and PCNA indices for all 165 tumours in the series (Pearson correlation coefficient, r = 0.302).

Mean cross-sectional tumour diameter for the 165 tumours examined in this section of the study was 0.96 mm (SD 1.31). Mean diameter for papillary tumours was 0.70 mm (0.38) and for solid tumours was 1.30 mm (1.90). Comparison of means showed solid tumours to be significantly larger than papillary tumours (t = 2.979, P = 0.0033).

Mean post-injection survival interval for all 165 tumours was 330.6 days (SD 94.7). The mean survival interval for solid tumours was longer (348.0 days, SD 96.5) than that for papillary tumours (318.7 days, SD 92.2) and the difference between the two groups just achieved statistical significance (t = 1.972, P = 0.05).

Discussion

The tumorigenic effect of streptozotocin in mice has been previously reported with tumours being divided into adenomas or carcinomas on the basis of size [8].

Light microscopic examination of the renal tumours in the present series showed them to have a morphological appearance similar to human RCC. The tumours exhibited a papillary or solid architecture with intermediate forms being identified. Larger solid tumours showed local invasion and contained areas of necrosis which, coupled with the presence of extra-renal extension in one tumour, suggests a malignant potential for streptozotocin-

Table 2 Significance (P) and (t statistic) obtained from comparison of mean AgNOR scores and mean PCNA indices between histological categories using Student's t-test

	Normal tubules untreated mice	Normal tubules treated mice	Dysplastic tubules	Papillary tumours	Solid tumours
Analysis of difference between mean PCNA indices for each histological category					
Normal tubules untreated mice	–	0.148* (1.487)	0.617* (1.899)	0.0001 (4.156)	<0.0001 (5.365)
Normal tubules treated mice	0.270* (1.125)	–	0.131* (1.528)	<0.0001 (5.010)	<0.0001 (6.982)
Dysplastic tubules	0.0001 (4.945)	<0.0001 (6.511)	–	<0.0001 (5.954)	<0.0001 (9.982)
Papillary tumours	<0.0001 (9.702)	<0.0001 (13.226)	<0.0001 (9.194)	–	<0.0001 (5.959)
Solid tumours	<0.0001 (12.271)	<0.0001 (16.850)	<0.0001 (13.707)	<0.0001 (5.622)	–
Analysis of difference between mean AgNOR scores for each histological category					

* Not significant

induced renal tumours. Similar growth characteristics have been ascribed to human RCC with small tumours rarely exhibiting overt malignant characteristics [2]. The rarity of metastasizing tumours in this series may be the result of the relatively short time interval over which the tumours were followed. If the behaviour of human RCC is extrapolated to the tumours in this series, then the time required for the development of metastatic potential in the majority of tumours in mice is likely to extend beyond the life expectancy of the host.

Both PCNA expression and AgNOR staining has been utilised to evaluate proliferative activity in a variety of premalignant and malignant lesions induced by chemical carcinogens in experimental animals [15, 17, 19–21, 23]. While these studies have shown that AgNOR numbers correlate with proliferative activity in various animal species, the results are not directly comparable between species, or with results obtained from silver-colloid staining of human tissue. Analysis of the sites of AgNORs on metaphase chromosome preparations in mice has shown greater numbers in non-neoplastic tissue than are seen in human metaphase preparations, with the distribution of AgNOR sites differing from those in human tissue. This interspecies variation in the site of AgNORs does not appear to be constant between different strains of mice, although AgNOR sites do show stable inheritance within individual strains [18]. This latter finding emphasises the necessity of utilising a single strain of mice when AgNOR scores are used to evaluate proliferative activity in series of carcinogen-induced premalignant and malignant lesions.

PCNA staining has been used to quantify proliferative activity in various experimental animal models and there is no current evidence to suggest an interspecies variation in PCNA expression [4, 7, 22].

Morita et al. [14] investigated the proliferation of colonic epithelium in mice using 19A2, a murine PCNA (IgM) antibody. In their results they reported low levels of background signal, presumably due to cross-reactivity of antibody staining, but were able to clearly identify those nuclei showing PCNA positivity. PC10 is also a mouse monoclonal antibody (IgG 2a, kappa isotope) and for this reason rabbit anti-mouse IgG 2a was employed as a cross-link in the staining reaction to minimise cross-reactivity which might be expected from the use of biotinylated rabbit anti-mouse secondary antibody with mouse tissue. Occasional sections showed very low levels of background stain; however, this did not inhibit identification of PCNA-positive nuclei.

Evaluation of the proliferative activity of neoplastic lesions in the present study has shown a stepwise progression for both mean AgNOR scores and mean PCNA indices between histologically normal tubules, dysplastic tubules, papillary tumours and solid tumours. These findings indicate a progressive increase in cell proliferation in these lesions and are consistent with tubular epithelial dysplasia being the premalignant lesion for streptozotocin-induced renal tumours in mice. This is reinforced by the observation that small papillary excrescences of tubular cells are occasionally observed within otherwise dysplastic tubules.

Neither of the markers of cell proliferation were able to differentiate between histologically normal tubules in treated and untreated animals, which is evidence that early development of neoplastic proliferation of renal tubular epithelium is associated with morphological changes detectable by histological examination.

Dysplastic tubules showed significant variation in AgNOR scores, compared with histologically normal tubules in streptozotocin-treated mice, which was not evident when the proliferative activity of these two histological categories was compared using PCNA indices. This lack of discrimination for PCNA expression is probably the result of sample size, as in all histologically normal or dysplastic tubules examined less than 1000 cells were available for counting purposes. PCNA is expressed in a relatively small compartment of the cell cycle and in slowly proliferating tissues PCNA-positive cells may be encountered only rarely. If small numbers of cells are examined, then it is possible that some sections are not representative of the population of cells expressing PCNA and as a consequence the proliferative activity of the tissue will be under-estimated. It has been shown that 1000 cells should be evaluated when tissues are examined for PCNA positivity if consistently reproducible results are to be obtained [11]. This is reinforced by the observation that in a number of dysplastic tubules no PCNA-positive cells were found. AgNOR scores appear to be more dis-

criminant as markers of cell proliferation in small population of cells as the numbers of silver-staining foci in each individual cell are evaluated.

Both mean AgNOR scores and PCNA indices showed the proliferative activity of papillary tumours to be lower than that for tumours with a solid architectural pattern. In this series solid tumours appeared to exhibit more malignant characteristics than papillary tumours on histological examination, with more pronounced tumour necrosis, a greater degree of nuclear pleomorphism and the presence of local renal infiltration and early metastasis being observed. In addition, the mean cross-sectional diameter of solid tumours in the series was significantly larger than that of papillary tumours. These histological differences, the variation in proliferative activity between the two tumour types and the observation that some tumours exhibited a dual architecture, containing both solid and papillary elements, suggest that papillary tumours are precursor lesions and have the ability to develop a solid architecture, which in turn is associated with more malignant behaviour. Further evidence for this may be deduced from the observation that solid tumours, as a group, appear to have developed over a longer time interval than papillary tumours, as the mean post-injection survival interval for animals with solid tumours was significantly longer than that for animals with papillary tumours. This observation of a temporal variation in tumour morphology was not absolute, however, as some animals were found to have co-existing tumours that exhibited both architectural patterns. This latter finding does not exclude the possibility that solid tumours were derived from papillary forms as the presence of dysplastic tubules within kidneys containing separate papillary and solid tumours indicates that streptozotocin-induced renal carcinogenesis is an on-going phenomenon not confined to a set interval following streptozotocin administration.

The relationship between papillary and solid tumours, as demonstrated in this model, is of interest as it suggests that papillary tumours have the ability to evolve into solid forms. While the murine model may not be fully applicable to human tumours, the observed morphological progression does raise the possibility of similar morphological transformation in human renal papillary "adenomas".

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