Proliferative activity of urothelial neoplasms: Comparison of BrdU incorporation, Ki67 expression, and nucleolar organiser regions

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Abstract

Aims: To evaluate the proliferative activity of urothelial neoplasms, compare it with that of the normal urinary tract epithelium, and determine its relation to morphological grade and presence of invasion.

Methods: Multiple biopsy specimens from 53 individuals—eight normal controls, five patients with severe urothelial atypia, and 40 with transitional cell carcinomas (TCCs)—were studied using *in vitro* bromodeoxyuridine (BrdU) incorporation, Ki67 antigen expression, and quantitation of the nucleolar organiser regions (NORs).

Results: The percentage of nuclei labelled by BrdU (BrdU index) correlated well with the percentage of nuclei expressing the Ki67 antigen (Ki67 index). These proliferation indices were very low (less than 0.1% in 60% of samples) in the urothelium of normal controls and the morphologically unremarkable epithelium of patients with TCCs. Non-invasive TCCs had increased proliferation (BrdU index 6.32 (SD 0.8)%, Ki67 index 5.04 (0.6)% but lagged behind the invasive tumours (BrdU 20.9 (3.2)%, Ki67 18.6 (2.8)%). The average NOR count was 1.57 (0.03) in morphological normal epithelium, which increased progressively with grade in non-invasive TCCs, but varied greatly in invasive tumours and did not correlate with the proliferation indices. The spectrum of values for both proliferation indices and NORs was particularly wide in grade 2 TCCs. Severe atypias without exophytic growth had an increase in BrdU and Ki67 indices comparable with that found in grade 3–4 invasive TCCs; these also had the highest NORs per nucleus.

Conclusions: The growth potential of urothelial neoplasms is an important indicator of their aggressive course. In particular, growth indices over 10% are strongly associated with the presence of invasion. Papillary grade 2 TCCs show heterogeneity in their growth characteristics which may relate to their diverse clinical course. The mitotic count underestimates the growth potential of papillary TCCs and the addition of proliferation indices such as BrdU incorporation or the Ki67 index may enhance the prognostic

accuracy of conventional morphological grading.

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The growth rate of tumours is an important determinant of their potential for progression and dissemination. Furthermore, knowledge of the specific growth characteristics of a neoplasm is a prerequisite for the development of effective therapeutic agents. In the case of urothelial neoplasia, information about the proliferative capacity of tumours at preinvasive stages may be important in predicting recurrences or progression in grade and stage.

Until recently, the study of the proliferation patterns of human tissues was restricted because of methodological difficulties and limited interaction between pathologists and basic research laboratories. Some of the constraints associated with the use of ³H thymidine have been obviated by the introduction of the *in vitro* incorporation of bromodeoxyuridine (BrdU) and the detection of labelled nuclei by immunohistochemical methods.¹ In addition, monoclonal antibodies against proliferation specific nuclear antigens have facilitated the evaluation of the growth rate of human tumours.² In particular, the monoclonal antibody Ki67 seems to detect a nuclear antigen expressed only in proliferating cells and is used as an alternative to nuclear labelling with nucleotide analogs.^{3,4} Another recent introduction to diagnostic pathology are the changes in the nucleolar organiser regions (NORs) associated with the cell cycle.⁵ Simplified methodology for the visualisation of the argyrophil proteins of the NORs (AgNORs)^{6,7} has made this popular and potentially useful for the detection of the malignant phenotypes in diagnostic pathology.^{8–10}

Because each of these indices of cell growth is associated with a different aspect of the cell cycle, the results obtained by the different approaches may be complementary rather than interchangeable. This is particularly true for neoplastic cells which often have multiple and diverse abnormalities of growth.

For these reasons, we investigated the proliferative patterns of the normal and neoplastic human urothelium using all three approaches in parallel. We then attempted to explore the interrelationships and relevance of the results

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to the degree of cytological and biological malignancy.

Methods

Tissues consisted of biopsy and resection specimens from 53 patients of the Minneapolis VA Medical Center, all men with a mean age of 62 years. Samples of normal urothelium from eight patients without a history of urothelial neoplasia were used as controls. Morphologically normal urothelium was also obtained from 17 of the patients who also had transitional cell carcinomas (TCCs). Five patients had severe epithelial atypia without an exophytic or invasive component. Among the 40 urothelial neoplasms, there were 29 papillary non-invasive TCCs, 10 invasive cancers, stages T1–T3, and one inverted papilloma.

After removal, the tissue was placed in RPMI (Gibco, Bethesda Research Laboratories, Grand Island, New York) and transferred immediately to the laboratory where it was divided for the following procedures: (1) incubation with BrdU followed by immunohistochemical detection of the labelled nuclei; (2) immunohistochemistry using the Ki67 monoclonal antibody on frozen tissues; (3) the AgNOR reaction on sections of paraffin wax embedded tissue; (4) conventional histopathological examination of sections stained with haematoxylin and eosin.

For *in vitro* BrdU incorporation, the tissue was diced into small fragments (greatest dimension 0.2–0.3 cm) using a tissue chopper, rinsed in RPMI medium placed in 2 ml of the same medium containing BrdU and incubated under 95% O₂–5% CO₂ under 2 atmosphere pressure with continuous shaking in a 37°C water bath.

After a series of pilot experiments the optimal conditions were set for the BrdU (Sigma B-5002) concentration at 0.25 mM and the length of incubation at 90 minutes. These conditions gave sharp definition of the labelled nuclei without background staining and a highly reproducible labelling index.

The incubated tissue was washed in phosphate buffered saline (pH 7.2); a portion was fixed in cold 70% ethanol to be processed in paraffin wax and the remainder was frozen for use in procedures requiring frozen sections.

The monoclonal antibody to BrdU (Becton-Dickinson, San Jose, California, USA) was used at a dilution of 1 in 100 and the Ki67 (Dako, Carpinteria, California) at 1 in 33. Biotinylated secondary antibodies and the avidin-peroxidase complex were purchased from Vector Laboratories (Burlingame, California). BrdU could be detected on either paraffin wax processed or frozen sections. The Ki67 was detectable on 6 µm frozen sections fixed in cold acetone and its expression was systematically evaluated in the immediately frozen portion of the tissue as well as in fragments frozen after incubation with BrdU.

The AgNOR reaction was performed on 6 µm sections of paraffin wax processed tissue, using a 2% solution of gelatin in 1% formic acid, mixed with a 50% solution of AgNO₃ at 1:2 ratio. The sections were incubated with this

mixture in the dark at 37°C for 50 minutes. No counterstaining was necessary because the dark brown NORs and the orange coloured nuclei were clearly visible against a pale yellow background.

All reactions were performed in duplicate and in parallel with their normal counterparts whenever these were available. Positive controls with an established level of reactivity were always included to assure quantitative reproducibility.

The counting of positive nuclei was performed with the help of an eyepiece graticule containing 1 cm² grid divided into 100 equal squares. Duplicate sections from the same block were thoroughly screened and representative fields were selected to reflect the overall cellularity and numbers of positive nuclei. At least 2000 cells were counted per section; the ratio of positive over total number of nuclei was expressed as the per cent BrdU or Ki67 index.

A similar set up was used to count the NORs; the number of nuclei and the number of argyrophilic nuclear dots were simultaneously recorded and the average number of NORs per nucleus was calculated for each biopsy specimen.

The results were analysed by applying the *t* test, the χ^2 test with the Yates' correction, and the Wilcoxon rank sum test, as indicated.

The morphological grading of the neoplasms was performed on haematoxylin and eosin stained sections according to established criteria.^{11,12}

Results

MORPHOLOGICALLY NORMAL UROTHELIUM

The normal mucosa from either the controls or the patients with TCCs had very low growth indices, as estimated by either the incorporation of BrdU or the expression of Ki67. These indices were below 0.1% in 50% to 60% of

Figure 1 Distribution of Ki67 and BrdU indices in morphologically normal urothelium from controls and patients with TCCs.

normal tissues and between 0.1 and 2% in 29% (fig 1). Rarely were indices over 2% found in morphologically normal transitional epithelium (the highest Ki67 was 3.6% and highest BrdU 4.2%). It should be emphasised that cases with inflammation or metaplastic changes were excluded from the normal category.

The counting of BrdU labelled nuclei was facilitated by the high contrast between the BrdU positive and negative nuclei; the results were highly reproducible. The counts of Ki67 positive nuclei were less consistent probably because of the variable intensity of the nuclear reactions within the same section which introduced an element of subjectivity and might have resulted in underestimation of weak reactions. Furthermore, some variability was noted in the performance of two different batches of Ki67. This was corrected by adjust-

ing the dilution of the primary antibody from 1 in 33 to 1 in 25 to obtain identical staining of the positive controls. There was a good overall correlation of the two indices (fig 1). With either method the positive nuclei were unevenly distributed along the mucosa and, sometimes, segregated locally. Positive nuclei were found more frequently but not exclusively in the deeper layers of the epithelium (fig 2). Mitotic figures were extremely rare in the normal mucosa and were found only in 12% of the cases.

AgNOR staining showed discrete, round intranuclear bodies, about 2 μ m in diameter (fig 3), on average 1.57 (0.03) per nucleus (fig 4).

No significant quantitative or qualitative differences were found between the AgNORs of normal controls and the morphologically normal epithelium of patients with TCCs.

Specimens with an average of 1.5 or fewer AgNORs per nucleus had Ki67 and BrdU indices below 0.1%, but samples with more AgNORs had variable growth indices.

Figure 2 Morphologically normal urothelium showing a rare BrdU labelled nucleus (arrow) in the basal layer.

Figure 3 AgNOR reaction on normal urothelium.

SEVERE ATYPIA

In five cases there was no exophytic growth or invasion, but the transitional epithelium was disorganised throughout all or most of its layers and consisted of dysplastic cells with large, irregular, and hyperchromatic nuclei. These cases were diagnosed as severe atypia-carcinoma in situ and showed noticeably increased cell proliferation with BrdU indices between 12% and 25% and Ki67 indices between 7% and 32%. Positively staining nuclei were randomly distributed throughout the thickness of the epithelium (fig 5). In all five cases the average AgNORs per nucleus exceeded the normal values and ranged between 2 and 3.6 with an average of 2.89 (0.3) (fig 4).

TRANSITIONAL CELL NEOPLASMS

The BrdU index was greater than 2% in all urothelial neoplasms and the Ki67 index also exceeded 2% in all but one case. The only exception was an inverted transitional cell papilloma which had a Ki67 index of 0.7%. In about 80% of non-invasive TCCs the two proliferation indices fell between 2% and 10%. In 90% of the invasive carcinomas the BrdU index was over 10% and in 80% of them the Ki67 index also exceeded 10%. A good correlation between the two indices was found in 25 cases of TCCs (15 non-invasive and 10 invasive) for which both indices were calculated (fig 6).

In non-invasive TCCs the average proliferation indices were 6.32 (0.8)% for BrdU and 5.04 (0.6)% for Ki67 which are significantly ($p < 0.01$) lower than the corresponding indices in invasive carcinomas (20.9 (3.2)% for BrdU and 18.6 (2.8)% for Ki67). The distribution of positive nuclei in the neoplastic cells was non-uniform. Specifically in the papillary TCCs the positive nuclei tended to segregate in some of the fields and were more frequent in the deeper strata (fig 7). The BrdU or Ki67 positive nuclei far outnumbered the mitotic count with a ratio

Figure 4 Distribution of the average NORs counts per nucleus in the various morphological categories of the urothelial biopsy specimens. Note the wide scatter of values in non-invasive grade 2 TCCs and in the invasive tumours.

Figure 5 Nuclear staining for Ki67 antigen in a case of severe urothelial atypia. Many positive nuclei are present in all layers.

ranging from 1:10 to 1:40. The mitotic figures also tended to segregate in the same microscopic fields as the labelled nuclei. Some invasive carcinomas had most of the positive nuclei arranged at the periphery of the infiltrating tumour islands (fig 8).

The cytological grades of non-invasive papillary TCCs ranged from 1 to 3, while the

Figure 6 Correlation of the Ki67 and BrdU indices in 25 urothelial neoplasms.

invasive TCCs were grade 3 or 4. No significant differences in the proliferation indices could be demonstrated in our material between the different grades of non-invasive TCCs, possibly because of the small number of cases classified as grade 1 (four cases) and the wide range of values among the grade 2 tumours.

The AgNORs in the TCCs varied greatly in numbers and morphology. Significant numerical differences ($p < 0.01$) were found between the grade 1 and grade 3 non-invasive papillary TCCs with average AgNORs of 1.54 (0.14) and 2.53 (0.18), respectively (fig 4). A wide scatter of AgNOR counts per nucleus was observed in grade 2 TCCs (fig 4). A similar scatter of AgNOR numbers was also observed in the invasive carcinomas with the highest—4.4 AgNORs per nucleus—found in an invasive tumour with extensive glandular differentiation and the lowest—1.2 AgNORs per nucleus—in a case with extensive squamous differentiation.

The morphology of the intranuclear argyrophilic bodies in the neoplastic cells was variable. The most common pattern was characterised by very small dots partly segregated into irregular clusters (fig 9).

The products of the positive reaction with anti-Ki67 sometimes resembled AgNORs (fig 10) which suggests that the Ki67 antigen is probably related to the NORs.

There was no correlation between the AgNORs and the proliferation indices of the tumours. When the invasive cancers were excluded some concordance could be demonstrated between the growth indices and the NORs, but it was not significant.

Discussion

The growth rate of neoplastic cells is a crucial factor in the clinical course of tumours and, for this reason, the mitotic count has long been used by pathologists as a criterion of malignant potential. The mitotic phase is too short compared with the duration of the cell cycle and therefore the mitotic count identifies a small fraction of the proliferating cells. Furthermore, it may be difficult to recognise mitotic nuclei other than in metaphase, or obtain reliable counts in tumours with irregularly scattered growth centres. In the case of TCCs some widely used classifications into grades are based on cytological criteria without reference to mitotic rate^{11 12} while others mention an increase in the frequency of mitoses with progression in grade.^{13 14}

While studying the evolution of TCCs in patients with long follow up, we observed that about 45% of the patients initially presenting with non-invasive, grade 2 tumours developed higher grade lesions of which 80% also became invasive.¹⁵ This observation prompted us to search for criteria other than cytological atypia which might have prognostic relevance to the clinical outcome. Among other variables we examined the mitotic activity in sequential biopsy specimens and noticed an abrupt increase preceding the progression in stage. We

Figure 7 Patterns of nuclear BrdU labelling in papillary non-invasive TCCs. (A) Localisation of positive nuclei in the deeper strata of the papillae along the basal lamina. (B) Clustering of positive nuclei in small foci indicating the presence of growth centres.

Figure 8 Nuclear BrdU labelling in an invasive TCC.

then explored the possibility that some of the grade 2 TCCs had a larger proliferating cell fraction prior to the recognition of an increased mitotic rate. To this purpose we studied the growth rate of TCCs using three different approaches: quantitation of the *in vitro* incorporation of BrdU which gives a close estimate of cells in S phase¹⁶; nuclear staining for the Ki67 antigen which marks the proliferating cells excluding those in G₀^{2,4,17}; and quantitation of the NORs which are related to the mitotic cycle.⁵ These studies set the guidelines of the interrelationships between BrdU, the Ki67 indices, and the NORs and the relevance of these parameters to the progression of urothelial neoplasia was then explored.

We found that the normal urothelium has a very low proliferation rate which in most cases is less than 0.1% by either the BrdU incorporation or the Ki67 expression. Similar low values were found when incorporated 3H-thymidine was used to identify cells in S phase.¹⁸ Both methods indicated the existence of growth

centres and showed that the proliferating capacity is predominantly, but not exclusively, localised in the basal layers. The AgNORs were consistent in the normal urothelium and fell, in 70% of cases, between 1.2 and 1.7 per nucleus. Because the results for the morphologically normal urothelium from patients with TCCs were similar to those for controls, no evidence for generalised cellular unrest was found in the bladder mucosas that harbour neoplasms. It is of course possible that the urothelium of patients with bladder cancer is capable of exaggerated response to growth stimuli despite its normal baseline proliferation rate. So far, our studies on the expression of the growth related receptors for transferrin and epidermal growth factor have shown that normal mucosas from patients with TCCs do not deviate from those of the controls.¹⁹ If a hyperresponsiveness exists it does not result from overexpression of these growth related receptors.

Specimens with inflammatory and neoplastic changes were excluded from the normal category and only biopsies containing well preserved, full thickness epithelium were considered suitable for quantitative evaluation. These selection criteria limited the variability of the normal values and reduced the possibility of deviations secondary to reactive conditions.

A noticeable increase in the proliferation indices as well as in the number of AgNORs was found in all specimens with severe atypia-CIS (also called intraepithelial neoplasia) despite the absence of an exophytic or infiltrative growth. This indicates that, in these conditions, there is a pronounced acceleration of the turnover rate of the epithelium. The rapidly proliferating epithelium does not pile up or break through the basal lamina but exfoliates on a much greater scale than normally and thus a large number of cells are found in the urine. The large number of exfoliated cells renders the cytological examination of the urine an excellent diagnostic tool in cases of

Figure 9 AgNOR reaction in a papillary, non-invasive, grade 2 TCC shows multiple small argyrophilic dots per nucleus.

Figure 10 An invasive carcinoma stained for the Ki67 antigen shows, in addition to the diffuse nuclear staining, darker intranuclear dots resembling NORs.

severe atypia-CIS. These cases had proliferation indices similar to those of invasive carcinomas and also had the highest average number of AgNORs per nucleus compared with all overt urothelial neoplasias. Despite the restricted topographical localisation, the morphological and biological characteristics in severe atypia-CIS meet the criteria for malignant neoplasia which explains the high frequency of progression to invasive carcinomas.^{20 21}

The proliferation indices of the papillary non-invasive TCCs were clearly beyond the normal range. Particularly interesting is the wide spectrum of values in TCCs with the morphological features of grade 2. This grade seems to include biologically heterogeneous tumours and the acceleration in growth seems to precede the appearance of overt cytological atypia. It may be clinically useful to evaluate systematically the proliferation index of the papillary non-invasive TCCs and take their

growth capacity into consideration when grading their invasive potential.

The heterogeneity of grade 2 TCCs was also apparent in the scatter of the average number of AgNORs per nucleus, from 1.2 to 3, which spans the spectrum between the lowest normal value to the highest among grade 3 tumours. Interestingly, the grade 2 TCCs show similar heterogeneity for the expression of other markers of aggressive behaviour, such as "loss" of the normally expected blood group antigen(s).²²

The presence of growth centres in many of the papillary tumours shows that cell growth in neoplasia is not uniform; sometimes it follows a distribution resembling the normal state with the proliferating cells distributed predominantly along the basal lamina of the papillary formations or invasive tumour islands, while, at other times, it appears chaotic with irregularly distributed foci of growth. Evidence that this is a genuine phenomenon and not a methodological artefact is provided by the similarity of the growth patterns for both BrdU and Ki67 reactions and by the concurrence in the same fields of clustered positive nuclei and mitoses. The fact that BrdU or Ki67 positive nuclei far outnumber the mitotic count confirms the much shorter duration of the actual mitosis compared with that of the S phase. Theoretically, Ki67 is expected to exceed the BrdU index because it identifies nuclei in several phases of the cycle and is not restricted to the S phase. This association was not demonstrated in our material possibly because we adjusted the antibody dilution to achieve a sharp cutoff point of the positive reactions and thus we might have identified mostly nuclei in late S-G₂ phase which express strongly the Ki67 antigen. On this basis we could also explain the correlation of the Ki67 with the BrdU index. Another explanation could be a cell type related difference in the degree of Ki67 expression in the various phases of the cycle. For example, urothelial cells may not behave like lymphoid cells or other tumour cells on which most of the studies relevant to Ki67 have focused.^{3 23}

In general, the mitotic count underestimates the growth potential of the papillary TCCs which far exceeds the normal state even in low grade tumours. Furthermore, the growth acceleration in preinvasive neoplasia appears to antedate the cytological changes on which the current grading system is based. We plan to follow the evolution of these tumours to clarify the time sequence of the three phenomena: proliferation, increased atypia, invasion.

Remarkably, the invasive carcinomas had much higher BrdU and Ki67 indices compared with non-invasive TCCs, a finding similar to that reported by Mellon *et al.*¹⁷ A detailed, sequential evaluation of the changes occurring in TCCs which present in non-invasive stages but later progress in stage is necessary to understand the relationship between accelerated and infiltrative growth. The results of this study indicate that a connection exists between growth indices over 10% and invasive potential.

Although the average number of AgNORs per nucleus increased with grade, it showed no correlation with the proliferation indices and was very variable in invasive carcinomas. The level and direction of cellular differentiation seem to affect the NORs. For example, we found that the AgNORs of well differentiated papillary TCCs fell within the normal range and that the AgNOR counts were highest in carcinomas with glandular differentiation and lowest in carcinomas with advanced squamous differentiation. Therefore, the clinical importance of AgNORs can only be evaluated within the context of cellular differentiation.

We conclude that the growth potential of urothelial neoplasms is an important variable in determining their aggressive course and that the recent improvement and simplification of the required methodology make the estimation of growth indices accessible to the up-to-date diagnostic pathology laboratory.

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