Experimental models of human bladder carcinogenesis

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Bladder cancer is the fifth most common cancer in the UK, yet human bladder carcinogenesis remains poorly understood and the response of bladder tumours to radio- and chemo-therapy is unpredictable. The aims of this article are to review human bladder carcinogenesis and appraise the different in vitro and in vivo approaches that have been developed to study the process. The review considers how in vitro models based on normal human urothelial (NHU) cells can be applied to human bladder cancer research. We conclude that recent advances in NHU cell culture offer novel approaches for defining urothelial tissue-specific responses to genotoxic and non-genotoxic carcinogens and elucidating the role of specific genes involved in the mechanisms of bladder carcinogenesis and malignant progression.

Bladder cancer and carcinogenesis

In developed countries, the majority (>90%) of bladder cancers are sporadic carcinomas that arise from the urothelium, the highly-specialized transitional epithelium that lines the urinary bladder. Urothelial cell carcinoma (UC or transitional cell carcinoma) is three times more common in men than in women and is predominantly a disease of the elderly (http://www.cancerresearchuk.org/aboutcancer/statistics/). As historically, the molecular pathways leading to the development and progression of muscle-invasive UCC were poorly understood, UCC has come to represent all urothelial neoplasms, irrespective of their malignant potential. It is now, however, evident that UCCs arise via two major pathways, each of which is defined by distinct biological phenotypes and underpinned by specific genetic lesions. The most common pathway, which is representative of ~70% of UCC at clinical presentation, leads to the development of low-grade papillary tumours. Despite their high frequency of recurrence (60–70%) following cystoscopic removal, these tumours have a relatively low rate of progression (10–20%) to muscle-invasive disease, although a proportion of tumours may recur at a higher grade (1). Papillary tumours represent an indolent form of UCC, yet there remain challenges in predicting the subset of tumours with malignant potential [e.g. (2)], in order to provide appropriate disease management. At clinical presentation, a further 20% of patients are found to have muscle-invasive UCC, which is thought to develop from flat, high grade dysplastic lesions of carcinoma in situ (CIS). This second pathway represents UCC of high malignant potential, which show high rates of progression and metastasis, poor response to therapy and five-year survival rates of only 50%.

A large number of genetic changes have been associated with the genesis and progression of UCC, many of which appear to abrogate the $G_1$/S cell cycle checkpoint [reviewed in (3)]. In particular, loss of p53 function has been linked to the development of muscle-invasive disease (4) and loss of retinoblastoma protein (pRb) has been associated with metastatic potential (5). However, no robust, unified model defining the two pathways has yet emerged. One model receiving current scrutiny is that activating point mutations in the FGFR3 receptor gene, which are associated with UCCs of low malignant potential, are mutually exclusive to alterations in the p53 gene. If verified, changes in FGFR3 and p53 genes, respectively, could epitomize the two alternative genetic/biological pathways of UCC (5,6).

An important feature of the biology of urothelium that may influence urothelial carcinogenesis relates to its function as a self-renewing urinary barrier. Normal urothelium from all species has an extremely low rate of turnover and virtual absence of mitoses (8,9), but a high regenerative capacity, showing rapid proliferation during development and in response to damage or injury (10). Excreted urinary carcinogens may promote carcinogenesis, not only by direct genotoxic damage, but also by damaging the epithelium and driving proliferation towards restoration of a urinary barrier.

A number of risk factors have been strongly linked to the development of bladder cancer. Tobacco smoking is the highest risk factor, accounting for at least one-third of bladder cancer cases (11). Typical smokers are two to three times more likely to develop the disease than non-smokers, and the risk increases with intensity of smoking (12–15). Tobacco smoke contains ~4000 chemicals, many of which are genotoxins, such as $N$-nitroso compounds and aromatic amines, e.g. 4-aminobiphenyl (4-ABP) and o-toluidine. The urine of smokers has been shown to be mutagenic, containing twice the total concentration of aromatic amines compared to non-smokers (16–18). DNA adducted to 4-ABP has been detected in significantly higher levels in both exfoliated cells in the urine and urothelium or UCC biopsies from smokers compared with non-smokers (19–23) [reviewed in (24)]. 4-ABP-haemoglobin adducts are also elevated in the blood of smokers; these adducts are potential biomarkers for estimating individual exposure to bladder carcinogens (25,26) [reviewed in (27)]. Mutation hotspots in the p53 gene of human bladder tumours correlate with the DNA-binding spectrum of 4-ABP, providing further evidence for the involvement of this important tobacco carcinogen in urothelial carcinogenesis (28). Smoking appears to influence p53 mutations and is associated with invasive, high grade UCCs; by contrast, FGFR3

Abbreviations: 4-ABP, 4-aminobiphenyl; HPV, human papillomavirus; NHU, normal human urothelium; MMC, mitomycin c; MNU, N-methyl-N-nitrosourea; RNAi, RNA interference; UCC, urothelial cell carcinoma.
mutations are not associated with smoking status and may result from endogenous DNA damage (29). Non-genotoxins in tobacco smoke may also contribute to bladder carcinogenesis by promoting tumour growth, for example by increasing cell proliferation or suppressing the immune response (30).

Domestic or occupational use of hair dyes has been suggested as a risk factor, particularly in women, who have a poorer survival rate than men (31). Exposure to industrial chemicals [reviewed in (32)] and drugs such as cyclophosphamide (33) and phenacetin (34) also increase bladder cancer risk. Chronic irritation or infection of the bladder has been implicated, possibly due to the production of nitrosocompounds or reactive oxygen species in the bladder, combined with increased proliferation in response to injury (35–37). In areas where infestation with the *Schistosoma haematobium* parasite is endemic, such as Egypt, infection is associated with an increased risk of developing squamous cell carcinoma (SCC), a histopathologically distinct form of bladder cancer (38) [reviewed in (39)]. The development of SCC may be related to dietary factors, e.g. Vitamin A levels, which can affect differentiation of the urothelium (40,41). Genetic factors, such as polymorphisms in genes encoding carcinogen metabolizing enzymes or DNA repair proteins, may contribute to inter-individual variation in bladder cancer susceptibility (42).

Paradoxically, genotoxins are also used to treat bladder cancer. Intravesical cytotoxic drugs, such as mitomycin C (MMC), a crosslinking antibiotic (43), are instilled into the bladder following transurethral resection of poorly differentiated, early stage tumours in order to reduce the risk of recurrence (44). Invasive bladder tumours may be treated with radiotherapy. However, the response of bladder tumours to both chemotherapy and radiotherapy is highly unpredictable. Resistance to chemotherapy may arise from over-expression of genes, such as the multidrug resistance gene *mdr1* (45,46). The p53 status of tumour cells is also implicated in therapy response, but its effect is unclear. p53-null UCC tumours have been reported to show increased sensitivity to some chemotherapeutic agents, but not others (47–49). Increased survival following radiotherapy of bladder tumours with altered p53 has been reported (50), whereas other studies have demonstrated the opposite effect (51); yet others have found no relationship between p53 status and response to radiochemotherapy (52).

**Animal models of bladder cancer**

Animal models of cancer have been important for demonstrating that carcinogenesis is a multistep process comprising three key stages: initiation, the irreversible fixation of a genetic alteration; promotion, the reversible expansion of the tumour cell population; and progression, the irreversible transition to a malignant, invasive tumour (53–55). In many models of bladder carcinogenesis, multiple doses of carcinogens have been instilled directly into the bladders of rodents and dogs. In 1972, Hicks and Wakefield used four doses of *N*-methyl-*N*-nitrosourea (MNU) to rapidly induce bladder cancer in rats, demonstrating that it is a complete carcinogen. However, the doses used were very high (~100 mM) and histological analysis of this dose in rat bladders revealed areas of necrosis, desquamation and haemorrhage followed by urothelial hyperplasia (56,57). This suggests that the compound induced cytotoxic damage to the urothelium, which would have invoked a 'wound response', driving rapid proliferation and allowing the accumulation and perpetuation of mutations. In the human bladder, it is likely that the urothelium is chronically exposed to sub-cytotoxic doses of genotoxins and, hence, acute exposure may not be representative of the process in man. Nevertheless, MNU has been used as the initiating agent in many rodent studies in order to test compounds for their ability to promote bladder tumorigenesis (58–61). Rodent models have also been used to study the contribution of DNA adduct burden to tumour incidence (62).

However, rodent models have produced false positive results. For instance, the artificial sweetener, sodium saccharin, was shown to induce bladder tumours in several studies (58,63,64). The mechanism was later shown to be due to the formation of calculi in the urinary tracts of rats, resulting in physical damage to the urothelium and a proliferative wound response that led to the promotion of coincidental or dormant mutations (65). Saccharin exposure does not lead to calculi formation in the human bladder due to differences in renal physiology, although the carcinogenic potential of the compound is still under debate. Thus, differences in physiology, metabolism and urinary pH between rodents and humans, together with the lower threshold for immortalization in rodent models due to constitutive telomerase activity in all somatic cells, strongly suggest that rodent models of bladder cancer are not ideal for studying mechanisms of human carcinogenesis (30,66).

In order to overcome the species differences inherent in rodent models, bladder carcinogenesis has also been studied in dogs. Owing to similarities with human metabolic processes, dogs are more suitable for experiments with carcinogens that require bioactivation. 4-ABP and its metabolites have been administered orally, intravenously and transurethrally to beagle dogs in order to study urothelial adduct formation, repair and tumour incidence (67–69). Although these studies have been useful in elucidating the mechanisms of bladder carcinogenesis mediated by 4-ABP, experiments on dogs incur both financial and ethical constraints.

The most useful animal model to emerge in recent years is the transgenic mouse, in particular the development of mice in which the transgene is driven from the tissue-specific uroplakin II gene promoter, so that expression is restricted to the urothelium (55,70–73). Mice expressing constitutively activated H-Ras developed urothelial hyperplasia and low-grade superficial papillary tumours (55), whereas mice expressing the SV40 T antigen, which functionally inactivates both p53 and pRb, developed CIS and invasive bladder tumours (70). These models afford insight into the molecular basis of urothelial cell tumorigenesis and the pathways of bladder cancer progression in man and provide direct experimental evidence that specific genetic lesions give rise to phenotypically distinct bladder tumours.

**Bladder cancer cell lines**

A number of established cell lines have been developed from rodent urothelial cells, facilitated by tissue accessibility and the propensity of rodent cells to undergo spontaneous transformation more readily than human cells (74). Rat urothelial cells have been used to show that agents such as MNU and hydrogen peroxide (H2O2) can cause malignant transformation of normal, non-tumorigenic and of low-grade tumour cells (75–77). However, and in light of the unsuitability of the rat
model for understanding human carcinogenesis discussed above, the relevance of the rodent cell lines to normal and malignant human urothelial cells is questionable. Many established cell lines have been derived from human UCC, reflecting access to tumour resection specimens and the immortalized nature of tumour-derived cells. Cell lines representing tumours of different grades and stage have been developed and variably characterized [see (78) for review of 22 cell lines] and have been shown to retain characteristics of the originating tumours. This is particularly exemplified in orthotopic nude mice models (79,80) and human in vitro organotypic systems (81,82), where UCC-derived cell lines have been shown to develop tumours that recapitulate the differentiation (grade) and invasion (stage) characteristics of the originating tumour. However, the success rates for generating cell lines from primary tumour material are very low and therefore the lines represent a very select subset of tumours with the potential to survive and adapt to a cell culture environment. Of all the human UCC-derived lines, only the RT4 cell line represents a well-differentiated papillary non-invasive (G1 pTa) tumour, which as discussed above is the most common form of human bladder cancer.

Bladder cancer cell lines have been invaluable for studying bladder tumour cell behaviour. For example, analysis of a panel of invasive UCC-derived cell lines helped define the functional significance and inter-relationships of pRb and p53 pathways in UCC development, and provided important insight into how the biology of UCC is governed by the mechanism of pathway inactivation (83). Nevertheless, UCC-derived cells are abnormal, with complex karyotypes reflecting accumulated genetic aberrations and although uncloned cell lines potentially represent the genetic and phenotypic biodiversity of the tumour population, they may exhibit genetic/phenotypic drift due to selection pressures imposed during extensive cell culture. Thus, data arising from studies using cancer cell lines may be idiosyncratic and in certain cases conflicting. For instance, p53-defective human bladder cancer cell lines have been reported as radiosensitive in one study (84), radioresistant in another (85), whereas a third study reported no correlation between p53 status and sensitivity to methotrexate, Adriamycin or cisplatin in nine bladder UCC cell lines (86). It is also worth highlighting the importance of pedigree, as cross-contamination between cell lines (of urothelial or non-urothelial derivation) can lead to confounding

Fig. 1. The NHU Cell Culture System. Schematic diagram summarizing the use of urothelial cell cultures for understanding urothelial carcinogenesis. Normal and malignant urothelial behaviour can be studied in situ (blue), for instance by immunohistochimical studies. Upon isolation and culture, NHU cells can be maintained as highly proliferative, finite cell lines. By means of genetic manipulation (viral transduction and induction of defined genetic alterations), ‘para-malignant’ cells can be established (yellow)—see text for detailed discussion. Studying these cells and comparing them to their well-characterized, malignant counterparts (RT4, RT112, EJ) has proven to be very useful in understanding the role of early genetic events in malignant transformation. The ability to induce full maturation/differentiation of urothelial cells and 3D reconstitution of normal and tumour cells in organ culture (green) both attests to the validity and strength of the urothelial system for such studies and renders the urothelial system unique among other human tissue culture models.
results and the use of cell lines with characterized marker expression or sourced from authenticated cell banks (e.g. European Collection of Cell Cultures at http://www.ecacc.org.uk/ or American Type Culture Collection at http://www.lgcromochem.com/atcc/) should be mandatory [discussed in (87–89)].

Normal human urothelial cells

Although urothelium has a low constitutive turnover rate, during urothelial repair, cells from all layers of the urothelium are recruited into the cell cycle (90). This regenerative 'wound response' is exploited in the isolation and growth of normal human urothelial (NHU) cells in vitro.

Traditionally, epithelial cells have been refractory to growth in monolayer cell culture, due to their propensity to become overgrown by competing stromal cell populations. A breakthrough was the development of low calcium, serum-free growth media to promote growth of normal human keratinocytes, at the expense of serum-dependent fibroblast growth. This led to the development of techniques to isolate and culture NHU cells from surgical specimens of bladder, ureter and renal pelvis (91–97).

NHU cells, cultured as monolayers in low calcium (0.09 mM) keratinocyte serum-free medium containing bovine pituitary extract, recombinant epidermal growth factor and supplemented with cholera toxin, have been characterized extensively (96,97). NHU cell cultures display a rapid (~15 h) proliferation rate during log phase growth and are contact-inhibited at confluence, but may be propagated as finite cell lines through at least 20 population doublings by serial passage. The removal of exogenous growth factors from the medium has been shown to make little difference to the exponential growth rate of NHU cell cultures, demonstrating that NHU cells stimulate their own proliferation in an autocrine or juxtacrine fashion (98), which is mediated through the EGF receptor (98–102). NHU cell cultures exhibit a basal/intermediate urothelial cell phenotype, but both stratification (96) and differentiation (103–105) may be induced, attesting to the normal differentiation potential of NHU cell cultures. Another feature of normal cells is that cultures derived from different donors retain the genotype of the tissue donor, so inter-individual variation can be studied. For example, DNA repair gene expression was shown to vary between six primary urothelial cell lines cultured under identical conditions; such differences may reflect differential susceptibility to bladder cancer (106). This inter-individual variation between cell lines also implies that several tissue donors should be used when assessing genotoxic response.

NHU cell lines have been used to investigate two important aspects of bladder cancer. First, studying the effects of carcinogens has elucidated possible mechanisms of bladder cancer development. Bladder carcinogens investigated include 4-ABP, the carcinogen implicated in tobacco-induced bladder cancer, MNU, a direct acting genotoxin used to develop rodent models of bladder cancer and H₂O₂, implicated in the development of bladder cancer due to infection. Typically, such studies have investigated the effects of genotoxic agents on cell morphology, proliferation, apoptosis and cell cycle arrest. For instance, Reznikoff et al. (107) showed that the response of NHU cells to MNU involved initial growth delay and subsequent recovery of morphologically normal cells, and Chien et al. (108) reported p53-independent G2 check-point arrest and induction of senescence after treatment with H₂O₂. Second, studying the effects of therapeutic agents such as γ-radiation and MMC on normal cells may help develop treatment strategies for bladder cancer. For example, MMC has been reported to induce cell cycle arrest with stabilization of p53 and upregulation of p21 proteins (109), whereas γ-radiation has been reported to induce apoptosis, pre-malignant changes and the expression of c-myc and bcl-2, with inter-individual variation in responses (110–112).

Genetically manipulated human urothelial cells

A disadvantage of using NHU cells is that they can only be subcultured a finite number of times before undergoing spontaneous replicative senescence (95). The role of telomere shortening in this process is unclear and it is thought that replicative senescence is due to cellular accumulation of the cyclin-dependent kinase inhibitor protein, p16INK4 (113,114). An understanding of the mechanisms leading to accumulation of p16INK4 protein in cultured cells is incomplete, although it may at least in part represent a culture artefact induced by stress, such as DNA damage or sub-optimal culture conditions (115). Nevertheless, it is likely that bladder tumorigenesis in vivo requires that this senescence block is overcome (116).

To establish long-term human urothelial cell lines, oncogenic viruses, such as SV40 and high-risk human papillomavirus (HPV), or their oncogenic products, large T antigen and E6/E7 oncoproteins, respectively, have been exploited as immortalizing agents (117–119). The derived cell lines have been used to investigate the role of genotoxins, including 4-ABP, in multistage carcinogenesis (120–123) and the effects of p53 loss on response of cells to chemo- and/or radio-therapies. Loss of functional p53 has been correlated with resistance to both radiation and methotrexate in HPV-immortalized urothelial cells in vitro (124,125). By contrast, Diggle et al. (109) reported that HPV16 E6 expression sensitized urothelial cells in vitro to treatment with MMC or γ-radiation.

Although these modified cell lines retain urothelial characteristics, it is clear that major signalling pathways controlling decision-making in the context of cell cycle progression, proliferation and/or apoptosis have been inactivated. Recent studies have demonstrated that HPV16 E6-transformed NHU cells exhibit increased susceptibility to growth inhibition and apoptosis signals such as those triggered by members of the Tumour Necrosis Factor (TNF) superfamily of proteins (126,127), the classical inducers of apoptotic cell death in both immunocytes and epithelial cells. Thus, in addition to offering the advantage of extended life-span, these immortalized cells have been useful in understanding how early events in malignant transformation may influence lympho-epithelial interactions in the context of tumour surveillance and how genetic changes affect urothelial cell responses to signals associated with proliferation and/or cell death.

Despite these advantages, however, the immortalization strategy has two inherent limitations. First, although the derived cells are non-tumorigenic, they are, by definition, no longer 'normal', as the oncogenic viral proteins disable both the critical p53 and pRb tumour suppressor pathways. For instance, loss of p53 function favours genetic instability and allows immortalized cells to accumulate chromosomal abnormalities, leading to the development of dominant, karyotypically abnormal transformed sub-populations (109,128–130).
Second, these cells have no ‘defined’ genetic changes and a number of signaling pathways have been inactivated simultaneously. For example, in addition to causing the degradation of p53 via ubiquitination, the HPV 16 E6 oncoprotein sensitizes cells to apoptosis (131) and activates the catalytic subunit of telomerase (hTERT) (132). Therefore, in order to understand how specific genetic changes influence urothelial cell homeostasis and to provide a better-defined in vitro model of urothelial carcinogenesis, more subtle genetic manipulations are required. A number of approaches for such studies have been developed, including the use of dominant-negative mutant proteins to disable specific tumour suppressor function.

NHU cells are susceptible to transduction by amphotropic retroviruses, which provides a highly efficient (>90%) method to introduce gene sequences of interest into NHU cells. This permits, for example, the effects of disabling key tumour suppressor genes, such as p53 and p16, to be studied. We have used this approach to transduce NHU cells and select stable ‘paramalignant’ human urothelial sublines expressing either a dominant-negative p53 miniprotein (HU-p53DD) or a p16-insensitive CDK4 mutant (HU-CDK4mut), respectively (127). The functionality of the sublines was demonstrated by the inability of HU-p53DD cells to transactivate p21 and hyperphosphorylation of retinoblastoma protein in HU-CDK4 cells (127). The use of retroviruses designed with different antibiotic selection cassettes offers the potential to look at combinations and sequences of genetic events, such as the loss of p53 from a p16-disabled background. In our hands, however, cells could not survive the simultaneous loss of p53 and p16 functions, suggesting that other changes, such as telomerase activation, may be required (127). We have also demonstrated that loss of p16, but not p53, function altered the susceptibility of NHU cells to apoptosis induced by CD40, a member of the TNF receptor family (127). Although the importance of p53 and p16 loss in bladder carcinogenesis is well established (133), such studies may provide an understanding of the underlying molecular mechanisms of progression to malignancy.

New developments and future prospects

Investigation of protein function by inhibition of activity has been critical to understanding a variety of biological processes, with antisense oligonucleotides, dominant-negative mutants and knockout transgenic animals being some of the most frequently employed methodologies. More recently, a new strategy has emerged, namely post-transcriptional gene silencing or RNA interference (RNAi), using small interfering RNA (siRNA) molecules (134). Combination of this powerful technology with the use of viral vectors for efficient delivery and stable siRNA expression (for an example see (135)) has opened new avenues for investigation, both in the field of functional genomics (136) and in clinical therapeutics. RNAi is characterized both by choice of targets and a high degree of specificity, and has been used successfully to study the consequences of inactivating a wide range of cancer-associated genes and signalling proteins, including Ras, Bcl-2 family members, cell cycle regulators, vascular endothelial growth factor and viral oncopgenes (137). Importantly, there are a number of examples where RNAi has shown efficacy at reducing the tumour burden in experimental cancer models in vivo (137), providing great promise for future RNAi-based cancer therapies.

The establishment and characterization of ‘paramalignant’ human urothelial cells with engineered genetic alterations (Table I) is a promising tool to understanding urothelial carcinogenesis. This approach will be revolutionized by the use of siRNA technologies to develop cells with ‘knocked-down’ candidate tumour suppressor gene activity in a NHU cell background. Such strategies will contribute significantly to our understanding of the influence of specific genetic changes in urothelial tumorigenesis and should have application in identifying novel, relevant chemo- and immuno-therapeutic targets.

Collectively, functional protein inhibition and phenotypic modification studies may provide a means to elucidate the multistep pathways leading from normal cells to cancer cells and identify potential therapeutic targets. As the genotypic and, consequently, phenotypic heterogeneity of tumours dictates that effective remedies may require patient-specific application, such in vitro models could prove invaluable for the development of more effective treatments on a tumour-specific basis.

In conclusion, the engineering of ‘designer’ human urothelial cells has, as yet, largely unexplored potential to improved specificity and physiological relevance over the experimental models of yesterday, although the clinical relevance of findings derived from these models will need to be determined by their validation in an in vivo setting.

Table I. Cell lines derived from genetically-manipulated human urothelial cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Protein expressed/modification</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV-HUC</td>
<td>Simian virus 40 T-antigen</td>
<td>(111)</td>
</tr>
<tr>
<td>UROtsa</td>
<td>Temperature-sensitive simian</td>
<td>(112)</td>
</tr>
<tr>
<td>HPV-16E6</td>
<td>HPV type 16 E6 protein</td>
<td>(104,113,120)</td>
</tr>
<tr>
<td>E6-HUC &amp; HU-E6</td>
<td>HPV type 16 E6 protein</td>
<td></td>
</tr>
<tr>
<td>E7-HUC</td>
<td>HPV type 16 E7 protein</td>
<td>(113)</td>
</tr>
<tr>
<td>HU-p53DD</td>
<td>Dominant-negative p53 mutant</td>
<td>(121)</td>
</tr>
<tr>
<td>HU-CDK4mut</td>
<td>p16-insensitive CDK4 mutant</td>
<td>(121)</td>
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