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Parathyroid hormone-related protein (1–34) and urothelial redifferentiation in the neuropathic urinary bladder

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Study design: A comparative study of immunostaining for parathyroid hormone-related protein (1–34) (PTHrP (1–34)) in the vesical epithelium of biopsies obtained from patients with non-neuropathic bladder and those with neuropathic bladder.
Objectives: To investigate the immunostaining for PTHrP (1–34) in the control cases and in neuropathic bladders showing (1) normal transitional epithelium, (2) hyperplastic transitional epithelium, and (3) squamous metaplasia.
Setting: Regional Spinal Injuries Centre, and Department of Cellular Pathology, Southport & Ormskirk Hospitals NHS Trust, Southport, Department of Pathology, Royal Liverpool University Hospital and the Departments of Clinical Chemistry and Cell Biology, The University of Liverpool, Liverpool, England.
Methods: Cold cup biopsies of bladder mucosa were taken from patients suffering from neuropathic urinary bladder when they were undergoing a therapeutic procedure in the urinary tract. Immunohistochemistry was performed on these biopsy specimens using a rabbit polyclonal antibody raised to a synthetic peptide corresponding to human PTHrP (1–34). Control group ($n=10$) consisted of archival biopsies taken from non-neuropathic bladders.
Results: In the control group, the transitional epithelium showed no immunostaining, or at the most, very faint positive staining was seen in the transitional epithelium of non-neuropathic bladder. Positive immunostaining to PTHrP (1–34) was seen in the normal transitional epithelium of neuropathic bladder in nine of 13 cases. Hyperplastic transitional epithelium showed positive immunostaining for PTHrP (1–34) in 11 of 13 biopsies from patients with neuropathic bladder. Immunostaining for PTHrP (1–34) was observed in the metaplastic squamous epithelium in 14 of 17 cases with neuropathic bladder.
Conclusion: The transitional epithelium of non-neuropathic bladder showed no immunostaining, or at the most, very faint positive staining for PTHrP (1–34). In contrast to this, positive immunostaining for PTHrP (1–34) was observed more frequently in the vesical epithelium of neuropathic bladder. This observation opens up avenues for innovative therapy with PTHrP or its analogues for possible modulation of urothelial differentiation in the neuropathic bladder.
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Introduction

Alternate differentiation pathways of urothelium
Epithelial tissues show a high degree of diversity of differentiation pathways, reflecting the unique functional specialisations of different epithelia. On the basis of cytokeratin subclass expression as a correlate of epithelial tissue development, morphology and organisation, a classification based on three major epithelial 'differentiation programmes', termed 'simple', 'stratified squamous', and 'complex' has been proposed.[1] Normal human urothelium, the stratified transitional epithelium of the urinary bladder, is a model example of the 'complex' epithelium. Urothelium can undergo at least

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three pathways of differentiation: (a) transitional; (b) squamous; and (c) glandular, characterised by the production of uroplakins, K1/K10 keratins, and secreted glycoproteins, respectively.[2,3,] Southgate and associates[4] suggested that stratified squamous epithelial phenotype is the default differentiation programme of urothelial cells. Urothelium shows a propensity for squamous redifferentiation in inflammatory or regenerative states.[5]

Squamous differentiation of urothelium in neuropathic bladder

In the neuropathic bladder, squamous differentiation of vesical epithelium is seen frequently in association with bladder stone(s), long-term bladder catheter drainage, or recurrent urinary infection. We observed in an earlier study, some degree of squamous metaplastic changes in 10 out of 40 (25%) consecutive bladder biopsies taken from patients with neuropathic bladder.[6] Spinal cord injury patients have a 16–28-fold higher risk of squamous cell carcinoma of the bladder.[6] Long-term follow-up studies of vesical squamous metaplasia demonstrate that 15% to 28% of patients ultimately develop carcinoma.[7]

The molecular mechanisms leading to squamous redifferentiation of vesical urothelium, and urothelial hyperplasia in neuropathic bladder need to be unravelled in order to develop novel therapeutic strategies for management of neuropathic bladder in spinal cord injury patients. Homozygous deletion of *p16/p19* was observed in squamous metaplasia from bladder cancer patients (five of 11; 45%), showing that this change occurred in preneoplastic cells.[8] Squamous-specific genes exhibit not only different patterns of tissue-specific expression but are also induced at different stages during differentiation, suggesting that transcription of individual genes is regulated by distinct mechanisms. Studies into these mechanisms will provide insight into the control of squamous metaplasia and the development of squamous cell carcinoma.[9]

PTHrP and epithelial differentiation

Parathyroid hormone-related protein (PTHrP), originally identified as a causative agent for hypercalcaemia of malignancy, has been implicated in the regulation of growth and differentiation of endochondral bone, hair follicle, and breast as an autocrine/paracrine factor.[10] PTHrP has been shown to regulate the rate of keratinocyte differentiation in the skin of adult mice.[11] The primary role of PTHrP is as a local paracrine or autocrine factor. PTHrP has putative powerful local regulatory effects.[12] *In situ* hybridization studies have shown that PTHrP mRNA is expressed typically in a focal pattern in surface epithelia, whereas the receptor mRNA is distributed diffusely in the adjacent mesenchyme. In non-neoplastic conditions of uterine cervix, the presence of PTHrP was correlated with the transformation of progenitor cells into squamous epithelia.[13] PTHrP-positive inner layer cells in pleomorphic adenomas of salivary glands were shown to represent a step in squamous differentiation.[14] Since squamous differentiation of the urothelium is not uncommon in neuropathic bladder, it is possible that PTHrP may be present in the epithelium of neuropathic bladder.

PTHrP in the urinary bladder

PTHrP immunoreactivity has been detected in the urinary bladder and increases in response to dilatation secondary to obstruction.[15] Increased PTHrP secretion in response to stretch of smooth muscle raises the possibility of an autocrine action to relax the bladder during filling. PTHrP may also exert a paracrine action on vessels regulating blood flow during bladder filling or it may modulate neural activity. Histochemical studies performed on distended bladder tissue indicated the presence of PTHrP immunoreactivity in smooth muscle cells.[16] PTHrP mRNA was expressed in the urothelium of the pelvis of the developing mouse kidney.[10] The physiology of PTHrP has been summarised in a previous publication in this journal.[17] To our knowledge, presence of PTHrP (1–34) in the human vesical urothelium has not been described.

PTHrP and urothelial redifferentiation in the neuropathic urinary bladder

We postulated that PTHrP (1–34) could play a role in urothelial redifferentiation in the neuropathic urinary bladder analogous to its role in epithelial differentiation elsewhere in the body. Therefore, we studied immunostaining for PTHrP (1–34) in the transitional epithelium, and in different urothelial redifferentiation programmes of neuropathic bladder.

Production of antibodies to PTHrP (1–34) for immunohistochemistry

Within the 1–34 molecule, there are hundreds of possible epitopes that could be used to produce antibodies if an 8 amino acid sequence is chosen and monoclonal antibodies are produced. We have chosen the peptides for injection based on postulated cleavage sites of the molecule, immunogenicity predictions and sequence differences from other known proteins in a protein data bank. This produces a type of antibody, which may be highly specific but may not react well with the whole (or part) PTHrP peptide because of protein conformation/folding, which could hide the sequence selected.

An alternative approach is to inject larger molecules such as the 1–34 sequence which is known to be biologically active and will expose an epitope(s) which is (are) likely to be available for binding in serum samples or in histological sections. The number of epitopes exposed/available will be significantly lower

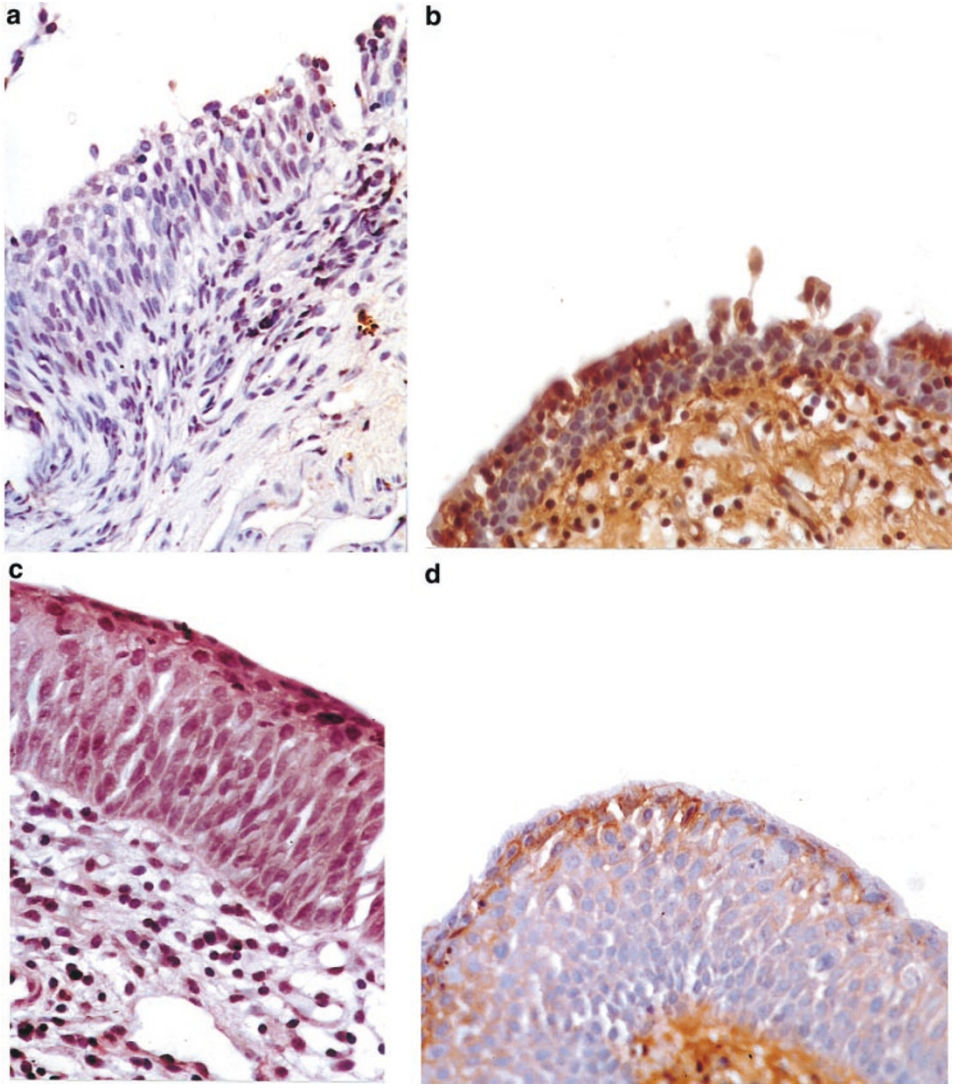
than the total possible from sequence analysis but of probable greater biological significance. The epitopes exposed are likely to raise antibodies against the most immunogenic sequence and so it is unlikely that multiple antibodies are produced to different portions of the molecule. We have not performed full characterisation of the sequence that the antibody recognises but in many ways this is irrelevant unless the molecule is cleaved into multiple small fragments.

From chromatographic studies, PTHrP is thought to circulate as a 34/36 amino acid N terminal fragment, a 19 amino acid N terminal fragment, an 84–90 amino acid fragment as well as possible 139, 141 and 173 amino acid molecules. All biologically active molecules that interact with the PTH/PTHrP receptor need to have the N terminal sequence intact and so a polyclonal antibody raised in a rabbit to (1–34) is very likely to reflect one aspect of biologically active PTHrP (N terminal activity).

Patients and methods

The North Sefton Research Ethics Committee approved this study. The criteria for inclusion were:

- Adult patients with neuropathic bladder who were registered with the Regional Spinal Injuries Centre, Southport, England.
- They should not be suffering from acute urinary infection.
- They should be undergoing an elective therapeutic procedure in the urinary tract such as endoscopic removal of bladder stone, insertion of a ureteric stent, etc.
- They were given a copy of the patient information sheet. A doctor from the spinal unit discussed this research project with the patient and his/her spouse/carer. If the patients were willing to participate in this study, they gave written informed consent to undergo bladder biopsy.



Cold cup biopsy of the bladder mucosa was taken from the trigone of the urinary bladder. Thereafter, the biopsy site was fulgurated with diathermy to achieve haemostasis. Indwelling urinary catheter drainage was maintained after the procedure. All patients stayed in the hospital for at least 24 h after the procedure. They were routinely given intravenous antibiotic prior to the procedure as prophylaxis against urinary infection.

The control biopsies were taken from the archival material of the Histopathology Department at the Southport General Infirmary. This control group represented patients with non-neuropathic bladder who had undergone routine bladder biopsy, and whose biopsies were either histologically normal or showed inflammation only, with no evidence of neoplasia of the transitional epithelium. None of the controls showed hyperplasia of transitional epithelium or metaplasia.

The biopsy specimens were fixed in 10% buffered formalin, and then embedded in Paraplast. The histopathology of each bladder biopsy was recorded by examining sections stained with haematoxylin and eosin.

Immunohistochemistry was performed using a rabbit antibody generated to a synthetic peptide representing the amino acid sequence of 1–34 of the human PTHrP molecule. This antibody was used in a dilution of 1 in 500. For each section, a negative control of bladder biopsy was included. This negative control section was treated with normal rabbit serum in strength of 1 in 100 in PBS instead of the PTHrP antibody. We followed the same methodology for immunohistochemistry as described in our previous study.[17] All tissue sections were stained contemporaneously.

Results of immunohistochemistry for PTHrP (1–34) in the control group

In the control group, the transitional epithelium of non-neuropathic bladder showed no immunostaining, or at the most, very faint positive staining was seen in

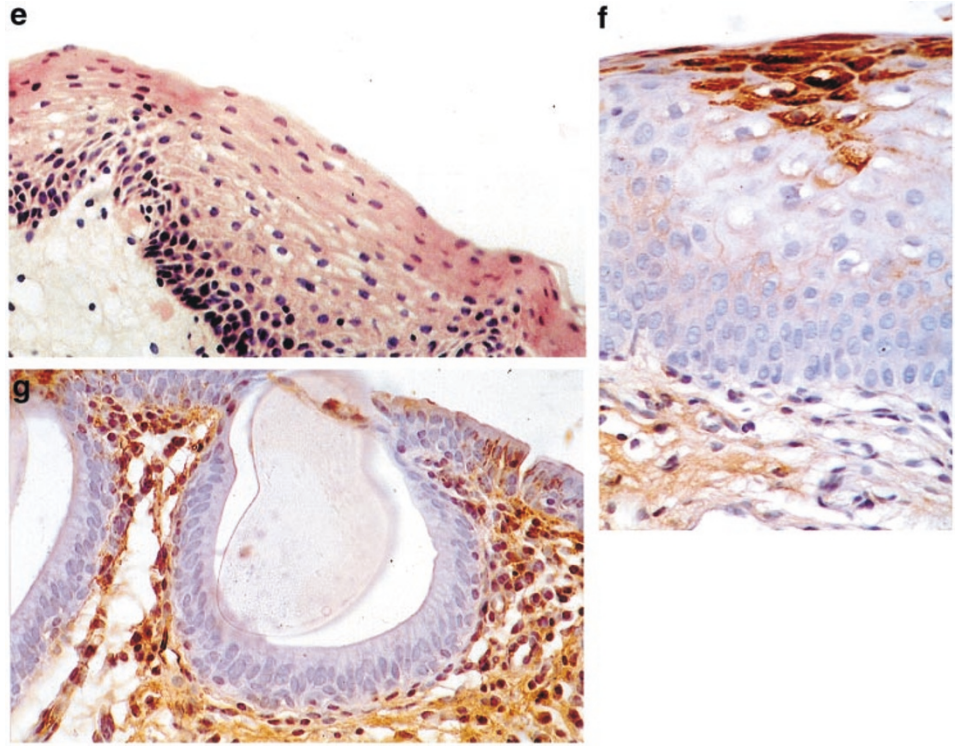


Figure 1 (**a**) Immunohistochemistry of bladder biopsy for PTHrP (1–34) in a control biopsy from non-neuropathic bladder shows very faint positive immuno-staining in the transitional epithelium. (**b**) Immunohistochemistry of bladder biopsy for PTHrP (1–34) in a tetraplegic patient shows cell membrane staining in the superficial layer of the transitional cell epithelium. (**c**) Histology of bladder biopsy in a tetraplegic patient shows hyperplasia of transitional epithelium. (H&E staining). (**d**) Immunohistochemistry of bladder biopsy in a tetraplegic patient shows cell membrane staining for PTHrP (1–34) in the superficial layer of hyperplastic transitional epithelium. (**e**) Histology of bladder biopsy in a male tetraplegic patient with multiple vesical calculi, shows squamous metaplasia. (H&E staining). (**f**) Immunohistochemistry for PTHrP (1–34) in a bladder biopsy from a SCI patient showing squamous metaplasia: Cytoplasmic immuno-staining is seen superficially, and a membrane-related positive staining is present in a deeper layer of squamous epithelium. (**g**) Bladder biopsy of a tetraplegic patient shows glandular metaplasia. Immunohistochemistry for PTHrP (1–34) shows no immunostaining in the glandular epithelium, although focal immunostaining is seen in the transitional epithelium

the transitional epithelium. (Figure 1a). In two biopsies, the lymphoid inflammatory cells in the epithelium stained positive for PTHrP (1–34). Lymphoid follicle showed strongly positive immunostaining in one case. Positive immunostaining was seen in the blood vessels present in the stroma in another biopsy.

Results of immunohistochemistry for PTHrP (1–34) in bladder biopsies taken from patients with neuropathic bladder

On the basis of histopathology of bladder biopsies, they were classified as follows:

- Group 1: Biopsies showing normal transitional epithelium ($n=13$)
- Group 2: Biopsies with urothelial hyperplasia ($n=13$)
- Group 3: Biopsies showing squamous metaplasia ($n=17$)
- Group 4: Biopsy showing intestinal metaplasia ($n=1$)

Group 1: Histopathology of these biopsies showed transitional epithelium with no evidence of urothelial hyperplasia, metaplasia, or neoplasia. Positive immunostaining for PTHrP (1–34) was seen in the vesical transitional epithelium in nine of 13 biopsies. (Figure 1b).

Group 2: Histopathology revealed hyperplasia of the transitional epithelium in these biopsies. For the purposes of this study, urothelial hyperplasia was defined as a flat mucosal lesion with more than seven cell layers of mature transitional epithelium.[18] Mitoses, where present, were limited to the basal cell layer. There was no cytologic atypia. (Figure 1c).

Eleven of the 13 biopsies with hyperplasia of transitional epithelium showed positive immunostaining for PTHrP (1–34) in the hyperplastic transitional epithelium. In a 20-year old ventilator-dependent tetraplegic male, histopathology of bladder biopsy showed urothelial hyperplasia. Positive immunostaining for PTHrP (1–34) was observed in the hyperplastic transitional epithelium (Figure 1d).

Group 3: In these biopsies, squamous epithelium was present either in part or in whole of the tissue section. (Figure 1e). Fourteen of 17 biopsies showed positive immunostaining for PTHrP (1–34) in the squamous epithelium. In a 34-year old tetraplegic male who had multiple stones in the urinary bladder, the surface urothelium showed squamous metaplasia. Immunohistochemistry demonstrated positive staining for PTHrP (1–34) in the squamous metaplastic epithelium (Figure 1f).

Group 4: This group consisted of a bladder biopsy from a 53-year old male who sustained fracture of C-5 and tetraplegia 23 years ago. Histology revealed glandular columnar cells, with occasional interspersed goblet cells; this represented a stage in the development of intestinal metaplasia. No immunostaining to PTHrP (1–34) was observed in the glandular epithelium, although focal immunostaining was seen in the transitional epithelium. (Figure 1g).

Discussion

We observed no immunostaining, or at the most very faint positive staining for PTHrP (1–34) in the transitional epithelium of non-neuropathic urinary bladder. In contrast to this, positive immunostaining for PTHrP (1–34) was observed more frequently in the transitional epithelium (nine of 13 cases), hyperplastic transitional epithelium (11 of 13 biopsies), and squamous metaplastic epithelium (14 of 17 cases) of neuropathic bladder.

The structure and function of the urothelium could be altered in spinal cord injury patients because of damage to the nerve supply to the urinary bladder. Injury to the spinal cord and consequent lack of trophic effect upon the urothelium may lead to a cascade of events involving urothelial proliferation, differentiation, maturation, and apoptosis.[19] Knowledge of the molecular mechanisms leading to urothelial hyperplasia, and squamous metaplasia in the neuropathic bladder is likely to provide guidance towards generating future therapeutic advances aimed at control of the cellular abnormalities occurring in the bladder mucosa. These denervation-associated changes in the bladder mucosa may represent important contributory factors for the increased susceptibility of spinal cord injury patients with neuropathic bladder for cystitis, and vesical neoplasia.

This study demonstrates that the vesical epithelium of neuropathic bladder shows positive immunostaining for PTHrP (1–34) more frequently than the transitional epithelium of non-neuropathic bladder. The role of PTHrP in the differentiation and proliferation of urothelium in the neuropathic bladder is not precisely known. Alipov and associates suggested that: (1) PTHrP acts as a cytokine for cell proliferation and tumour progression, and (2) over-expression of PTHrP may be involved in the malignant transformation and progression of gastric carcinoma.[20]

Induction of PTHrP is mediated via the *ras* signalling pathway. It is possible that in future, innovative therapy with molecular medicine may help to modify neoplastic transformation of the urothelium in spinal cord injury patients with neuropathic bladder. If expression of PTHrP is contributory to the malignant transformation of the urothelium in the neuropathic bladder, Ras processing inhibitors may be potential candidate therapeutic agents to decrease PTHrP expression, and hopefully achieve normalisation of urothelial proliferation. The scientific basis for such novel therapeutic regimes in spinal cord injury patients may be analogous to the role of *ras*

farnesylation inhibitors as potential therapeutic agents against the syndrome of malignancy-associated hypercalcemia for which PTHrP is a major causal agent.[21]

The expression of PTHrP may be amenable to modulation in future by systems capable of localised, direct plasmid gene delivery *in vivo*. An example of this kind of innovative therapy is the implantation of gene activated matrices at sites of bone injury. This was associated with retention and expression of plasmid DNA for at least 6 weeks, and with the induction of centimetres of normal new bone in a stable, reproducible, dose- and time-dependent manner.[22] It may be possible that in future, growth-regulating molecules may come into the clinical arena for treatment of vesical urothelial abnormalities in spinal cord injury patients, similar to the potential use of recombinant, human keratinocyte growth factor (rhKGF) in the prevention of chemotherapy-induced mucositis.[23]

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