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Epithelial mucin expression in bladder cancer: correlation with pathological and clinical parameters

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Abstract Recently, attention has been drawn to the role of polymorphic epithelial mucin (PEM) as a possible target for cancer immunotherapy. To investigate the expression of this molecule in bladder tissue, we used two mouse monoclonal antibodies (HMFG1 and HMFG2) raised against the core protein of the PEM. The localization of these two anti-PEM antibodies was examined in normal ($n = 10$), inflammatory ($n = 10$) and malignant ($n = 67$) bladder tissue samples with the use of a three-step avidin-biotin method. For HMFG1 and HMFG2 localization was successful in 78% and 60% of the bladder cancer samples, respectively, where as they were localized only in 30% and 40% of normal bladder tissue samples, respectively. Staining of either antibodies did not correlate with the grade, stage, or survival of bladder cancer patients. We conclude that PEM is frequently overexpressed by bladder cancer cells and HMFG1 is the antibody of choice to be used as a carrier of a cytotoxic agent for application of intravesical targeted therapy of bladder cancer.

Keywords Bladder cancer · MUC1 · Polymorphic epithelial mucin (PEM) · HMFG1 · HMFG2 · Immunotherapy

Introduction

Bladder carcinoma is a common human malignancy and in the United Kingdom, an estimated 12,900 cases are diagnosed and 5400 deaths from the disease occur each year [7]. The lifetime risk of bladder cancer is 3.38% among men and 1.18% among women. The lifetime risk of dying from bladder cancer is 0.70% for men and 0.35% for women [19]. Like many solid tumors, bladder cancer is a disease of the elderly, with a peak incidence in the seventh decade of life. Although bladder cancer is a chemotherapy responsive disease, there is significant scope for improving the results of treatment, that lead to response rates of up to 50%, but few cures. There are limits to the intensity of chemotherapy that can be given because of the relative frailty of this population, while issues of chemotherapy induced toxicity are even more complicated because of the comorbidity of these elderly patients. Furthermore, age adjusted death rate remains unchanged over the last 50 years, indicating that the effectiveness of the combination of traditional therapeutic schemes has reached a plateau [20]. Consequently, the need for novel therapeutic strategies is apparent.

There has been considerable progress recently towards the development of novel therapeutic strategies such as the application of monoclonal antibodies, gene therapy and cancer vaccines. Several molecules have been considered as potential targets for vaccination, including the polymorphic epithelial mucin (PEM), a high molecular weight glucoprotein (Mr > 400,000), member of the mucin family [11, 13, 21].

In this study we have investigated the expression of PEM in bladder cancer and normal bladder tissue, as the first step in the development of an effective antibody directed therapy.

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Materials and methods

Patients and tumor specimens

Ninety-one human formalin-fixed, paraffin embedded bladder samples were obtained from the Histopathology Department of Hammersmith Hospital, London. These consisted of ten normal bladder samples (six males and four females of mean age 64.3 years), ten inflammatory bladder samples (five males and five

females of mean age 65.2 years), four in situ carcinomas of the bladder (three males and one female of mean age 69.3) and 67 transitional cell carcinomas (49 males and 18 females of mean age 67.9 years) obtained by cystoscopic biopsy. The malignant tumors were classified and re-graded by one pathologist (MP) using the 3-grade system of the WHO classification [16]: grade I, $n = 20$; grade II, $n = 20$; grade III, $n = 27$. The T stage of the primary tumor was defined by cystoscopy, biopsy, bimanual examination and radiological imaging (CT and MRI). The nodal status was not assessed. The presence or absence of metastatic disease was assessed by CT scanning and chest radiography in all patients. Nuclear medicine bone scans were performed if clinically indicated. Treatment and length of survival following diagnosis were obtained from hospital and general practice records.

Monoclonal antibodies

The expression of PEM was investigated with the use of two anti-PEM mouse monoclonal antibodies, the HMFG1 and HMFG2 [21]. Both antibodies are class IgG_1 and are directed to similar but distinct polypeptide epitopes of the core protein of PEM [2]. The core protein of this molecule consists of 20 amino acid residues repeated in tandem (designated tandem repeats), which are rich in serine and threonine glycosylation sites. The amino terminus contains a putative transmembrane sequence and a 69 amino acid cytoplasmic tail. It has been suggested that tyrosine residues in the cytoplasmic tail may facilitate the uptake and recycling of PEM, allowing continued post-translational modification (glycosylation), even after newly synthesized mucin molecules have been transported to the cell surface [4, 5, 8, 9]. These antibodies were supplied by Imperial Cancer Research Fund (London, U.K.).

Immunohistochemistry

Fifty microlitres of each primary antibody (HMFG1 and HMFG2) were added to each section and incubated overnight at 4 °C. An avidin-biotin complex immunoperoxidase technique was used to amplify epitope recognition (ABC kit, *Dako* Ltd, High Wycombe, UK) and subsequent colorific visualization was achieved by 50 µl DAB solution, at a concentration of 0.3 mg/ml (*Dako* Ltd, High Wycombe, UK). Slides were then washed and mounted for microscopic examination. Positive control tissue sections were used to ensure accurate and reproducible staining and they included breast cancer tissue. Normal epithelial bladder tissue present in the tumor slides was used as an internal control. Negative controls were duplicate sections similarly stained in which the primary antibody was omitted and replaced by normal mouse immunoglobulins.

Evaluation

Sections were examined by three independent observers (KS, AK, MP) using light microscopy. The proportion of stained cells and the cellular localization of immunostaining were used as criteria for the evaluation.

Statistical analysis

For statistical analysis, antigen expression was considered either as normal or abnormal. Correlations between antigen expression and clinicopathological variables were evaluated by Fisher's exact test. A P value of less than 0.05 was accepted as statistically significant.

Results

Table 1 demonstrates the expression of PEM in the specimens studied.

Table 1 PEM expression in the various specimens of bladder tissue that we studied. $*P < 0.001$, $**P < 0.01$

	Number of cases investigated	HMFG1 positive 9 > cases	HMFG2 positive cases
Normal bladder tissue	10	3 (30%)	4 (40%)
Inflammatory bladder tissue	10	5 (50%)	4 (40%)
Bladder cancer tissue	67	52 (78%)*	40 (60%)**
In situ carcinoma of the bladder	4	3 (75%)*	1 (25%)
Total	91	63 (69%)	51 (56%)

With regard to normal urothelium samples only 3/10 (30%) and 4/10 (40%) normal bladder specimens showed homogenous membranous localization of HMFG1 and HMFG2 respectively, mainly in the lateral epithelial surface membrane of the upper layers, while the cell membrane and cytoplasm of the underlying cells did not localize either of the antibodies.

Membranous staining was also observed in 5/10 (HMFG1) and 4/10 (HMFG2) samples of inflammatory tissue samples, as well as at the intercellular borders of histologically normal bladder epithelium present in the bladder tumor specimens, although no staining was seen in the most superficial umbrella cells.

With regard to the specimens with malignancy, there was a variation in the number of cancer cells that showed membrane and/or cytoplasmic staining. Both HMFG1 and HMFG2 predominantly stained the tumor surface (surface of the papillae), when present. Deeper cells also showed a reaction, mainly in the tumors of advanced grade. In general, tumors of the same histological grade showed antigenic heterogeneity. In some tumors of grade II/III a large number of neoplastic cells demonstrated a strong membranous and cytoplasmic reaction, mainly for HMFG1, while for HMFG2 immunostaining was less intense (Fig. 1). The intensity of the staining and the number of positive cells increased with grading of the tumors, for both monoclonal antibodies, although the difference was not statistically significant ($P = 0.5$ for HMFG1 and $P = 0.6$ for HMFG2) (Table 2). In addition, cells in the areas of muscle invasion were successfully targeted by both HMFG1 and HMFG2. There was no statistically significant correlation with the stage of the disease ($P = 0.6$), and the prognosis of the patients ($P = 0.6$), for either of the antibodies studied (Fig. 2). All the specimens that reacted with HMFG2 also localized HMFG1.

One out of four in situ carcinomas positively stained (25%) for HMFG2, while two additional specimens (3/4, 75%), successfully localized HMFG1.

Discussion

Polymorphic epithelial mucin is the only mucin that has a transmembrane localization [6, 8, 9]. It is thought to

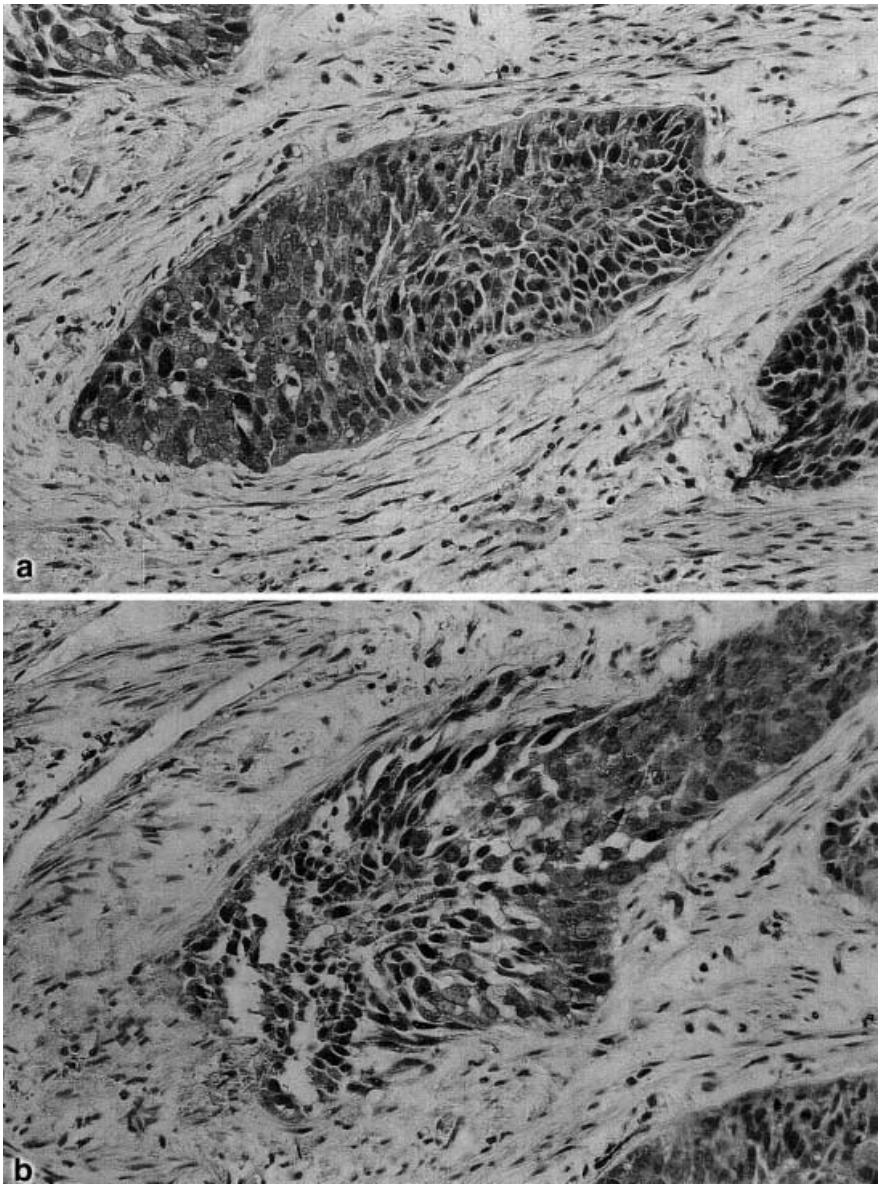

Fig. 1 HMFG1 (**a**) and HMFG2 (**b**) immunoreactivity in transitional cell carcinoma of the bladder. Membranous and cytoplasmic expression of polymorphic epithelial mucin (PEM) in poorly differentiated transitional cell carcinoma (avidin-biotin indirect immunoperoxidase staining; *Bar* 150 μm). All the specimens that reacted with HMFG2 also localized HMFG1

function in adults as a lubricant, and in fetal development is thought to play an important structural role in forming the lumen of a duct, orthe cavity of the bladder, by keeping apart cells located opposite one another. Polymorphic epithelial mucin belongs to a group of molecules called "sequestered" antigens, which are always expressed but under normal circumstances not presented to the immune system.

When the integrity of the normal urothelium is compromised by transformation of one of its cells, several changes take place that make immune recognition of PEM possible; the architecture of the epithelium is destroyed, expression of mucin on tumor cells becomes non-polarized and finally, mucin expression by the malignant cells is upregulated and reaches the surface of the cell incompletely glycosylated.

It is now widely recognized that, in human epithelial tumors, altered mucin expression and glycosylation may play a crucial role in tumor cell biology [1, 3, 6, 14]. In vitro studies have shown that mucinous human colon cancer cell lines demonstrate a more aggressive and invasive phenotype, presenting greater tumorigenicity and metastatic ability, when installed in athymic nude mouse models [11, 14]. In vivo, mucinous colon cancers are

Table 2 There is no statistically significant correlation between PEM expression and grading of the bladder cancer tumors for both monoclonal antibodies studied ($P = 0.5$ for HMFG1 and $P = 0.6$ for HMFG2)

Tumor grade	Number of cases investigated	HMFG1 positive cases	HMFG2 positive cases
I	20	16 (80%)	13 (65%)
II	20	17 (85%)	13 (76%)
III	27	19 (70%)	14 (52%)
Total	67	52 (78%)	40 (60%)

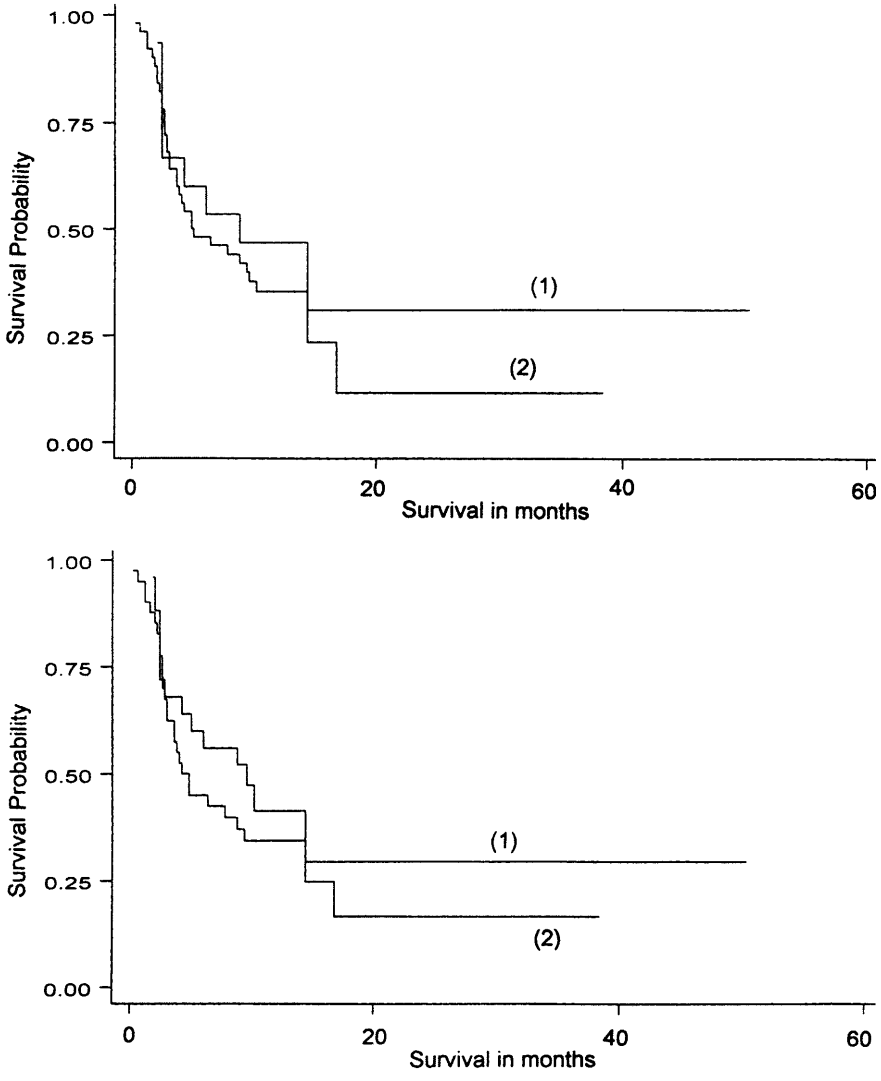

Fig. 2a, b Overall survival for 68 patients with transitional cell carcinoma of the bladder with regard to the normal or abnormal expression of PEM. There was no statistically significant correlation of PEM expression with the prognosis of the patients ($P = 0.6$), for either of the antibodies studied: HMFG1 (**a**) and HMFG2 (**b**)

generally diagnosed at a more advanced stage compared with other histological classifications of a colon cancer and patients with mucinous types of colon cancer usually have a worse prognosis [10].

In our study we were able to demonstrate that PEM is overexpressed in 78% of the bladder cancer cases and 75% of in situ carcinomas. We have also shown that PEM is uniformly overexpressed, regardless of the grade and the stage of the disease, indicating that this is an early event in the process of tumorigenesis. Staining characteristics ranged from membrane and cytoplasmic staining at the tumor surface to patchy, isolated reaction of deeper cells. These observations are in accordance with previous studies [5]. Furthermore, according to our findings, 30–50% of the non-malignant tissue samples (normal or inflammatory) localized successfully either of the antibodies studied. This particularly high percentage could hamper the efforts of using PEM as a target for antineoplastic treatment.

Although the mechanisms responsible for these biological effects are poorly understood, it seems that mucins such as PEM may alter the function of immune effector cells, while overexpression of mucins may alter the cellular adhesion properties [15] In vitro studies have demonstrated that a high level of expression of MUC1 by the malignant cells can make them much less adherent to each other, by inhibiting cadherin mediated adhesion, while loss of apical polarity further contributes to a lack of cellular cohesion. Thus, overexpression of MUC1 by neoplastic cells may facilitate their metastasis. This is in line with our own observation that

Table 3 There was no statistically significant correlation of PEM expression with the stage of the disease ($P = 0.6$ for both antibodies studied: HMFG1 and HMFG2)

Tumor grade	Number of cases investigated	HMFG1 positive cases	HMFG2 positive cases
T1	15	11 (73%)	7 (47%)
T2	9	6 (67%)	6 (67%)
T3	27	21 (78%)	15 (56%)
T4	16	14 (88%)	12 (75%)
Total	67	52 (78%)	40 (60%)

cells in the areas of muscle invasion were successfully targeted by both HMFG1 and HMFG2.

Recently, several attempts have been made to target PEM for antineoplastic treatment, with the application of several strategies, such as radioimmunotherapy, cancer vaccination, gene therapy and antibody directed enzyme prodrug therapy (ADEPT). The use of anti-PEM antibodies as vehicles of a cytotoxic agent are currently being investigated in several Phase I/II ongoing clinical trials, of breast, colon and pancreatic cancer [12, 17, 18, 22]. Our study provides the stimulus to organize similar studies of bladder cancer patients, based on high PEM expression. Comparing the two antibodies that we used, we concluded that HMFG1 is more suitable for developing targeted therapeutic strategies, because it is less frequently localized to non-malignant cell, while the percentage of positive bladder cancer samples is higher for HMFG1 than for HMFG2. Furthermore, within the same tumor sample, the percentage of HMFG1 positive cells is higher than the percentage of HMFG2 positive cells, mainly because they recognize a different epitope of the core protein.

Conclusions

Overexpression of PEM by bladder cancer cells occurs frequently and induces increased anti-adhesive properties. The antibody HMFG1 can be used successfully as a vehicle of a cytotoxic agent for application of intravesical targeted therapy of bladder cancer. Finally, PEM immunogenicity and provocation of anti-MUC1 immune response may be used in the near future for the development of a vaccine strategy.

References

1. Ajioka Y, Watanabe H, Jass JR (1997) MUC1 and MUC2 mucins in flat and polypoid colorectal adenomas. J Clin Pathol 50: 417
2. Burchell J, Durbin H, Taylor-Papadimitriou J (1983) Complexity of expression of antigenic determinants, recognized by monoclonal antibodies HMFG-1 and HMFG-2, in normal and malignant human mammary epithelial cells. J Immunol. 131: 508
3. Bresalier RS, Niv Y, Byrd JC, Duh QY, Toribara NW, Rockwell RW, Dahiya R, Kim YS (1991) Mucin production by human colonic carcinoma cells correlates with their metastatic potential in animal models of colon cancer metastasis. J Clin Invest 87: 1037
4. Byrd JC, Yan P, Sternberg L, Yunker CK, Scheiman JM, Bresalier RS (1997) Aberrant expression of gland-type gastric mucin in the surface epithelium of *Helicobacter pylori*-infected patients. Gastroenterology 113: 455
5. Conn JG, Crocker J, Emtage LA, Wallace DM (1988) HMFG-2 as a prognostic indicator in superficial bladder cancer. J Clin Pathol 41: 1191–5
6. Gendler SJ, Spicer AP, Lalani EN, Duhig T, Peat N, Burchell J, Pemberton L, Boshell M, Taylor-Papadimitriou J (1991) Structure and biology of a carcinoma-associated mucin, MUC1. Am Rev Respir Dis 144: S42
7. Heiken JP, Forman HP, Brown JJ (1994) Neoplasms of the bladder, prostate and testis. Radiol Clin North Am 32: 81
8. Hilkens J, Ligtenberg MJ, Vos HL, Litvinov SV (1992) Cell membrane-associated mucins and their adhesion-modulating property. Trends Biochem Sci 17: 359–63
9. Ho JJ, Cheng S, Kim YS (1995) Access to peptide regions of a surface mucin (MUC1) is reduced by sialic acids. Biochem Biophys Res Commun 210: 866
10. Ho SB, Ewing SL, Montgomery CK, Kim YS (1996) Altered mucin core peptide immunoreactivity in the colon polyp-carcinoma sequence. Oncol Res 8: 53
11. Ho JJ, Bi N, Siddiki B, Chung YS, Yuan M, Kim YS (1993) Multiple forms of intracellular and secreted mucins in a pancreatic cancer cell line. Cancer Res 53: 884
12. Hodge JW, McLaughlin JP, Kantor JA, Schlom J (1997) Diversified prime and boost protocols using recombinant vaccinia virus and recombinant nonreplicating avian pox virus to enhance T cell immunity and anti-tumor responses. Vaccine 15: 759
13. Kim YS (1992) Altered glycosylation of mucin glycoproteins in colonic neoplasia. J Cell Biochem Suppl 16G: 91
14. Kuan SF, Byrd JC, Basbaum CB, Kim YS (1987) Characterization of quantitative mucin variants from a human colon cancer cell line. Cancer Res 47: 5715
15. Ligtenberg MJ, Buijs F, Vos HL, Hilkens J (1992) Suppression of cellular aggregation by high levels of episialin. Cancer Res 52: 2318
16. Mostofi FK, Sobin LH, Torloni H (1983) Histological typing of urinary bladder tumors. In: International histological classification of tumors, 10th edn. World Health Organisation, Geneva
17. Reddisch MA, MacLean GD, Koganty RR (1998) Anti-MUC1 class I restricted CTLs in metastatic breast cancer patients immunised with a synthetic MUC 1 peptide. Int J Cancer 76: 817
18. Reddisch MA, MacLean GD, Poppema S (1996) Preimmunotherapy serum CA 27.29 (MUC1) mucin level and CD69+ lymphocytes correlate with the effects of Theratope sialyl-Tn-KLH cancer vaccine in active specific immunotherapy. Cancer Immunol Immunother 42: 303
19. Silverman DT, Rothman N, Devesa SS (1999) Epidemiology of bladder cancer. In: Syrigos KN, Skinner DG (eds) Bladder cancer: biology, diagnosis and management. Oxford University Press, London, p 18
20. Stein JP, Skinner DG (1999) Clinical presentation and investigation of bladder cancer In: Syrigos KN, Skinner DG (eds) Bladder cancer: biology, diagnosis and management. Oxford University Press, London, p 141
21. Taylor-Papadimitriou J, Peterson JA, Arklie J, Burchell J, Ceriani RL, Bodmer WF (1981) Monoclonal antibodies to epithelium-specific components of the human milk fat globule membrane: production and reaction with cells in culture. Int J Cancer 28: 17
22. Zbar AP, Lemoine NR, Wadhwa M, Thomas H, Snary D, Kmiot WA (1998) Biological therapy approaches in colorectal cancer. Strategies to enhance carcinoembryonic antigen (CEA) as an immunogenic target. Br J Cancer 77: 68