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Bladder cancers respond to intravesical instillation of HAMLET (human α-lactalbumin made lethal to tumor cells)

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We studied if bladder cancers respond to HAMLET (human α-lactalbumin made lethal to tumor cells) to establish if intravesical HAMLET application might be used to selectively remove cancer cells *in vivo*. Patients with nonmuscle invasive transitional cell carcinomas were included. Nine patients received 5 daily intravesical instillations of HAMLET (25 mg/ml) during the week before scheduled surgery. HAMLET stimulated a rapid increase in the shedding of tumor cells into the urine, daily, during the 5 days of instillation. The effect was specific for HAMLET, as intravesical instillation of NaCl, PBS or native α-lactalbumin did not increase cell shedding. Most of the shed cells were dead and an apoptotic response was detected in 6 of 9 patients, using the TUNEL assay. At surgery, morphological changes in the exophytic tumors were documented by endoscopic photography and a reduction in tumor size or change in tumor character was detected in 8 of 9 patients. TUNEL staining was positive in biopsies from the remaining tumor in 4 patients but adjacent healthy tissue showed no evidence of apoptosis and no toxic response. The results suggest that HAMLET exerts a direct and selective effect on bladder cancer tissue *in vivo* and that local HAMLET administration might be of value in the future treatment of bladder cancers.

Key words: bladder cancer; apoptosis; therapeutics; α-lactalbumin; protein folding

Bladder cancers are common and remain a challenge, despite significant therapeutic advances. The prevalence is about 1/4000, making this the fourth most common malignancy in the United States and the fifth in Europe.[1] Surgery or combinations of surgery and cytostatic drugs are used successfully, but therapy-resistant tumors still cause significant morbidity and mortality.[1] Superficial papillary nonmuscle invasive tumors may be removed by transurethral resection and the short-term prognosis is excellent but the patients need life-long follow-up because of a high recurrence rate combined with a risk for dedifferentiation.[2] Invasive tumors are eventually treated by radical cystectomy and some patients receive adjuvant systemic chemotherapy but these tumors have a poor prognosis with 5-year survival rates of 40% or less.[3] Focal or multifocal ''cancer *in situ*'' (CIS) is restricted to the urothelium initially, but may also progress and require cystectomy. A variety of topical treatments are used to prevent or delay cystectomy, and the drugs used for intravesical instillation include Thiotepa, Epirubicin, Mitomycin and Bacille-Calmette-Guerin (BCG).[4,5] BCG treatment is efficient and results in a recurrence free interval of at least 2 years in about 70% of the patients with superficial bladder cancers.[6] BCG causes significant side effects, however, including granulomatous prostatitis, local discomfort, dysuria and may require systemic antituberculous therapy. The incidence of life-threatening complications of BCG therapy is relatively low (0.4%),[7] but a significant number of deaths have been reported, especially in immunosuppressed patients. The problems with BCG therapy nowadays are more due to noneffectiveness than to toxicity, however.

HAMLET (human α-lactalbumin made lethal to tumor cells) is a complex of α-lactalbumin and oleic acid that kills tumor cells *in vitro*.[8,9] To form HAMLET, α-lactalbumin is first unfolded by the removal of the tightly bound Ca^{2+} ion, which coordinates the native conformation of the protein. The partially unfolded protein is subsequently bound to oleic acid on an ion-exchange matrix, and the complex is eluted with high salt.[10] The biological properties of HAMLET are defined by the partially unfolded state of α-lactalbumin,[8,11] and by the fatty acid, as neither the protein alone nor the fatty acid show this tumoricidal activity. The tumor cells die by an apoptosis-like mechanism but healthy, differentiated cells, are resistant to the lethal effects of HAMLET.[9,12] The antitumor activity of HAMLET is maintained also *in vivo*, as shown in experimental animal models and clinical studies. Local HAMLET infusion was shown to delay the development of tumors and to prolong survival in a human glioblastoma xenograft model.[13] In a randomized, placebo-controlled study, topical HAMLET administration was shown to efficiently remove or reduce human skin papillomas.[14] These observations suggested that topical HAMLET administration might be useful in cancer patients.

In vitro studies have shown that kidney and bladder carcinoma cell lines undergo a rapid death response to HAMLET.[15] In our study, we used an intravesical instillation protocol to investigate if HAMLET triggers apoptosislike death in bladder cancer cells, *in vivo*. This approach was based on the established practice of local instillation of antitumor substances such as BCG bacteria in patients with bladder cancer.[7]

Methods

Patients

Nine male patients awaiting transurethral surgery for newly diagnosed or recurrent superficial bladder cancer were invited to participate in the study and received HAMLET instillations (Table I). Seven patients had nonmuscle invasive tumors diagnosed by cystoscopy and urine cytology and 2 patients had CIS diagnosed by mucosal biopsies and urine cytology. Patient P1 had multiple solid tumors, patients P2, P3 and P5–P7 had newly diagnosed papillary tumors and P1 and P4 had recurrences of previously diagnosed and treated nonmuscle invasive tumors. Patient P8 had a recurrence after BCG treatment of multifocal CIS and patient P9 had a newly diagnosed CIS, which had not been treated with BCG. Patients P2, P5–P9 and P9 were previously healthy.

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TABLE I – CLINICAL STATUS AND RESPONSE TO HAMLET

Diagnosis			Outcome						
				Shed cells		Remaining tumour			Benign adjacent tissue	
Patient age (years)	Tumor characteristics	Cytology	Exposure time (h)	Fold increase	% TUNEL positive	Morphology	Histology	TUNEL	Histology	TUNEL
P1 (82)	Multiple, solid (r)	Atypical cells	5.6	15	13	Dry, not bleeding	TA grade 2	Pos.	Moderate focal dysplasia	Neg.
P2 (38)	Solitary, pepillary (p)	No data	8.2	100	45	Tumor necrosis	T1 grade 2	Pos.	Sample missing	n.s.
P3 (72)	Solitary, pepillary (p)	Cancer grade 2	12.4	1.2	0	Partial tumor necrosis	TA grade 2	Neg.	n.s.Mild urothellal hyperplasia but no atypic cells	Neg.
P4 (84)	Solitary, pepillary (r)	Atipical cells	8.5	30	n.s	Surface atrophy	TA grade 1	Pos.	Normal urothelium	Neg.
P5 (73)	Solitary, pepillary (p)	Cancer grade 2	10.3	30	65	Tumor necrosis	TA grade 2	n.s	Sample missing	n.s.
P6 (61)	Solitary, pepillary (p)	Susp. cancer grade 1	21.2	180	4	Surperficial tumor atrophy	TA grade 1	Neg.	n.s.Normal urothelium	Neg.
P7 (64)	Solitary, solid (p)	Cancer grade 3	4.7	2.8	41	Surface atrophy	TA grade 2	Pos.	Sample missing	n.s.
P8 (62)	3/4 biopsies positive for CIS	Cancer grade 3	10.6	6	71	Not relevant	1/4 biopsies positive		n.s.Normal urothelium	
P9 (80)	3/4 biopsies positive for CIS	Cancer grade 3	8.3	7	61	Not relevant	No remaining tumor		One fragment with atypic cells, remaining biopsies normal	

n.s, no sample available; (p), primary diagnosedtumors; (r), recurrence of previously diagnosed tumor.

Study design

At inclusion, the patients were subjected to cystoscopy to assess the tumor size and to document the lesions with endoluminal photography (Nikon, Tokyo, Japan). During the week preceding scheduled surgery, intravesical instillations of HAMLET were given daily for 5 days. The patients were asked to avoid fluid intake for 4 hr before, and immediately after the instillation of HAMLET, to delay the dilution effect. To avoid urinary tract infection, low dose, per oral antibiotic prophylaxis was used (Ciprofloxacin or Trimetoprim). At the time of surgery, cystoscopy with endoluminal photography was repeated and the tumor size and morphology were reassessed. The instillations were performed in the outpatient clinic where the patients were under close surveillance to detect any subjective or objective side effects of the HAMLET administration.

HAMLET was instilled into the bladder through a soft polyethylene catheter (Lowfric® Ch 12, Astra Zeneca, Södertälje, Sweden). Prior to instillation, the bladder was completely emptied and the urine was collected for preinstillation analysis. HAMLET (30 ml, 25 mg/ml) was deposited in the bladder, the catheter was removed, and the patients were encouraged to postpone voiding to maximize the exposure to HAMLET. The HAMLET concentration (25 mg/ml) was based on the skin papillomas study,[14] where a concentration of 10 mg/ml of HAMLET was found to be efficient. We calculated that 25 mg/ml would be required to reach a similar concentration in the bladder, as newly produced urine would be diluting the instilled HAMLET concentration.

Control instillations were performed in 5 patients. Three of them were included in the HAMLET study. C1 = P5 (native α-lactalbumin) and C2 = P7 (PBS) were given control instillations before HAMLET treatment and C3 = P9 (NaCl) after completed HAMLET instillations. Two patients were subjected only to control instillations with NaCl before scheduled surgery. C4 had a recurrence with a single small papillary tumor and C5 was a female patient with a newly diagnosed large papillary tumor.

IRB approval was obtained from the Medical Ethics Committee of the Lund Medical Faculty, Lund, Sweden, and informed consent was obtained from each patient (LU 454-00).

HAMLET

HAMLET was prepared from human milk α-lactalbumin, as described.[12] The starting material was excess milk from the hospital milk bank of the quality required for feeding of premature babies (HIV and Hepatitis B negative, microbial burden was $\leq$1CFU/mg protein). The milk was stored at −20°C until used. Briefly, α-lactalbumin was purified from milk by ammoniumsulphate precipitation followed by hydrophobic interaction chromatograpy. EDTA treatment was used to unfold the protein and oleic acid was incorporated on an ion-exchange matrix, forming HAMLET. For intravesical instillation, HAMLET was dissolved in PBS (25 mg/ml, 30 ml) under sterile conditions.

Urine cytology

To examine if voided tumor cells showed a cell death response to HAMLET, urine samples were obtained prior to and after each HAMLET instillation and cells in uncentrifuged urine were counted by light microscopy (Nikon Eclipse E800, Tokyo, Japan) using a Bürker chamber. Cell viability was determined by trypan blue exclusion. Cell morphology was determined after hematoxylin- and eosin staining. Apoptotic cells were identified by the TUNEL assay (Roche, Basel, Switzerland) and examined by fluorescence microscopy using the LSM META 510 software package (Carl Zeiss, Jena, Germany). Representative cells were photographed using a CCD camera (Diagnostic Instruments, MI). In addition, the urine samples were examined at the Department of Pathology at Lund University Hospital. All of the patients were subjected to urine cytology, which was used as a diagnostic tool. For urine cytology, cells concentrated from 30 ml urine are used. Cell shedding in response to HAMLET was examined in uncentrifuged urine. Urine cytology is based on 30 ml of urine, and cells are cen-

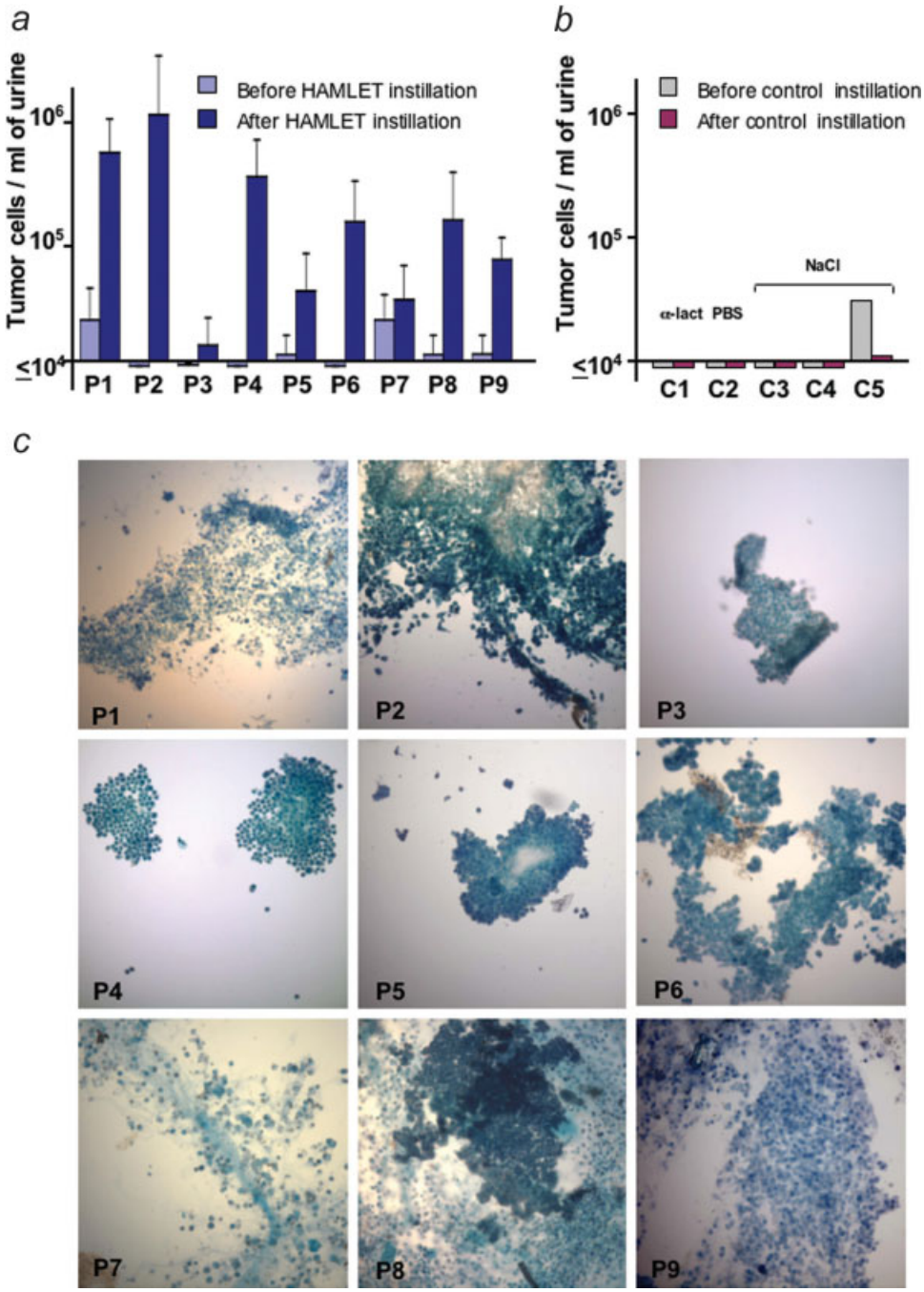

Figure 1 – Shedding of tumor cells following intravesical instillation of HAMLET (30 ml, 25 mg/ml). Urine samples were obtained from each patient ($n = 9$) before and about 2 hr after each HAMLET instillation (88 samples). (*a*) Number of cells in the urine of each patient, before or after HAMLET instillation (individual means ± SDs of 5 daily instillations). (*b*) Patients who received PBS, NaCl or α-lactalbumin instillations showed no increase in cell shedding (number of cells excreted after one instillation). C1 = P5 received one instillation with native α-lactalbumin and C2 = P7 received one instillation of PBS one day before the HAMLET instillations. C3 = P9 received one instillation of NaCl (0.9%) after the HAMLET instillation period. C4 and C5 were not exposed to HAMLET but received one instillation each of NaCl before their scheduled surgery. (*c*) Cells and tissue aggregates in urine after HAMLET instillation. Light microscopy of trypan blue stained, unfixed cells. The aggregates were not included in the numbers in (*a*).

trifuged onto appropriate glass slides. Cells excretion in response to HAMLET was examined in uncentrifuged urine.

Tissue biopsies

Tumor biopsies were collected at surgery, fixed in 4% paraformaldehyde in PBS for 24 hr, treated with 10, 20 and 25% sucrose in PBS solution, embedded in Tissue-Tec (Sakura Finetek, Torrance, CA, USA) and frozen in propane alcohol chilled with dry ice. Biopsies were stored in −80°C until use. Serial 10-μm sections were cut on a Microm HM500M (Microm Microtech, Francheville, France). Apoptotic cells were detected by the TUNEL assay. Paraffin-embedded tissue samples from benign adjacent tissue were obtained from pathology.

Statistical analysis. The analysis of cell shedding used the Mann-Whitney U test (InStat 3, GraphPad Software, San Diego, CA, USA).

Results

Patients with superficial bladder cancers were subjected to intravesical HAMLET instillations on 5 consecutive days, during the week before transurethral surgery. The patients did not report any symptoms of the HAMLET instillations, other than those caused by the catheterization procedure *per se*. The study examine if bladder tumor cells respond *in vivo* to topical HAMLET instillation but was not a treatment trial. This was not a treatment trial,

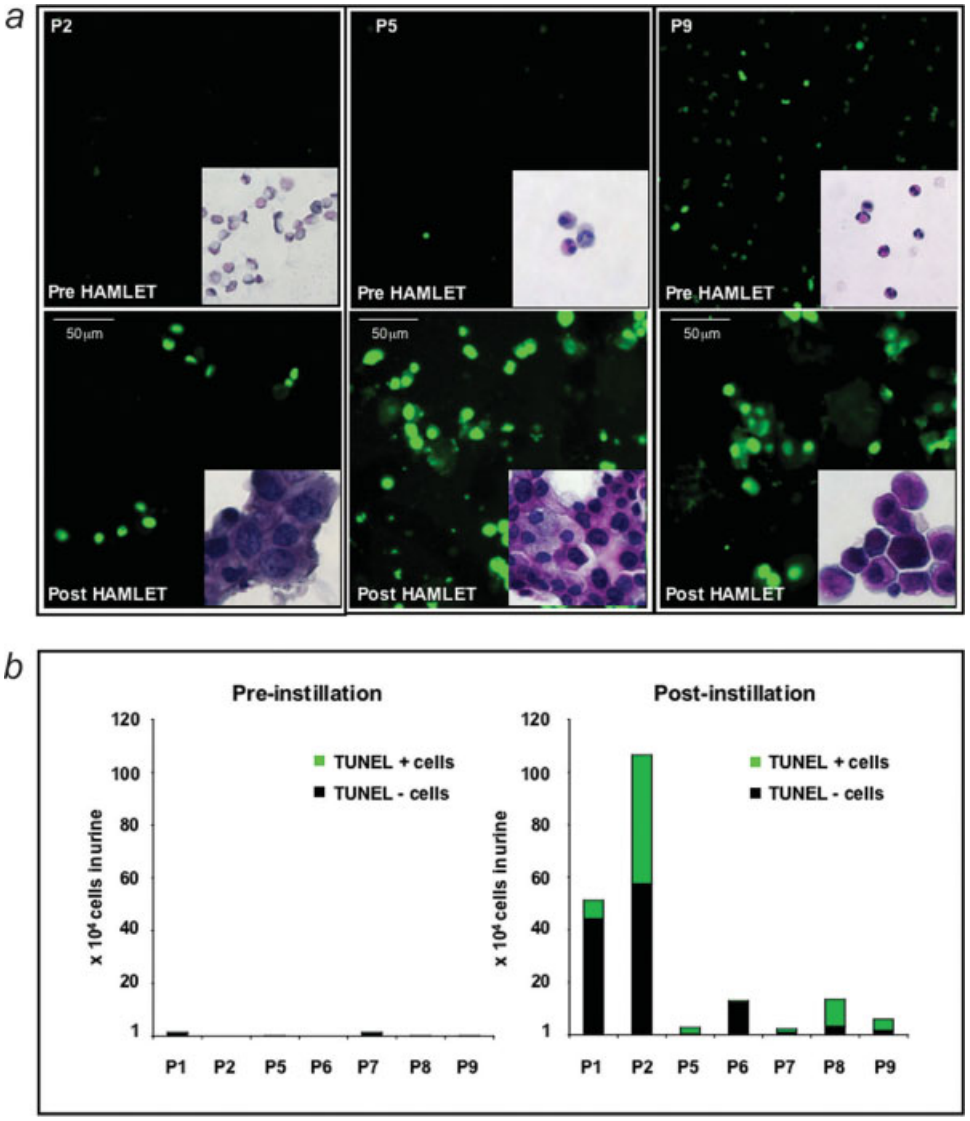

FIGURE 2 – Shed apoptotic cells as defined by TUNEL staining. (*a*) Overview of pre- and postinstillation samples from 3 representative patients. Cells excreted from patients were cyto-centrifuged onto poly-L-lysine coated glass slides, stained with hematoxylin-eosin and examined by the TUNEL assay. Lower right panels show the morphology of shed cells by hematoxylin-eosin staining (magnification ×40). In the preinstillation samples occasional TUNEL positive neutrophils were observed. In the postinstillation samples, the TUNEL positive cells were shed tumor cells. Patient numbers are in the upper left corners. (*b*) Frequency of shed TUNEL positive tumor cells in each patient. Pre- and postinstillation samples were not available for TUNEL staining in P3 and P4.

but a study examining if bladder tumor cells respond *in vivo* to topical HAMLET instillation by undergoing apoptosis.

HAMLET triggers shedding of tumor cells

The HAMLET instillations caused massive shedding of cells into the urine in all patients except P3 (Fig. 1*a*). The mean number of single cells increased from levels below 10^4/ml to a mean of $2.9 \times 10^5 \pm 1.3 \times 10^5$ in the postinstillation samples ($p < 0.0001$, Mann Whitney). In addition, most of the posturine samples contained large cell aggregates (Fig. 2*c*). The number of cells in these aggregates could not be quantified but they added considerably to the total number of shed cells, as there were no aggregates in the preinstillation samples. The increase in cell shedding occurred daily, during the 5 days of instillation ($p < 0.01$, compared to the preinstillation cell numbers), and cell shedding occurred in all patients except patient P3 (Table I). Before HAMLET instillation, the spontaneous shedding of tumor cells was $<10^4$/ml of urine in most of the patients (Table I), and cell aggregates or tissue fragments were not detected.

To examine if cell shedding was caused by HAMLET, 5 patients received control instillations with buffer or α-lactalbumin (Fig. 1*b*). There was no increase in cell shedding after intravesical instillation of buffer or native α-lactalbumin, and aggregates were not observed in the urine samples of those patients (Fig. 1*b*, $p > 0.05$). As prophylatic antibiotic treatment was given to both HAMLET treated and control patients, the influence of this variable on cell excretion could be excluded.

TUNEL staining of shed cells

After HAMLET instillation, more than 90% of the shed cells and cells aggregates were dead, as determined by trypan blue exclusion (Fig. 1*c*). Apoptotic changes in the shed cells were detected using the TUNEL assay (Fig. 2*a*). In the preinstillation samples, tumor cells were scarce but some samples from patients P5 and P9 contained a few apoptotic neutrophils. After HAMLET instillation, TUNEL positive tumor cells were detected in 6 patients (4 with papillomas and 2 with CIS). The highest proportion of TUNEL positive cells was found in patients P2, P5, P7 and P9 (Fig. 3*b*, Table I).

The morphology of the shed cells is shown in Figure 4*a*. Many cells had large amorphous nuclei, little cytoplasm and condensed chromatin suggesting that they originated from the tumors. Other cells showed no definitive tumor characteristics, but were multinucleated or with degenerative changes. TUNEL staining was in the nuclei of the shed cells (Fig. 4*b*). There was no significant

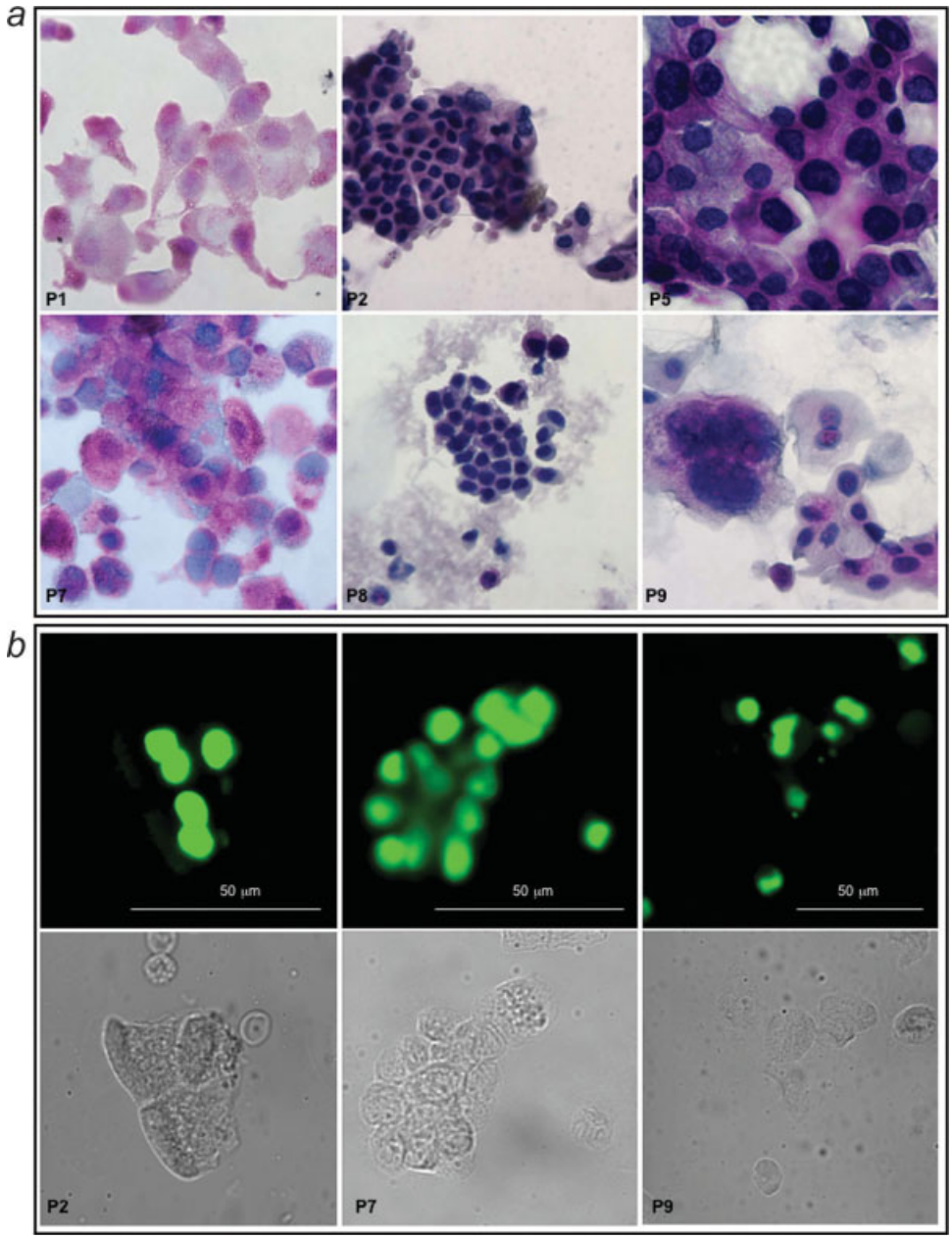

Figure 3 – Morphology of shed tumor cells after HAMLET instillation. (*a*) Hematoxylin-eosin staining of tumor cells from 6 patients. Patient numbers are in the lower left corner. (*b*) TUNEL staining and morphology of shed tumor cells from patients P2, P7 and P9, with the corresponding light microscopy image.

increase in the shedding of squamous or transitional epithelial cells with normal morphology.

Effects of HAMLET on tumor size and morphology

The response of the papillary tumors to HAMLET was examined by endoscope and documented by photography (Fig. 4). The clinical outcome is summarized in Table I. Patients P2 and P5 (TA grade 2) showed a nearly complete resolution of the tumor after the HAMLET instillations. Most of the papillary structure had been lost and the base of the tumors appeared fractured. Patient P3 (TA grade 2) carried a papillary tumor on the left bladder wall, which was too large to be captured in 1 photograph (Fig. 4). The HAMLET instillations caused a reduction in tumor size of about 50% and the tumor was fractured and appeared fragile. Patient P4 had 2 small papillary tumors on the left bladder neck (TA grade 1). HAMLET caused a minor reduction in tumor size but a marked change in tumor character with surface atrophy (Fig. 4). Patient P1 (TA grade 2, not shown) showed no apparent reduction in tumor size but a change in tumor character from brittle and bleeding on contact to ''dry.'' Patient P6 had a fairly large papillary tumor on the right side of the bladder (TA grade 1, not shown) that showed signs of peripheral atrophy after HAMLET exposure. Patient P7 had 2 solid tumors that did not change appearance significantly after HAMLET exposure (not shown).

Apoptotic cells in tumor biopsies but not in adjacent healthy tissue

Biopsies were obtained from the tumors in connection with surgery, and the presence of tumor cells in the biopsies was confirmed by pathology. Sections without diathermia damage and with an intact epithelial lining were selected for analysis. Apoptotic cells were detected by the TUNEL assay (Fig. 5, Table I). TUNEL positive tumor areas were detected in 6 of 9 patients (Fig. 5*a*); 4 of the patients had papillomatous tumors (P1–P4 and P6) and 2 had CIS (P8, P9). Patient P5 did not deliver a sample, as most of the tumor was lost due to cell shedding. TUNEL positive areas were not found in patients P3 and P6 and those patients lacked TUNEL positive cells in urine (Table I).

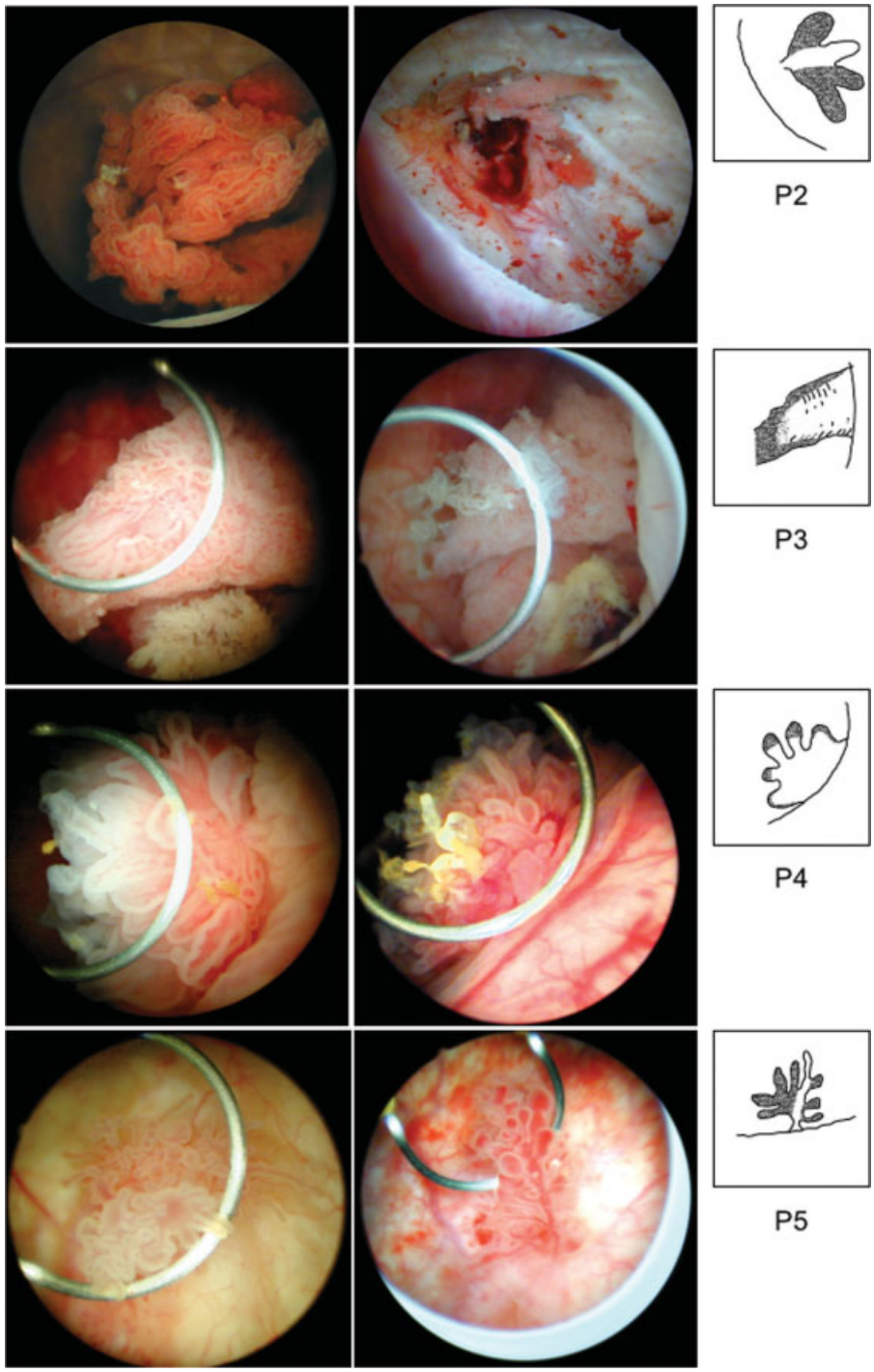

FIGURE 4 – Macroscopic changes in papillary tumors after HAMLET exposure. The tumor is shown before (left panels) and after (right panels) the 5 HAMLET instillations. Changes in tumor size in individual patients are illustrated by the drawings next to each pair of photographs. The diathermia loop is 5-mm wide. Patient numbers are below each schematic.

In 4 patients, biopsies were also obtained from benign tissues distant from the tumor. The pathologist confirmed that tumor cells were absent from those samples. TUNEL positive cells were not detected in this urothelium (Fig. 5*b*, Table I).

Discussion

Our study shows that HAMLET triggers tumor cell death *in vivo* in patients with bladder cancers. The tumors were exposed to HAMLET topically, by intravesical instillations on 5 consecutive days during the week preceeding scheduled surgery and the tissue response was evaluated at surgery. Rapid shedding of dead tumor cells occurred after each HAMLET instillation, but control instillations with inactive α-lactalbumin or salt solutions did not trigger shedding. The HAMLET instillations caused a reduction in tumor size in four papillomas, and a slight change in character in an additional two. Biopsies from the remaining tumor showed abundant apoptotic changes, but there was no evidence of apoptosis in healthy benign tissues surrounding the tumor. We conclude that HAMLET triggers tumor cell death *in vivo*.

HAMLET kills tumor cells and embryonal cells *in vitro*, but healthy differentiated cells survive high concentrations of HAMLET.[12] This relative selectivity is a surprising and potentially very useful property and has been confirmed in a limited

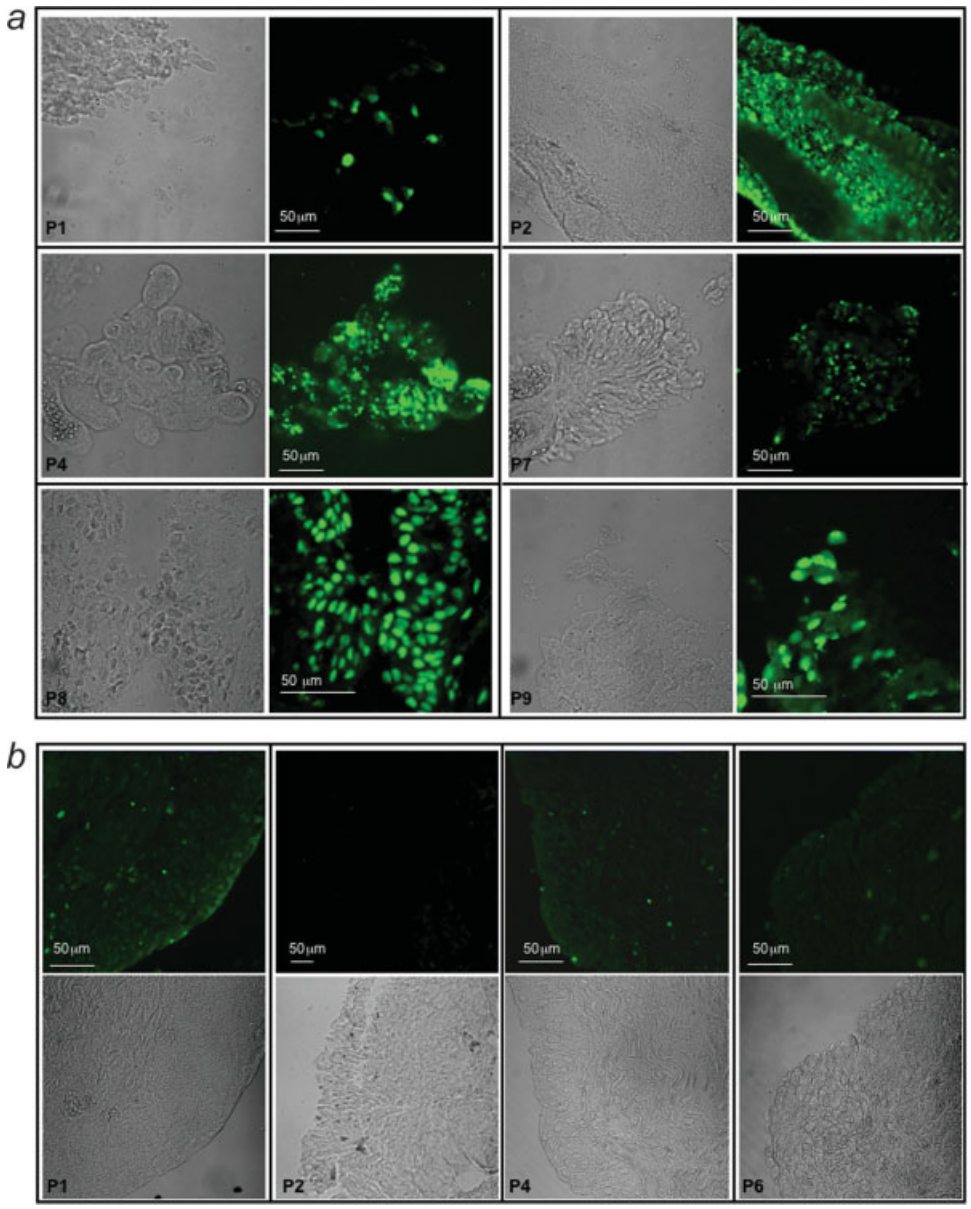

FIGURE 5 – Apoptotic response to HAMLET *in vivo* as shown by TUNEL staining of tumor biopsies. (*a*) Tissue sections were obtained at surgery, after 5 HAMLET instillations. Tissue sections are shown by light microscopy (left panels) and by fluorescence microscopy, with TUNEL positive cells (right panels). (*b*) Lack of apoptosis in tissue sections from healthy tissues adjacent to the tumor. The TUNEL-stained image is shown in the upper panels and light microscopy images in the lower panels. The patient number are shown in the lower left corner.

number of *in vivo* studies. Patients with skin papillomas, who received HAMLET topically during 3 weeks, did not report adverse effects or show signs of irritation in the skin surrounding the lesions.[14] In a rat xenograft model of human glioblastoma, tumor cell death was observed after injection of HAMLET into the brain, but there was no apoptotic response in healthy brain tissue surrounding the tumor and no evidence of toxicity after injection of HAMLET into healthy rat brains.[13] The present study provided further evidence in support of selectivity. There was an apoptotic response in tumor biopsies from HAMLET-treated patients, but there was no evidence of apoptosis in the biopsies from benign bladder tissue in the same patients. HAMLET thus appears to trigger a death response preferentially in malignant bladder cells, although the influence of variables such as the glucosaminoglycan layer has not been considered in our study. There is little concern that HAMLET would cause systemic toxicity. HAMLET is inactivated in as the fatty acid is lost and the protein reverts to serum, because of the loss of the fatty acid and reversion of the protein to the native state, which is inactive in the apoptosis assay. The fatty acid is ''stolen'' by other high-affinity. Serum albumin is one of the fatty acid binding proteins that successfully compete with α-lactalbumin for oleic acid, like albumin. Furthermore, studies of breast-fed children have shown that the absorbtion of α-lactalbumin across the intestinal mucosa is harmless, and thus the presence of α-lactalbumin in the systemic circulation is considered physiological.

Topical tumor therapy is widely used in urologic oncology. BCG treatment has been shown to reduce papillary tumor recurrences by up to 40%.[7] The therapeutic effect has been attributed to the local production of inflammatory mediators including TNF.[16] Several differences between BCG and HAMLET were noticed. HAMLET appears to mainly act directly on cancer cells *in vivo*, and not through the recruitment of immune-effector cells. In the skin papilloma study, immunocompetent and immunocompromised individuals showed a similar response, suggesting that HAMLET does not act through specific immunity. Furthermore, the effect of HAMLET was more rapid than BCG, as seen by tumor cell excretion within a few hours after HAMLET instillation. Finally, the patients did not develop symptoms from the instillations, other than the irritation because of frequent catheterizations. Ongoing studies in a murine bladder cancer model have confirmed

that tumor progression is limited by intravesical instillations of HAMLET (Mossberg *et al*., in preparation). Thus, topical HAMLET administration represents a new and potentially useful approach to bladder cancer therapy. Controlled trials are needed to evaluate the potential of HAMLET as a topical agent in bladder cancer patients.

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