Bioluminescence imaging to monitor bladder cancer cell adhesion *in vivo*: a new approach to optimize a syngeneic, orthotopic, murine bladder cancer model

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OBJECTIVE

To improve the orthotopic murine bladder cancer model by using bioluminescent (BL) MB49 tumour cells for noninvasive *in vivo* monitoring of tumour growth and to examine the efficacy of integrin receptor-blocking oligopeptides on preventing tumour cell adhesion in this improved bladder cancer model.

MATERIALS AND METHODS

The capacity of oligopeptide combinations to interfere with tumour cell adhesion was assessed *in vivo* in a syngeneic, orthotopic, murine bladder cancer model. Tumour outgrowth was monitored noninvasively by bioluminescence imaging (BLI) after administration of luciferase-expressing MB49^{LUC} bladder cancer cells. The presence of tumour cells was verified histologically and immunohistochemically on paraffin wax-embedded sections of excised bladders.

RESULTS

Anti-adhesive oligopeptides effectively inhibited tumour outgrowth. BLI detected tumour cells at an early stage when there were no clinical signs of cancer in any of the mice. The technique has high sensitivity in detecting tumour cell implantation, but is less reliable in assessing tumour volume in advanced-stage disease due to light attenuation in large tumours.

CONCLUSIONS

Peptides targeting adhesion molecules prevent attachment of bladder cancer cells to the injured bladder wall. BLI is a sensitive method for detecting luminescent bladder cancer cells in an orthotopic mouse model.

KEYWORDS

bladder cancer, bioluminescence imaging, cell adhesion, integrins, oligopeptides

INTRODUCTION

Recurrences of nonmuscle-invasive urothelial cell carcinoma after transurethral resection (TUR) are a major clinical problem in urological oncology. Several reports have indicated that the risk of recurrence is 50–70% [1,2].

Endoscopic transurethral electroresection under continuous flow irrigation, the primary therapy for nonmuscle-invasive urothelial cell carcinoma, results in release of large numbers of tumour cells [2]. The re-implantation of floating viable tumour cells to the injured bladder wall might account for the high rate of tumour recurrences.

Several trials have investigated this initial step in tumour formation by instillating cytotoxic drugs immediately after TUR to inhibit cell viability and implantation. These trials resulted in impressively reduced recurrence rates, which matched the results seen with standard intravesical treatment courses with cytotoxic drugs [3]. An alternative treatment method would be to directly interfere with components of the extracellular matrix (ECM).

The importance of ECM proteins, particularly fibronectin, in promoting cellular attachment to the surrounding tissues was recognized in the early 1980s [4]. Several cell-surface receptors, called integrins, were identified, which mediate adhesion by binding to specific peptide epitopes residing on ECM proteins [4]. For fibronectin, the arginine-glycine-aspartic acid (RGD) tripeptide appears to be the crucial sequence for ligand–receptor interaction [5].

In the ECM of the bladder wall fibronectin, collagen, and laminin are the main promoters of cell attachment [6]. Previous studies have shown more effective inhibition of tumour cell adhesion by combining integrin receptor-blocking oligopeptides with different binding specifities as compared with using single oligopeptides [7]. In the present study, our primary goal was to evaluate a novel mixture of oligopeptides for its anti-adhesive properties. The mixture included GRGDS, which has affinity mainly to the fibronectin receptor, but also to the collagen and laminin receptors [8,9]. Furthermore we administered EILDV, which is known to interact with the fibronectin receptor [10], and YIGSR, which has a high affinity for laminin receptors [11]. Our second objective was to use the sensitive and noninvasive bioluminescence imaging (BLI) technique to measure *in vivo* the efficacy of these oligopeptides in inhibiting adhesion of MB49^{LUC} cells to the injured bladder wall in an orthotopic murine model of bladder cancer.

MATERIALS AND METHODS

The cell cultures of murine MB49^{LUC} cell clones, stably expressing luciferase, were

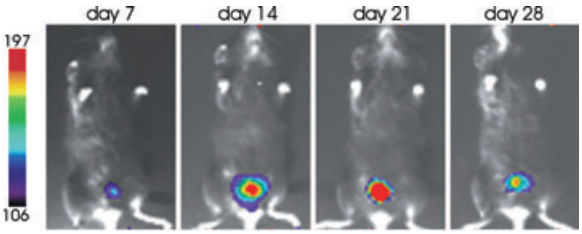
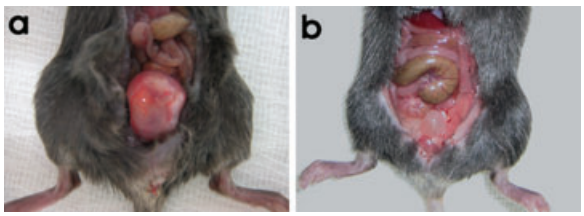


FIG. 1.
Serial BL images of a control mouse. Counts on days 7, 14, 21, and 28 were 2054, 6789, 13406, and 6092, respectively. Bladder weight at day 28 was 130 mg. Histology showed extensive necrosis and haemorrhage in the tumour.

TABLE 1
Efficacy of anti-adhesive peptides in the murine orthotopic BL MB49^{LUC} bladder cancer model.

Variable	Treatment group	Controls	P
Group size, n	12	10	
Mean light emission on day 7, counts	0	8177	<0.001
Tumour take, %	16.7	90	<0.001
Mean bladder weight at death, mg	35	186	0.005

FIG. 3. Surgically exposed bladder of a control mouse (a) and a mouse treated with anti-adhesive peptides (b), photographed on day 28 after tumour implantation. Bladder weights at day 28 were 430 mg (a) and 30 mg (b), respectively.



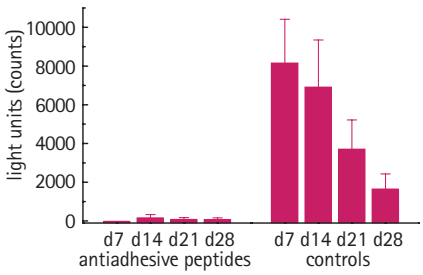
generated by selection with geneticin as described previously [12].

The single letter code of the peptide sequences corresponds to the following amino acids: D, aspartic acid; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; P, proline; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine. For the oligopeptide mixture the therapeutic peptides used were GRGDS, EILDV, and YIGSR (Bachem Biochemica, Heidelberg, Germany), which mimicked sites of fibronectin/collagen/laminin, fibronectin, and laminin, respectively [8,9,13]. Three pentapeptides (Bachem) with unrelated specifities, DRVYI (angiotensin I/II), YIHPI (angiotensin I/II), and RYLPT (proctolin) were applied as a control.

All animal protocols were approved by the Animal Care and Use Committee of Martin Luther University Halle-Wittenberg. After cauterization of the bladder 5×10^4 MB49^{LUC} cells in 100 μL PBS were intravesically instilled into female C57/BL6 mice as described previously [7], followed by intravesical administration of 100 μL oligopeptide solution. Mice in the treatment group (12 mice) were given the combination of GRGDS, YIGSR, and EILDV (3 mg/mL each) while controls (10) received the nonspecific peptides DRVYI, YIHPI, and RYLPT (3 mg/mL each). Except for three control mice, which had to be killed on days 19, 21, and 24 after tumour implantation, respectively, the mice were killed on day 28 and bladder weights were determined.

In vivo BLI with a VisiLuxx Imager (Visitron, Puchheim, Germany) was done weekly as described previously [12], starting on day 7 after tumour cell instillation. Acquisition time was 10 min and binning 6. Bioluminescent (BL) signals were quantified using Metamorph software (Visitron). All values are reported as the mean (SEM) of the integrated counts (light units) obtained from all mice within one group. In most cases excised bladders were also imaged to verify the origin of light emission.

FIG. 2. Light emission over time in the treatment group (12) and the control group (10) as quantified by BLI. The mean (SEM) values are reported and differed significantly between the groups at all four time points (d, day).



Immunohistochemistry was done on paraffin-wax sections of explanted bladders using a rabbit polyclonal anti-luciferase antibody (CR2029RAP; Europa Bioproducts, Cambridge, UK), following the method described by Lee *et al.* [14]. The haematoxylin and eosin (H&E) staining was done according to standard protocols.

Statistical analysis was done using the ANOVA and the Student's *t*-test, with $P < 0.05$ considered to indicate statistical significance.

RESULTS

Mice from the treatment group and control mice were given intravesically therapeutic or unrelated control peptides, respectively, immediately after instillation of MB49^{LUC} cells (day 0). In a preliminary study we had shown that control mice given saline and mice treated with *unspecific* oligopeptides showed no difference in bladder weight and survival. As we had found previously that no BL signals were detectable immediately after intravesical administration of 5×10^4 MB49^{LUC} cells, mice were first imaged on day 7 after tumour cell implantation (Fig. 1). While no BL signals were detectable in any of the 12 treated mice, 9 of 10 control mice showed photon emission on day 7 ($P < 0.001$). The mean counts (light units) in the control group were 8177 (Table 1, Fig. 2). There were no clinical signs of cancer in any of the mice at this time, underscoring the high sensitivity of BLI in detecting tumours at this early stage of disease.

BL signals, which decreased over time, were detected in one of 12 mice in the treatment group on days 14, 21, and 28 while light emission continued to be negative in the

remaining mice of this group. The bladder weight in this mouse was 80 mg on day 28 while the bladder weights in the remaining mice were 20–50 mg (Fig. 3b). Histology and immunohistochemistry for luciferase on paraffin wax-embedded sections of explanted bladders confirmed the presence of a small tumour in the mouse that had shown light emission. A very small tumour cell nest was also identified by histology and immunohistochemistry in another mouse of the treatment group, that had not had any photon emission throughout the study (bladder weight on day 28 was 30 mg). All the other bladders were histologically tumour-free, indicating that tumour cell implantation was successfully inhibited by anti-adhesive peptides in 10 of the 12 mice in the treatment group.

All control mice with detectable light signals on day 7 (nine mice) showed continuous photon emission from the bladder region at all subsequent imaging time points while signals in the mouse that had not demonstrated light emission on day 7 continued to be negative, suggesting that tumour cell implantation in this mouse had not been successful. The mean counts in the control group on days 14, 21, and 28 were 6973, 3728, and 1670, respectively, and differed significantly from the respective mean counts (178, 101, and 95) in treated mice at all time points ($P = 0.006$, 0.009, and 0.018, respectively, Figs 1 and 2). The mean bladder weight in the control group was 186 mg vs 35 mg in the treated mice ($P = 0.005$), and bladder weights ranged from 25 mg (in the mouse with no BL signals) to 430 mg (Fig. 3). Within the control group there was no significant correlation between bladder weight and BL signals, indicating that light emission confirms the presence of viable tumour cells but, especially at later time points, does not necessarily accurately reflect tumour volume. Explanted bladders were cut and re-imaged with the cut-side facing the camera. BLI indicated reduced light emission from necrotic and haemorrhagic areas as compared with vital tumour areas (Fig. 4a). Histological analysis confirmed deep muscle-invasive cancer, extensive necrosis, haemorrhage, and infiltration of host cells in large tumours (Fig. 4b,c); areas which contribute to tumour volume but not to BL. Immunohistochemistry showed scarce staining for luciferase in these regions while vital tumour tissue strongly expressed luciferase (Fig. 4b,c). Luciferase-labelled tumour cells were identified by immunohistochemistry in all mice showing photon emission by BLI before death.

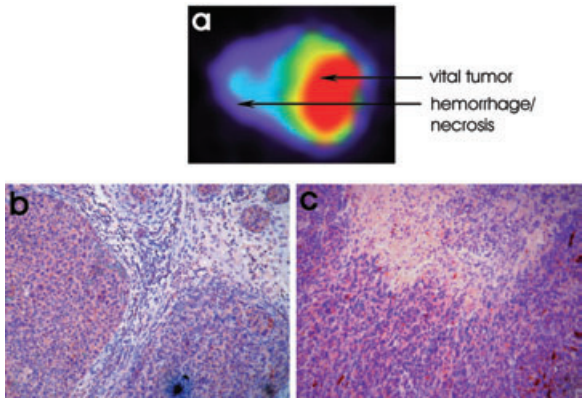


FIG. 4.
Ex vivo *BLI (a) of the explanted and cut bladder shown also in Fig. 2(a). Obviously necrotic tumour areas emitted less light than vital tumour tissue. Immunohistochemistry for luciferase (b and c) on corresponding paraffin wax-embedded sections show luciferase-positive tumour cells surrounded by infiltrating host cells (b) and large areas of necrosis (c).*

DISCUSSION

The ECM of the bladder consists of adhesion molecules, such as fibronectin, laminin, and collagen, for which a multitude of receptors are expressed on the cell surface [5,15,16]. Thus, blockage by single *monospecific* anti-adhesive peptides can lead to ineffective inhibition of tumour cell adhesion. Based on our previous findings, showing that combinations of oligopeptides blocked cell attachment to the ECM more effectively [7], we examined a novel polyvalent fibronectin/collagen/laminin-related peptide mixture consisting of GRGDS, EILDV, and YIGSR [8–11,13]. As fibronectin seems to be a key player of the ECM in mediating cell adhesion, we used two different oligopeptides, GRGDS and EILDV, to block different fibronectin receptor epitopes. See *et al.* [16] showed that GRGDS significantly decreased bladder cancer cell adhesion to fibronectin *in vitro*. However, *in vivo*, GRGDS alone failed to inhibit cell attachment and tumour outgrowth. Hyacinthe *et al.* [17] used an adhesion assay with mixed matrices: GRGDS inhibited cell attachment *in vitro* and also significantly reduced cell adhesion in a murine bladder cancer model when given intravesically. These authors administered GRGDS at a 10-fold higher concentration compared with See *et al.* [16].

EILDV had shown efficacy in inhibiting bladder cell adhesion on a fibronectin monomatrix, while in the natural environment of the murine bladder EILDV alone was ineffective in inhibiting tumour outgrowth [7]. Yamamoto *et al.* [18] reported a significant anti-metastatic effect of YIGSR and an even stronger effect of EILDV in a melanoma model *in vivo*. EILDV and/or RGD also significantly reduced endodermal cell migration [19], while YIGSR and RGD inhibited adhesion of gastric cancer cells to ECM and prolonged survival in a murine i.p. gastric cancer model [20]. YIGSR also reduced the invasive potential of human lung cancer cells *in vitro* and inhibited lung metastasis in a murine model [13]. Altogether, these data prompted us to test a novel combination of the anti-adhesive peptides GRGDS, EILDV, and YIGSR for its potency in inhibiting bladder cancer cell attachment *in vivo*. This peptide mixture successfully inhibited bladder cancer outgrowth in 10 of 12 mice, suggesting that direct blockade of adhesion receptors might be a valuable treatment option for reducing recurrences after TUR.

Due to the impossibility of measuring tumour size by calliper in orthotopic bladder cancer, therapeutic effects are usually assessed by clinical symptoms and 'endpoint variables' such as survival time, bladder weight, and histological analysis of explanted bladders [21]. In the present study, we examined whether BLI is a suitable method for detecting orthotopically implanted bladder cancer cells at an early time point and might be used to monitor tumour growth and therapeutic efficacy noninvasively over time. In the present study there was effective inhibition of tumour cell implantation to the injured bladder wall by anti-adhesive oligopeptides as early as day 7 after tumour implantation. To our knowledge, this is the first syngeneic, BL bladder cancer model to be reported. BLI allows for repetitive imaging of multiple mice in parallel, which reduces statistical variability and the number of mice needed per

experiment. In a previous BLI study we showed that emitted photons tightly correlate with tumour volume up to a certain tumour size, beyond which correlation appears to be much less reliable [12]. Features such as necrosis, haemorrhage, muscle invasion of cancer cells, and tumour infiltration of host cells contribute to tumour size but not to light emission, as we clearly show also in the present study (Fig. 4). In addition, intratumoral distribution of vessels, oxygen, and the luciferase substrate luciferin becomes more heterogeneous with increasing tumour size.

The sensitivity for detecting the weak BL light originating from tumour cells in a living body is mainly influenced by cellular luciferase activity and localization of the luciferase-expressing tumour cells. The MB49^{LUC} cells we used had high luciferase activity as determined luminometrically. In cell culture experiments the lower detection limit for BLI was 1000 MB49^{LUC} cells (data not shown).

The main obstacles for reliably quantifying photon emission *in vivo* are scattering and absorption of light by tissues, predominantly caused by haemoglobin [22]. The histological features described above might explain why in the present study all mice that had BL signals on day 28 also showed the lowest light emission on this day, while photon emission had been up to 10-fold higher on days 7 or 14, respectively (Fig. 2). Light absorption by tissue, dark fur, and dark pigmented skin as seen in the C57/BL6 mice we used might have contributed to the fact that BLI failed to detect tumour cells on day 0. Recently, the use of *human* BL bladder cancer cells in orthotopic tumour models has been reported [23–25]. Smakman *et al.* [25] administered i.p. a cyclooxygenase-2 inhibitor while intravesically instilled bisphosphonates and polo-like kinase small interfering RNAs, respectively, were tested by Sato *et al.* [24] and Nogawa *et al.* [23].

Although experimental differences limit comparability with the present data, these studies corroborate our main findings: therapeutic efficacy corresponds to significantly less mean photon emission in treated mice as compared with control mice. Both Sato *et al.* [24] and Nogawa *et al.* [23] implanted 2×10^6 (40-fold our dose) UM-UC-3^{LUC} cells. BL signals were first detectable on day 4, underscoring that BLI cannot detect luminescent cells immediately after intravesical administration. Both studies are terminated at day 21. Nogawa *et al.* [23] show a strong linear increase in light emission from day 12 to day 18, but do not present data thereafter, while Sato *et al.* [24] report a strong increase between days 15 and 18, followed by a plateau phase until day 21. This plateau also suggests that tumour growth and BL signals correlate less well in advanced disease. Thus, BLI loses some of its power as a *quantitative* method for assessing tumour burden as cancer progresses, but still retains its power as a *qualitative* technique.

Smakman *et al.* [25] injected 6×10^5 human BL T24 TCC cells into the bladder dome of nude mice. Notably, T24 cells are thought to have only limited use for *in vivo* experiments as they lack tumorigenicity [26]. Control mice showed a linear increase in mean photon emission until closure of the study on day 56, which differed significantly at all time points from that of the treatment group. BLI was *first* done on day 28, a time point at which the present study had to be closed for ethical reasons and three of our control mice (bladder weights: 310, 370, and 380 mg) were already dead. Histological analysis did not reveal any invasion of the muscle layer by T24 cells, which indicates that the xenogeneic T24 tumour grows far less aggressively than bladder cancer in humans and the syngeneic model we used and thus allowed for a longer observation period.

In summary, BLI is a valuable tool for assessing therapeutic efficacy in murine models of orthotopic bladder cancer. BLI confirms intravesical implantation of bladder cancer cells at an early stage, which also allows randomization of mice into different groups before starting anticancer therapies. The present data verify that specific combinations of anti-adhesive peptides effectively prevent cell attachment and thus subsequent invasion and growth of bladder cancer cells, indicating that this therapeutic approach might contribute in reducing the recurrence rate of bladder cancer after TUR.

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CONFLICT OF INTEREST

None declared.

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Abbreviations: **TUR**, transurethral resection; **ECM**, extracellular matrix; **BL**, bioluminescent; **BLI**, bioluminescence imaging.